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Self-assembly of multi-component mitochondrial nucleoids via phase separation

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1st Editorial Decision 5th Jan 2021

Thank you for submitting your revised manuscript along with the referees' reports from Review Commons to The EMBO Journal. Please accept my sincerest apologies for the delay in getting back to you with our decision. We have sent the revised study to original referee #1 (mitochondrial expert) and referee #3 (phase separation expert) for re-review. While the former submitted his/her comments in due time, the latter has remained unresponsive, even after repeated chasing. Therefore, we have contacted an external advisor, who was asked to evaluate your response to the referees' points from the phase-separation angle.

Our advisor states "I find the authors have done an excellent and careful job in the response and addressed the comments. The authors did not answer: "what the degree of labeling (or DOL) was of DyLight labeled TFAM?" but this is usually not very high degree of labelling with this technique (NHS ester labeling - I went to look up the methods from the preprint supplementary material) so I doubt it is a problem. (It is simple to test label efficiency by measuring the UV absorbance at the 280 nm (and 260 nm) and the dye absorbance wavelength and using the extinction coefficients to check). The authors have taken the reviewer's suggestion to avoid claiming "liquid-liquid" phase separation for in cell and sticking with "phase separation" which I think is a good move and they have convincing data to suggest phase separation. So, I think there is no issue I would have with 1. I think the data in Figure 1 especially are interesting and evocative and well explored by the subsequent data presented. I think the observation of phase separation of mt nucleoid is important finding".

Given these comments and the positive feedback received from Review Commons referee #1 (pasted below), we would like to pursue publication of your study in our journal. Even if not strictly necessary for publication, we feel that adding the control experiment suggested by the advisor would strengthen the manuscript.

In addition, there are a few editorial issues concerning the text and the figures that I need you to address.

Referee #1:

In this revised manuscript the authors have in my opinion professionally and adequately dealt with the reviewers earlier comments, resulting in a very interesting and convincing paper.

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New_manu_number: EMBOJ-2020-107165
Corr_author: Misteli

Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this article the authors use biochemical, biophysical and cell biological approaches to study mtDNA nucleoid organization. They show that the major mtDNA packaging factor TFAM has intrinsic properties to phase separate in liquid-like droplets and in combination with PCR amplified large mtDNA fragments self-assembles in multiphase liquid-like particles containing both TFAM and mtDNA. They have examined various TFAM mutants for their phase-separation properties and examined whether nucleoids in cultured cells behave as phase-separated particles. Finally, they show altered nucleoid morphology in fibroblasts from patients with the Hutchinson-Gilford Progeria Syndrome (HGPS) and use this as further proof of phase separation properties of mtDNA nucleoids. This is an interesting paper that provides novel insights into the organization of mtDNA in mitochondria and provides novel lines of research.

Comments:

1.For the first in vitro part of their studies the authors use DyLight594 labeled variants of TFAM. I have some concern here. DyLight fluorophores are negatively charged and hydrophilic and are typically conjugated to pos charged lysine residues in labeled proteins. TFAM as the authors themselves also point out is very strongly positively charged at neutral pH. One may assume not only that the conjugation to the e-amino group of lysine removes the net pos charge but that in addition a negative charge is added. This raises an important question which is what the degree of labeling (or DOL) was of DyLight labeled TFAM? This is very straightforward to measure and calculate. After writing this I now read in the M&M the following: 'prior to buffer exchange, protein was thawed and labelled protein was mixed with unlabeled protein at a ~1:100 mass ratio.' Does this mean that all measurements such as in Fig 2 were done with a 1:100 ratio mix of labeled versus non-labelled protein? If true, this would take away some of my concern but nonetheless a high DOL for the labeled protein could clearly affect it biophysical properties such as DNA binding. In figure 3 it is argued that increasing the mtDNA concentration at a constant TFAM concentration shows de-mixing mtDNA and TFAM thereby showing that TFAM/mtDNA structures behave as

multiphase structures. Apart from the fact that I find this very hard to judge based on this figure, the TFAM that is visible is only the TFAM that is labeled by the DyLight594 dye and thus possibly not representative of all TFAM. In fact based on the 1:100 ratio, the majority of TFAM is unlabeled and would be invisible. One could perhaps also argue, since only labeled TFAM is visible that the droplets observed in Figure 2 are droplets formed by a subpopulation of molecules, namely those that have the label. So a simple question (and experiment) would be: do the droplets observed in Fig 2 also form in the absence of fluorophore?

2.Concerning the observation of altered nucleoid morphology in HGPS fibroblasts and its relation to mitochondrial dysfunction. I find this an overstretched conclusion and possibly even contradicted by the observations in the paper although this might be a misunderstanding on my part. In Figure 1 it is concluded that fibroblasts from young patients do not show a significant increase in damaged mitochondria whereas those from older patients do. In the main text it is commented that: 'In morphologically aberrant mitochondria, mt nucleoids clustered together into structures.....etc' leading me to understand that in the younger patient fibroblasts nucleoids by and large appear normal. However, in Figure 6 it appears that OXPHOS dysfunction (based on Seahorse respirometry) is observed in all 4 patient cell lines, both young and old, leading me to conclude that the reduced basal and maximal respiratory capacity in the patient fibroblasts does not correspond with enlarged nucleoids in patient fibroblasts.
3. I understand that for live cell imaging a fluorescent protein fusion is necessary but for the experiment in Fig 5 I would be more convinced if overexpression with a small epitope tag had been used.
4. I find it surprising that in none of the experiments hexanediol treatment has been used to disrupt phase separated structures, this can to my knowledge be used both in vitro and in vivo and would add additional evidence that nucleoids show liquid phase separation.

Minor comments:

1.In figure 6 K/L HGPS young and old are separated. Does this mean lines HGPS1+2 are separated from 3+4?

2.What do the dotted-line arrows indicate in Fig 1L (compared to the solid-line arrows).

3. Significance:

Significance (Required)

Phase separation has not yet been considered for mitochondrial nucleoids, even if it has been considered for so-called mtRNA granules. If proven correct and confirmed by other studies it would open up novel lines of research and increase our understanding of mtDNA and its expression.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Cannot tell / Not applicable

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This study by Feric et al studies the properties of mitochondrial nucleoids and associated proteins. It combines biochemical work with live cell assays and high-resolution structured illumination microscopy of fixed cells, including samples from patients with a premature aging disorder. In general, I find that this study is very interesting and contains high-quality data. However, I have doubts about the main conclusion that multi-component mitochondrial nucleoids self-assemble via liquid-liquid phase separation. As outlined below, my doubts are related to the fact that TFAM droplets do not seem to be liquid, and that the observed properties of the mitochondrial nucleoid might be explained by DNA condensation that is distinct from liquid-liquid phase separation. If the authors refer to another type of phase separation than liquid-liquid phase separation, this has to be explicitly specified to not mislead the reader.

Major points

1.Figure 1 shows that mitochondria can fuse, which is consistent with previous studies. The authors call these fusions "liquid-like fusion events" and point out that this behavior is consistent with that of coalescing droplets. As mitochondria are membrane-bound organelles, presumably filled with liquid like most of the rest of the cell, I am wondering what "liquid-like" means in this context and why coalescing droplets are invoked. Are the observations different from what you would expect if two membrane-bound organelles, say, vesicles, fuse? I think their (liquid) content should mix in a very similar way, without any phase separation, shouldn't it?

2.Figure 2 shows that recombinant TFAM can form droplets (Fig. 2). These droplets are found to be gel-like, i.e., they show slow protein dynamics and contain a substantial immobile protein fraction. This seems to be at odds with LLPS that should produce two liquid phases. Nevertheless, the authors use these results to support the model that mitochondrial nucleoids are formed by LLPS. How does this fit together? Is the idea that these droplets are "aged" condensates that are liquid before they become gels? In this case, the authors should provide some additional data that show this, as gels can form in multiple ways, e.g., by regular gelation resulting from molecular interactions that do not

drive LLPS.

3.Figure 3 shows that gel-like droplets with irregular morphology are obtained in TFAM:mtDNA mixtures, similarly to mixtures containing TFAM and ssDNA/dsDNA/RNA/dNTPs. In these droplets, mtDNA and TFAM do not co-localize, which is interpreted as demixing in a multi-component phase separating system. I am wondering if these results could also be explained by DNA condensation, similar to "psi-condensation" of DNA molecules induced by divalent cations and crowders (polyethylene glycol), or to "polymer-collapse" as recently suggested for HP1-bound heterochromatin? Such a process would arguably be distinct from LLPS and would be consistent with the phases not being liquid, which is what seems to be observed (if I interpret the data correctly, neither the TFAM "phase" nor the "mtDNA" phase is liquid). In contrast to TFAM/mtDNA, the multiphase system consisting of nucleolar proteins (NPM1/FIB1) that is mentioned yields structures with different morphology that seem to be rather liquid, suggesting different underlying biophysics.

4.The authors analyze enlarged mitochondria in HGPS cells to test whether the phase-separation properties of mt-nucleoids are related to mitochondrial function. Albeit interesting, I do not see how this analysis tells us much about phase separation and its functional relevance, as the relationship between transcriptional activity, metabolic function and structure/phase separation remains unknown. Maybe the authors could address this question in a different way, e.g., by replacing TFAM with a mutant that does not phase-separate and then assessing transcriptional/metabolic activity? This would add to the study.

5.The authors imply in their Discussion that the nuclear genome is organized by (liquid) phase separation, although several studies that are not cited have questioned this view. I think that the Discussion would profit from a more balanced representation of our current knowledge and the caveats.

Minor points

1.Fig. 2C: What do we learn from the inset? Droplet length and relaxation time seem to be pretty uncorrelated.

2.Fig. 2D: While large dextrans are excluded from droplets, small dextrans do not only access the droplets (expected partition coefficient = 1?) but are even enriched in it (partition coefficient > 1). Does this suggest that TFAM and dextrans interact? What about other probes with different chemical properties but similar size? These data could contain more information than just the characteristic length scale.

3.Fig. 2G: FRAP of the different droplets could be interesting to see which ones are rather liquid and which ones are rather gel-like. This could be instructive to decide if the proteins undergo liquid phase separation or gelation.

4.Fig. 3A: Droplets are observed in TFAM:mtDNA mixtures - however, not at the concentration and TFAM:mtDNA ratio deemed "physiological" in the text (10 M TFAM, 1:1000 ratio = 10 nM mtDNA). While I suspect that droplets can also be obtained for

these parameters if the right buffer conditions are chosen, I think it would be good to either show this or otherwise comment in the text why you think that it will phase-separate in the cell.

5.Fig. 3C-G: Wouldn't it make sense to do these experiments at the "physiological" TFAM concentration (10 M)?

6.Fig. 5A-C: It looks like the number of nucleoids in the mitochondria changes upon TFAM overexpression. Could you quantify this so that it is easier to understand these images (multiple points in the green channel = multiple nucleoids or substructures of the same nucleoid?)?

3. Significance:

Significance (Required)

In the current state, the significance is not clear to me, as the relationship between a potential phase separation mechanism and the functional relevance has not been worked out.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Reviewer Summary

The authors report that mitochondria and their associated nucleoids are enlarged in cells of patients with the disease HGPS. The authors find that the mitochondrial nucleoid behaves as a phase-separated compartment, in which the protein TFAM forms liquid-like condensates with the mtDNA genome. Overall, this is a well-written paper with excellent in vivo and in vitro data. While we find the evidence for nucleoid phase separation convincing, we have some concerns about the link between nucleoid phase separation and enlarged, dysfunctional mitochondria in HGPS cells. We suggest that the authors provide data that can bolster this link, or describe limitations to this work that

will necessitate future studies that link HGPS to aberrant nucleoid phase separation. We provide detailed comments below

Comments to the Authors

1)To supplement Fig. 1I, please provide a quantification of droplet fusion from multiple fusion events, to show how the aspect ratio relaxes. This would more strongly support the hypothesis that nucleoids are liquid-like, and motivate the in vitro experiments in the next figure.

2)How many replicates are there for each construct in Fig. 2 E-G? How reproducible are the differences in morphology and phase behavior among constructs? Some quantification of droplet morphology, such as aspect ratio, would be helpful to show how reproducible these morphology observations were.

3)On page 10, the authors say that the delta C-tail mutant "influenced the wetting behavior of the droplets as indicated by a decreased smoothness along the droplet perimeter." The term "wetting behavior" is a little confusing - is this term referring to how droplets wet the surface of the imaging chamber, or are they referring to droplet surface tension? Please clarify. Also, what specific molecular interactions within the droplet phase do the authors believe the C-tail is regulating to control droplet morphology?

4)In the next sentence, the authors say "Consistent with this notion, removal of the HMGA domain (ΔHMGA), also resulted in droplet formation, but at slightly higher saturation concentrations." Please elaborate a bit on how this observation is consistent with removal of the disordered C-tail described in the previous sentence.

5)The data in the lower inset in Fig. 2C look uncorrelated, but the authors fit it with a line anyway to estimate the inverse capillary velocity, which is likely not appropriate. Instead, remove the linear fit, and say that there was no clear correlation between droplet size and relaxation time, which supports the idea that droplets are highly viscoelastic and do not follow simple rheological predictions.

6)What is the dashed green line in Figure 3B? Is it a fitted function? If so, describe the function.

7)In Figure 3B, the term "gelation" in the figure panel suggests that higher DNA/protein ratios drives some kind of aging process that drives condensate gelation. But later in 3J it becomes clear that DNA is actually just highly immobile within condensates, and forms a viscoelastic network that likely fixes droplets into non-spherical shapes. Please change the phrasing of the term "gelation" on this panel to something else which makes it more clear how DNA drives this aspect ratio increase with increasing DNA/protein.

8)Are the black lines in Figure 3J single exponential recovery models? If so, what are the time constants and mobile fractions of protein and DNA?

9)In the explanation of the FRAP data in Figure 4D, the authors write "Based on observed recovery times of ~1 min, we estimate diffusivities of ~1x10^-3 μm^2/s."

This is very qualitative, and the images seem to show that recovery is still incomplete after 3 min. What is this recovery time of 1 min based on? Please provide a quantification of these data with associated fit and description.

10)The images in Fig 4E are really nice, but it is a little difficult to see droplet fusion clearly with only three frames spaced apart by 8 and 12 min. Please add more frames from this time series to show the sequential events of the fusion process more clearly.

11)The text suggests that TFAM droplet size correlates with the level of protein over-expression. If so, please provide a quantification of droplet size as a function of expression level in Fig. 5. Further, do the images of increasingly larger droplets in Fig. 5 A-C come from cells with increasing expression level of TFAM? If so, please provide values of the respective expression level (e.g. quantified in terms of the average cytosolic protein fluorescence intensity) with each column of images.

12)Does expression level of the different TFAM constructs only affect droplet size, or is the localization of TFAM with mtDNA and TFB2M also affected as a function of expression level? Please provide some control data to show whether TFAM expression level affects localization with other nucleoid components.

13)In a related question, are the data in Figure 5F from cells with relatively similar expression levels of the different TFAM mutant constructs? If not, then the control data requested above is important to show that expression level does not affect localization with other nucleoid components.

14)The HMGB + C-tail localization to the lining of the mitochondrial membrane is very interesting. This suggests that in addition to disrupting interactions with mtDNA, the HMGB + C-tail mutant also has increased affinity for TFB2M, which is supported by the quantification in 5F. Indeed, the images in Fig. 5E suggest that TFB2M is more enriched at the membrane with the HMGB + C-tail mutant compared to Fig. 5D with full-length TFAM. Please describe in the text whether this TFAM mutant also has increased affinity for TFB2M.

15)As a follow-up to this, do the authors have any in vitro data that include TFB2M in addition to TFAM and mtDNA? That would be a great addition to the paper to show i) whether different TFAM mutants have altered affinity for TFB2M and ii) whether TFB2M shows multiphase behavior within droplets in vitro, similar to in vivo observations.

16)As mentioned in the summary above, our main concern is that the paper is highly motivated by enlarged mitochondria and nucleoids, and associated mitochondrial dysfunction, in HGPS cells. However, this disease is caused by a mutation that causes progerin production, a protein that is not involved with mitochondria or the nucleoid. Therefore, it is still very unclear how the HGPS disease and aberrant nucleoid phase separation are linked. If possible, please provide in vivo data with some of the TFAM mutants to show whether altering nucleoid architecture with disrupted TFAM phase separation leads to mitochondrial dysfunction. Alternatively, the authors should more clearly state that the link between HGPS and phase separation is still unclear and remains a topic for future investigation.

Minor comment

1)When possible, please change channel colors from red/green to magenta/green, for colorblind friendliness.

3. Significance:

Significance (Required)

Expertise in phase separation and cell biology.

Point-by-point response RC-2020-00390 (Feric)

Reviewer #1

This is an interesting paper that provides novel insights into the organization of mtDNA in mitochondria and provides novel lines of research.

We would like to thank the reviewer for describing our work as interesting and novel and that our findings will open new avenues of research.

1.For the first in vitro part of their studies the authors use DyLight594 labeled variants of TFAM. I have some concern here. DyLight fluorophores are negatively charged and hydrophilic and are typically conjugated to pos charged lysine residues in labeled proteins. TFAM as the authors themselves also point out is very strongly positively charged at neutral pH. One may assume not only that the conjugation to the e-amino group of lysine removes the net pos charge but that in addition a negative charge is added. This raises an important question which is what the degree of labeling (or DOL) was of DyLight labeled TFAM? This is very straightforward to measure and calculate. After writing this I now read in the M&M the following: 'prior to buffer exchange, protein was thawed and labelled protein was mixed with unlabeled protein at a ~1:100 mass ratio.' Does this mean that all measurements such as in Fig 2 were done with a 1:100 ratio mix of labeled versus non-labelled protein? If true, this would take away some of my concern but nonetheless a high DOL for the labeled protein could clearly affect it biophysical properties such as DNA binding. In figure 3 it is argued that increasing the mtDNA concentration at a constant TFAM concentration shows de-mixing mtDNA and TFAM thereby showing that TFAM/mtDNA structures behave as multiphase structures. Apart from the fact that I find this very hard to judge based on this figure, the TFAM that is visible is only the TFAM that is labeled by the DyLight594 dye and thus possibly not representative of all TFAM. In fact, based on the 1:100 ratio, the majority of TFAM is unlabeled and would be invisible. One could perhaps also argue, since only labeled TFAM is visible that the droplets observed in Figure 2 are droplets formed by a subpopulation of molecules, namely those that have the label. So a simple question (and experiment) would be: do the droplets observed in Fig 2 also form in the absence of fluorophore?

As suggested by the reviewer, we show now in Fig. S2E that TFAM phase separates in the absence of any fluorophores. We observe identical behavior under these conditions for unlabeled and labelled TFAM. As the reviewer points out, to minimize any artifacts associated with labelling of TFAM, we fluorescently labelled in all our experiments only a small fraction of TFAM (1:100) using a small molecule (DyLight). Since labeled and unlabeled species of TFAM behave identically, it is highly unlikely that the TFAM-lean regions of the compound droplets (TFAM+mtDNA) observed in Fig. 3 represent a sub-population of TFAM. We are also reassured by the fact that we find throughout our study indistinguishable behavior of TFAM (untagged, tagged with a small dye, or tagged with a fluorescent protein) both *in vitro* and *in vivo*, very strongly suggesting that the tags do not influence the observed phase behavior of TFAM.

2.Concerning the observation of altered nucleoid morphology in HGPS fibroblasts and its relation to mitochondrial dysfunction. I find this an overstretched conclusion and possibly even contradicted by the observations in the paper although this might be a misunderstanding on my part. In Figure 1 it is concluded that fibroblasts from young patients do not show a significant increase in damaged mitochondria whereas those from older patients do. In the main text it is commented that: 'In morphologically aberrant mitochondria, mt nucleoids clustered together into structures.....etc' leading me to understand that in the younger patient fibroblasts nucleoids by and large appear normal. However, in Figure 6 it appears that OXPHOS dysfunction (based on Seahorse respirometry) is observed in all 4 patient cell lines, both young and old, leading me to conclude that the reduced basal and maximal respiratory capacity in the patient fibroblasts does not correspond with enlarged nucleoids in patient fibroblasts.

We agree with the reviewer's comment and have now clarified and de-emphasized this conclusion throughout the manuscript, including in the abstract. We mention in the results section on p. 15-16 that our data suggest that mitochondrial morphological changes are preceded by mitochondrial dysfunction in HGPS. This observation is in line with our conclusion that swollen mitochondria represent damaged/stressed mitochondria as confirmed with UPRmt markers (Figs. 1 and S1). Furthermore, when cells are stressed under phototoxic conditions, we note that mitochondrial swelling, indicative of dysfunction, appears first, followed by fusion events of the nucleoids (Fig. 1H,I). Regardless, we have now made these descriptions clearer in the text and moved the functional experiments to supplementary materials to prevent misinterpretation of the results.

3. I understand that for live cell imaging a fluorescent protein fusion is necessary but for the experiment in Fig 5 I would be more convinced if overexpression with a small epitope tag had been used.
We now demonstrate in Fig. S6B,C that untagged TFAM induces the same changes in nucleoid morphology as mKate2-tagged TFAM. To this end, we co-expressed untagged TFAM and mKate2 from the same plasmid. As observed for tagged-TFAM, the untagged protein led to the formation of enlarged TFAM droplets to which multiple mtDNA foci were associated, similar to our observations with tagged TFAM (Fig. 6). Furthermore, we now show in a new Fig4/S4 similar *in vivo* localization patterns of several mKate2-labelled TFAM mutants and the corresponding DyLight-labelled mutants *in vitro*, which suggests that TFAM-specific behavior is determining the interactions, not the tag. Overall, we are reassured by our finding that the *in vitro* and *in vivo* behaviors of TFAM either untagged, tagged with a small dye, or tagged with a fluorescent protein are indistinguishable, very strongly suggesting that the tags do not influence the observed phase behavior of TFAM.

4. I find it surprising that in none of the experiments hexanediol treatment has been used to disrupt phase separated structures, this can to my knowledge be used both in vitro and in vivo and would add additional evidence that nucleoids show liquid phase separation.
While hexanediol is commonly used in phase separation studies, we find that hexanediol, even at relatively low concentrations, is highly toxic to mitochondrial function in cells, leading to rapid loss of membrane potential and dramatically altered mitochondrial morphology. We were thus unable to interpret these experiments. However, as requested, we have now performed hexanediol experiments *in vitro* with TFAM and include these data in Fig. S2G and mention them on p. 7. We find that hexanediol reduces the droplet number and size in a concentration dependent matter. As many in the field, we have concerns regarding use of hexanediol and find other assays such as salt concentration dependence of phase separation (Fig. 2A, Fig. S2D), reversibility of phase separation (Fig. S2F) and *in vivo* observations (Fig. 5,6) more indicative of phase separation behavior TFAM than hexanediol treatment.

Minor comments:
1.In figure 6 K/L HGPS young and old are separated. Does this mean lines HGPS1+2 are separated from 3+4?
We now use color coding in original Fig. 6 (now Fig. S7) to clearly separate the young and old HGPS cell lines and have clarified the text and figure legend.

2.What do the dotted-line arrows indicate in Fig 1L (compared to the solid-line arrows).
The solid and dotted arrows were meant to distinguish between pairs of coalescing droplets. We have now made all the arrows solid and changed the placement of the arrows to help with clarity (Fig. 1H,I). This data can also be visualized in our Supplementary Video S1.

Reviewer #1 (Significance)

Phase separation has not yet been considered for mitochondrial nucleoids, even if it has been considered for so-called mtRNA granules. If proven correct and confirmed by other studies it would open up novel lines of research and increase our understanding of mtDNA and its expression.

We appreciate the referee's support.

Reviewer #2

This study by Feric et al studies the properties of mitochondrial nucleoids and associated proteins. It combines biochemical work with live cell assays and high-resolution structured illumination microscopy of fixed cells, including samples from patients with a premature aging disorder. In general, I find that this study is very interesting and contains high-quality data. However, I have doubts about the main conclusion that multi-component mitochondrial nucleoids self-assemble via liquid-liquid phase separation. As outlined below, my doubts are related to the fact that TFAM droplets do not seem to be liquid, and that the observed properties of the mitochondrial nucleoid might be explained by DNA condensation that is distinct from liquid-liquid phase separation. If the authors refer to another type of phase separation than liquid-liquid phase separation, this has to be explicitly specified to not mislead the reader.

We thank the reviewer for considering our data to be of high-quality and of interest and now clarify the type of phase separation mitochondrial nucleoid undergo (see below).

1.Figure 1 shows that mitochondria can fuse, which is consistent with previous studies. The authors call these fusions "liquid-like fusion events" and point out that this behavior is consistent with that of coalescing droplets. As mitochondria are membrane-bound organelles, presumably filled with liquid like most of the rest of the cell, I am wondering what "liquid-like" means in this context and why coalescing droplets are invoked. Are the observations different from what you would expect if two membrane-bound organelles, say, vesicles, fuse? I think their (liquid) content should mix in a very similar way, without any phase separation, shouldn't it?

We have modified the text to make it clear that we are observing fusion events between *mitochondrial nucleoids* within the same mitochondria not fusion of multiple *mitochondria*. The fusion of mitochondrial nucleoids occurs within the mitochondrial lumen and does not require fusion of membranes.

To more clearly determine the type of phase separation event, we have now further quantified the fusion events for nucleoids in live cells (Fig. S1Q). Based on standard criteria used in the biophysics field, we describe these fusion events as "liquid-like" because the aspect ratio of the nucleoids converges to 1 as they rearrange to take on a spherical shape. The same nomenclature was used in the original description of coalescing liquid droplets for RNP-bodies [1, 2]. We have now eliminated the term "liquid-liquid" from the text and use the more appropriate term "liquid-like" when describing the fusion behavior and droplet morphologies.

The term "liquid-like" is standard terminology in the field used to describe the behavior of many non-membrane bound bodies. Despite the term "liquid", these structures are not simple Newtonian liquids due to the complex nature of the components (various biopolymers in an active, crowded environment), and tend to be viscoelastic – meaning the behavior is a function of the time-scale on which they are observed. We now refer to them as viscoelastic where appropriate to better reflect the time and length scale dependent complexity of their behavior and to avoid confusion.

2.Figure 2 shows that recombinant TFAM can form droplets (Fig. 2). These droplets are found to be gel-like, i.e., they show slow protein dynamics and contain a substantial immobile protein fraction. This seems to be at odds with LLPS that should produce two liquid phases. Nevertheless, the authors use these results to support the model that mitochondrial nucleoids are formed by LLPS. How does this fit together? Is the idea that these droplets are "aged" condensates that are liquid before they become gels? In this case, the

authors should provide some additional data that show this, as gels can form in multiple ways, e.g., by regular gelation resulting from molecular interactions that do not drive LLPS.

We agree that the terminology in this still emerging field can be confusing. Please see point 1 for our discussion of the nature of the observed droplets. To be more accurate in our description of the complex time and length-scale dependent responses of TFAM droplets, we now refer to the droplets as undergoing "phase separation" since the system undergoes a phase transition upon reduced salt and increased protein concentration to generate a second condensed state that is different in composition from the dilute, aqueous phase. To avoid confusion, we now refrain from calling the droplets "gel-like", but we describe their behavior as "viscoelastic" where appropriate (incomplete FRAP, size-dependent dextran exclusion, etc.) as is commonly done in the literature.

3.Figure 3 shows that gel-like droplets with irregular morphology are obtained in TFAM:mtDNA mixtures, similarly to mixtures containing TFAM and ssDNA/dsDNA/RNA/dNTPs. In these droplets, mtDNA and TFAM do not co-localize, which is interpreted as demixing in a multi-component phase separating system. I am wondering if these results could also be explained by DNA condensation, similar to "psi-condensation" of DNA molecules induced by divalent cations and crowders (polyethylene glycol), or to "polymer-collapse" as recently suggested for HP1-bound heterochromatin? Such a process would arguably be distinct from LLPS and would be consistent with the phases not being liquid, which is what seems to be observed (if I interpret the data correctly, neither the TFAM "phase" nor the "mtDNA" phase is liquid). In contrast to TFAM/mtDNA, the multiphase system consisting of nucleolar proteins (NPM1/FIB1) that is mentioned yields structures with different morphology that seem to be rather liquid, suggesting different underlying biophysics.

Based on several observations, we can rule out that the observed multi-component droplets are generated by DNA condensation such as psi-condensation. In contrast to heterochromatin, mitochondrial genomes are only 16kb in size, and as such, the mtDNA foci are detected as diffraction limited small structures (≤100 nm; see figures), even in our Structured Illumination Microscopy (SIM) imaging experiments, regardless of their condensation state. We would not be able to detect differences in compaction of mtDNA (16 kb) at this scale *in vitro* or in live cells. Since the droplets we form are >>1 micron, the droplets represent a true macroscopic, cohesive phase. In addition, we also show in Fig. 3A and Fig. S3C that mtDNA on its own does not precipitate using a simple buffer but requires concentrations of TFAM that can already phase separate to concentrate into phase-dense droplets that markedly contrast with the structures formed by DNA psi-condensation. It is also important to keep in mind that TFAM does not act as a crowder, but rather envelopes mtDNA into proteinaceous droplets. Moreover, we see that the large droplets cease to form at extremely high DNA/protein ratios, suggesting a type of reentrant behavior into a single dilute phase, which is not in agreement with how psi-condensation would favor further compaction.

In addition, the polymer-collapse model does not fit our observations on the mtDNA-TFAM *in vitro* system nor live mitochondrial nucleoids. TFAM very robustly forms droplets *in vitro* under simple buffer conditions (20 mM Tris-HCl, 150 mM NaCl, pH 7.5), and forms very large droplets (>1 micron) in live cells after overexpression or other perturbation conditions, unlike the polymer collapse behavior observed with HP1 in mouse cells where it only has a weak capacity for droplet formation [3]. Moreover, we observe dynamic exchange within droplets after photobleaching, both *in vitro* and *in vivo*, which is in contrast to the lack of internal mixing seen in mouse chromatin globules [3]. Furthermore, TFAM recovers at similar time scales in the absence or presence of mtDNA, which supports that unbound molecules of TFAM are still recruited into the TFAM-mtDNA droplets, unlike the poor ability of mouse HP1 to recruit more HP1 molecules when bound to DNA [3]. As mtDNA is incrementally added to these droplets *in vitro*, the

droplets change in morphology and concentration of TFAM, but still maintain a highly proteinaceous composition unlike that observed in the polymer collapse model.

Instead, our FRAP data and irregular droplet morphologies demonstrate that mtDNA is highly immobile in our TFAM-mtDNA droplets. As a result, mtDNA appears to be kinetically trapped, which explains the heterogeneous localization within the droplets. Very long times would be needed to observe how mtDNA samples more configurations and ultimately rearranges inside the droplet. Taken together, our system shows that these are multi-component, phase-separated viscoelastic droplets, both *in vivo* and *in vitro*, and are consistent with behavior observed in other biomolecular condensates. The features observed in our TFAM-mtDNA droplets are not consistent with those for systems of polymer collapse nor psi-condensation.

4.The authors analyze enlarged mitochondria in HGPS cells to test whether the phase-separation properties of mt-nucleoids are related to mitochondrial function. Albeit interesting, I do not see how this analysis tells us much about phase separation and its functional relevance, as the relationship between transcriptional activity, metabolic function and structure/phase separation remains unknown. Maybe the authors could address this question in a different way, e.g., by replacing TFAM with a mutant that does not phase-separate and then assessing transcriptional/metabolic activity? This would add to the study.
We appreciate the referee's point. While we find a strong relationship between mitochondrial dysfunction and phase behavior of mt-nucleoids, it has been challenging to demonstrate direct causality, mostly because we have not been able to identify any TFAM mutants, as suggested by the referee, that have a dominant negative effect on phase separation of endogenous TFAM. As a consequence, we have now de-emphasized this aspect of the study by re-organizing the relevant data in Fig. 7 (former Fig. 6) and changes to the text, including in the abstract.
It is worth pointing out that our observations highlight that normal cells maintain their nucleoids at a homogeneous size of ~100 nm in diameter under normal conditions, but fail to maintain this size under conditions that promote damage, such as phototoxic stress (Fig. 1H,I) or age-associated damage (Fig. 1,7), suggesting a link between nucleoid size and mitochondrial function. We have now made changes to the text to discuss this topic on p. 15-16 and 19 to more clearly draw the connection between lack of mt-nucleoid homeostasis and mitochondrial dysfunction.

5.The authors imply in their Discussion that the nuclear genome is organized by (liquid) phase separation, although several studies that are not cited have questioned this view. I think that the Discussion would profit from a more balanced representation of our current knowledge and the caveats.
We have now balanced the discussion and added citations on this topic on p. 21 in the Discussion.

Minor points:
1.Fig. 2C: What do we learn from the inset? Droplet length and relaxation time seem to be pretty uncorrelated.
We have removed the inset to improve clarity as suggested by the reviewer.

2.Fig. 2D: While large dextrans are excluded from droplets, small dextrans do not only access the droplets (expected partition coefficient = 1?) but are even enriched in it (partition coefficient > 1). Does this suggest that TFAM and dextrans interact? What about other probes with different chemical properties but similar size? These data could contain more information than just the characteristic length scale.
The behavior of small dextrans is most likely due to physicochemical interactions. TFAM is highly positively charged, and dextran-FITC has a negative charge, explaining why small dextran could be favorably attracted into droplets, similar to how negatively charged dNTPs also partition in our droplets (see Fig.

S3). This enrichment or exclusion behavior of dextran has been well documented in other biomolecular condensate systems [4, 5].

3.Fig. 2G: FRAP of the different droplets could be interesting to see which ones are rather liquid and which ones are rather gel-like. This could be instructive to decide if the proteins undergo liquid phase separation or gelation.

While FRAP is routinely used to probe the dynamics of exchange within droplets it is not the best way to characterize the bulk material properties because in FRAP the protein is not necessarily an inert probe and its diffusion may be length-scale dependent, especially in complex environments or phases containing multiple length and time scales. Instead, we have quantified the droplets' morphological properties using the aspect ratio as a marker of their propensity to relax into spherical shapes as is commonly done [6]. We now show these data in Fig. S2. In depth analysis of the material properties using methods such a microrheology would be rather interesting and informative but is beyond the scope of the simple biophysical assays used in this work and will be the focus of future work.

4.Fig. 3A: Droplets are observed in TFAM:mtDNA mixtures - however, not at the concentration and TFAM:mtDNA ratio deemed "physiological" in the text (10 M TFAM, 1:1000 ratio = 10 nM mtDNA). While I suspect that droplets can also be obtained for these parameters if the right buffer conditions are chosen, I think it would be good to either show this or otherwise comment in the text why you think that it will phase-separate in the cell.

5.Fig. 3C-G: Wouldn't it make sense to do these experiments at the "physiological" TFAM concentration (10 M)?

We apologize if our wording was unclear, and we have now improved the writing throughout on this matter by indicating that we are referring to "order of magnitude estimates". In the SI (page 13) we describe how we estimated dilute physiological concentrations based on information in the literature. Note that estimates of *in vivo* TFAM and DNA concentration vary in the literature and depending on cell type, so they are at best "order-of-magnitude" estimates (~1 mtDNA molecule and ~1000 TFAM molecules per nucleoid, which corresponds to molar ratios of ~0.001) [7]. We estimate a dilute concentration of ~20 nM mtDNA and ~20 uM TFAM inside a unit mitochondrion. As it is difficult to know the exact concentrations, we describe these order of magnitude estimates as ~10 nM mtDNA and ~10 uM TFAM and, which correspondingly give rise to a ~0.001 molar ratio. In our simple system, we find that droplets form over a range of starting conditions from 5-25 uM TFAM, well within the range of physiological TFAM, and we observed droplets for DNA/TFAM ratios on the low end of 4E-6 (ex: 0.1 nM mtDNA and 25 uM TFAM) and on the high end 4E-4 (ex: 10 nM mtDNA and 25 uM TFAM) , which are indeed very close to the estimated physiological range of ~1E-3 (~1 nM mtDNA and ~10 uM TFAM). Furthermore, the ratio of TFAM to mtDNA *inside the droplet* may be more reflective of physiological ratios than the ratio before mixing from our *initial, dilute condition*, but it is also experimentally challenging to precisely determine both the mtDNA and TFAM concentrations within the droplet.

Additionally, other mt-nucleoid associated proteins and the crowded environment within the mitochondria most likely also contribute to the overall phase behavior. In an attempt to better capture the crowded nature within the mitochondria, we have gone to higher DNA/protein (10 nM mtDNA and 5 uM TFAM, ratio ~0.002) ratios under crowded conditions with PEG and show droplet formation (Fig. S3D). The fact that we see such strong agreement between our *in vivo* and *in vitro* measurements strongly suggests that our *in vitro* system and observations are meaningful and physiologically relevant.

6.Fig. 5A-C: It looks like the number of nucleoids in the mitochondria changes upon TFAM overexpression. Could you quantify this so that it is easier to understand these images (multiple points in the green channel = multiple nucleoids or substructures of the same nucleoid?)?

The referee is correct that the number of nucleoids increases upon TFAM overexpression. The higher number of mtDNA foci in larger TFAM droplets is precisely what we would expect based on our model. Segmentation and quantification of the number of foci has been challenging due to the highly variable shapes inside of the TFAM structures, but we now include additional quantification of the colocalization of mtDNA with mt-nucleoid markers (Fig. S6 and see Reviewer 3's comments). Specifically, we plot the integrated intensity of anti-mtDNA as a function of mt-nucleoid cross-sectional area, and we see these are strongly positively correlated, suggesting that there is an increase of mtDNA content with increasing mt-nucleoid size, consistent with our model. We have also clarified the description of the images in the figure legend.

Reviewer #2 (Significance)

In the current state, the significance is not clear to me, as the relationship between a potential phase separation mechanism and the functional relevance has not been worked out.

Our primary goal of this study was to demonstrate the biophysical properties of mitochondrial nucleoids. This is a novel observation. As is the case for most phase separated structures, demonstration of functional relevance of the phase separation properties will be the focus of future work. We have now de-emphasized the functional implications, particularly as it relates to HGPS, throughout the text.

Reviewer #3

Overall, this is a well-written paper with excellent in vivo and in vitro data. While we find the evidence for nucleoid phase separation convincing, we have some concerns about the link between nucleoid phase separation and enlarged, dysfunctional mitochondria in HGPS cells. We suggest that the authors provide data that can bolster this link, or describe limitations to this work that will necessitate future studies that link HGPS to aberrant nucleoid phase separation. We provide detailed comments below

We would like to thank the reviewer for describing our data to be excellent and the paper to be well-written. We have now clarified and de-emphasized the link to mitochondrial dysfunction in HGPS (see below for details)

1)To supplement Fig. 1I, please provide a quantification of droplet fusion from multiple fusion events, to show how the aspect ratio relaxes. This would more strongly support the hypothesis that nucleoids are liquid-like, and motivate the in vitro experiments in the next figure.

We have now performed analysis on the fusion events of the mt-nucleoids from experiments in Fig. 1 to be included in Fig. S1Q to show that they in fact relax into spherical shapes. The average relaxation time was 20$\pm$10 s and the average size of the nucleoids was 0.8$\pm$0.1 microns (n=8 nucleoids, error = s.e.m). The inverse capillary velocity was 30$\pm$10 s/micron, which is on the same order of magnitude as what we measured for our in vitro TFAM droplets (~80 s/micron). We now mention these data on p. 7.

2)How many replicates are there for each construct in Fig. 2 E-G? How reproducible are the differences in morphology and phase behavior among constructs? Some quantification of droplet morphology, such as aspect ratio, would be helpful to show how reproducible these morphology observations were.

We describe in the methods the replicates for each experiment. We always performed at least three independent experiments for each high-throughput spinning disc confocal microscopy experiment (Fig. 2A,F, Fig. S2). Each mutant concentration (1, 5, 10, 15, 20, 25 and 50 uM in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5) was imaged with 12 fields of view and each field of view had hundreds to thousands of droplets in the two-phase region. We analyzed mean droplet size and mean droplet morphology for each field of

view, and then determined the average for each experimental replicate. We have now included aspect ratio and droplet size at a fixed concentration for each mutant based on high-throughput imaging (Fig. S2).

The images shown in Fig. 2 were taken on a laser-scanning microscope and contained representative sections of the droplets, which are well supported by our quantification. Consistently, we saw similar droplet size and aspect ratio with our mutant + mtDNA droplets shown in the new figure (Fig. 4), and these data are consistent with our *in vivo* experiments.

3)On page 10, the authors say that the delta C-tail mutant "influenced the wetting behavior of the droplets as indicated by a decreased smoothness along the droplet perimeter." The term "wetting behavior" is a little confusing - is this term referring to how droplets wet the surface of the imaging chamber, or are they referring to droplet surface tension? Please clarify. Also, what specific molecular interactions within the droplet phase do the authors believe the C-tail is regulating to control droplet morphology?
We have now modified the text on p. 9 to more clearly describe the observed effect. The wetting behavior refers to the contact line of the droplet on the glass coverslip, which is a function of the interfacial tension at the contact between the droplet, glass coverslip and aqueous buffer.

The C-tail has previously thought to be instrumental in recruiting and interacting with other proteins that are part of the transcriptional machinery and the C-tail is necessary for activation of mt-transcription [8, 9]. Our data further supports that the C-tail is primarily important for protein-protein interactions and not mtDNA interactions because our mutants which lack the C-tail have increased affinity for mtDNA. First, we find that removal of the C-tail leads to increased colocalization of the mutant with mtDNA in live cells (Fig. 6/S6). Similarly, in vitro, we find more uniform localization of mtDNA inside the droplet and higher degree of co-localization within delta-C mutant and mtDNA compared to wild-type (Fig. 4). We now discuss in more detail on p. 17/18 the molecular interactions of TFAM in phase separation, which also suggest wetting behavior of mt-nucleoids along the mitochondrial membrane.

4)In the next sentence, the authors say, "Consistent with this notion, removal of the HMGA domain (ΔHMGA), also resulted in droplet formation, but at slightly higher saturation concentrations." Please elaborate a bit on how this observation is consistent with removal of the disordered C-tail described in the previous sentence.
We apologize if the wording was unclear. We have removed the ambiguity with the phrase “consistent with this notion.”

5)The data in the lower inset in Fig. 2C look uncorrelated, but the authors fit it with a line anyway to estimate the inverse capillary velocity, which is likely not appropriate. Instead, remove the linear fit, and say that there was no clear correlation between droplet size and relaxation time, which supports the idea that droplets are highly viscoelastic and do not follow simple rheological predictions.
The droplets were within a narrow size range under our experimental conditions, so we are not able to capture the time scales for a large enough range of droplet sizes needed to fully complete the trend. We have now removed the inset to avoid confusion.

6)What is the dashed green line in Figure 3B? Is it a fitted function? If so, describe the function.
Yes, the dashed green lines in Fig. 3B correspond to single exponential fits to the data. We have placed the equations in the legend.

7)In Figure 3B, the term "gelation" in the figure panel suggests that higher DNA/protein ratios drives some

kind of aging process that drives condensate gelation. But later in 3J it becomes clear that DNA is actually just highly immobile within condensates, and forms a viscoelastic network that likely fixes droplets into non-spherical shapes. Please change the phrasing of the term "gelation" on this panel to something else which makes it clearer how DNA drives this aspect ratio increase with increasing DNA/protein.
To improve clarity, we have changed the labelling of the figure and results section to make the description of the results clearer. See also our response to referee 2, points 1 and 2, for clarification of terminology.

8)Are the black lines in Figure 3J single exponential recovery models? If so, what are the time constants and mobile fractions of protein and DNA?
Yes, the solid black lines in Fig. 3B correspond to exponential fits to the data. We have placed the fits as well as time constants and immobile fractions for TFAM in the legend. The error on the time constants and mobile fractions (based on the confidence intervals on the fit) for mtDNA was very large, as there was very little recovery under the observed time period.

9)In the explanation of the FRAP data in Figure 4D, the authors write "Based on observed recovery times of ~1 min, we estimate diffusivities of ~1x10^-3 µm^2/s." This is very qualitative, and the images seem to show that recovery is still incomplete after 3 min. What is this recovery time of 1 min based on? Please provide a quantification of these data with associated fit and description.
We have now modified this section in the main text on p. 13 and in the figure legend. We performed the analysis in Fig. 5D on n=18 nucleoids after bleaching a R≈0.2 micron spot and saw on average 70±20% recovery of area under the curve upon 6 minutes, suggesting a majority of molecules exchange within that timeframe. Now, using τ = 6 minutes, we obtain an order of magnitude estimate for the diffusion coefficient ($D \sim R^2/\tau \sim (0.2um)^2/(6\ min) \sim 1x10^{-4}\ um^2/s$), which is still within close agreement of our *in vitro* measurement.

10)The images in Fig 4E are really nice, but it is a little difficult to see droplet fusion clearly with only three frames spaced apart by 8 and 12 min. Please add more frames from this time series to show the sequential events of the fusion process more clearly.
We have now added more frames to capture the coalescence events (Fig. 5E). Also, this image is represented in Supplemental Video S10.

11)The text suggests that TFAM droplet size correlates with the level of protein over-expression. If so, please provide a quantification of droplet size as a function of expression level in Fig. 5. Further, do the images of increasingly larger droplets in Fig. 5 A-C come from cells with increasing expression level of TFAM? If so, please provide values of the respective expression level (e.g. quantified in terms of the average cytosolic protein fluorescence intensity) with each column of images.
The images of nucleoids in Fig. 6A-C (former Fig. 5) come from a single cell overexpressing TFAM (Fig. S6A). We commonly find a range of sizes even within a single cell, as mitochondria themselves span a variety of sizes and may have different levels of TFAM, making reliable quantification exceedingly difficult.

We have now compared the integrated intensity of mKate2 from the z-slice analyzed for each nucleoid as a function of cross-sectional area ($A=\pi r^2$) for TFAM, as well as all mutants, in Fig. S6. We would expect a linear trend as integrated intensity in a cross-section (number of molecules, y) scales linearly with area (x) to obtain a constant concentration, C, ($y \approx C * x$), and on a log-log scale, the slope should be 1. In fact, we do see a roughly linear trend, suggesting larger nucleoids have proportionally higher amounts of TFAM, which is consistent with our phase separation model.

12)Does expression level of the different TFAM constructs only affect droplet size, or is the localization of

TFAM with mtDNA and TFB2M also affected as a function of expression level? Please provide some control data to show whether TFAM expression level affects localization with other nucleoid components.
We did not see a dependence on the correlation coefficient as a function of mKate2 intensity. To quantify this, we took the correlation coefficient for each nucleoid and normalized it by dividing each measurement by the average for correlation coefficient measured for each mutant ("normalized correlation coefficient"). We analyzed this for each pair of interactions (TFAM-mtDNA, TFAM-TFB2M, and mtDNA-TFB2M). We saw very weak dependence (slope ~0 on a log-log plot and correlation of ~ 0) of normalized correlation coefficient on nucleoid size which we showed scales proportionally with mKate2 intensity (Fig. S6).

13)In a related question, are the data in Figure 5F from cells with relatively similar expression levels of the different TFAM mutant constructs? If not, then the control data requested above is important to show that expression level does not affect localization with other nucleoid components.
As we showed above, the amount of mKate2 intensity was proportional to the size of the nucleoid. We also show that the nucleoids we measured across conditions were of similar size (between r=0.25 – 1 um, Fig. S6).

14)The HMGB + C-tail localization to the lining of the mitochondrial membrane is very interesting. This suggests that in addition to disrupting interactions with mtDNA, the HMGB + C-tail mutant also has increased affinity for TFB2M, which is supported by the quantification in 5F. Indeed, the images in Fig. 5E suggest that TFB2M is more enriched at the membrane with the HMGB + C-tail mutant compared to Fig. 5D with full-length TFAM. Please describe in the text whether this TFAM mutant also has increased affinity for TFB2M.
Yes, this mutant has increased affinity for TFB2M as seen by the correlation coefficient between HMGB+C-tail and TFB2M. We have now made the distinction in the text clearer.

15)As a follow-up to this, do the authors have any in vitro data that include TFB2M in addition to TFAM and mtDNA? That would be a great addition to the paper to show i) whether different TFAM mutants have altered affinity for TFB2M and ii) whether TFB2M shows multiphase behavior within droplets in vitro, similar to in vivo observations.
We agree that testing the phase behavior of other nucleoid proteins including TFB2M will be interesting and is the focus of our ongoing work. However, purifying large amounts of soluble, non-aggregating TFB2M has proven challenging in our hands and will require additional biochemical work beyond the scope of this current study. We have now included additional data in Fig. 4 on our set of mutants *in vitro* in combination with mtDNA, and we find consistent behavior with our *in vivo* results.

16) As mentioned in the summary above, our main concern is that the paper is highly motivated by enlarged mitochondria and nucleoids, and associated mitochondrial dysfunction, in HGPS cells. However, this disease is caused by a mutation that causes progerin production, a protein that is not involved with mitochondria or the nucleoid. Therefore, it is still very unclear how the HGPS disease and aberrant nucleoid phase separation are linked. If possible, please provide in vivo data with some of the TFAM mutants to show whether altering nucleoid architecture with disrupted TFAM phase separation leads to mitochondrial dysfunction. Alternatively, the authors should more clearly state that the link between HGPS and phase separation is still unclear and remains a topic for future investigation.
We included the HGPS data in this study since this project originated by the observation of enlarged nucleoids in HGPS cells prompting us to explore the biophysical properties of mitochondrial nucleoids. We agree with the referee that the mechanistic link between progerin and mitochondrial dysfunction or phase behavior of nucleoids remains to be determined. As a consequence, we have de-emphasized this aspect

of the study, including by re-organizing some of the functional HGPS data in figure 7 (former Fig. 6) and changes to the abstract. Please also see our comments to referee 1, point 2.

Minor comment

1)When possible, please change channel colors from red/green to magenta/green, for colorblind friendliness.

We have taken care to now include wherever possible single channel images.

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
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- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
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- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	At least three independent experimental replicates were performed for each biological experiment. Each biological experimental had at least three technical replicates, as well as multiple fields of view for each imaging experient, as indicated in the figure legend and/or methods section.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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5. For every figure, are statistical tests justified as appropriate?	Yes, the name of the sample size and test statistic are listed in the caption and described in more detail in the methods.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We performed standard statistical analysis on data, including mean, standard deviation or standard error, and ANOVA analysis, where approproriate. We also checked for statistical outliers.
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Is the variance similar between the groups that are being statistically compared?	We used the exact standard deviation pertaining to each cell line/mutant when performing ANOVA analysis and we report the standard deviation or standard error for each cell line/mutant in all graphs.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies are described in the methods. IF: anti-DNA (EMD Millipore, clone AC-30-10), anti-TOMM20 (Sigma, HPA011562), anti-TFAM (Sigma, HPA063684), anti-TFB2M (Sigma, HPA028482), anti-SSBP1 (Sigma, HPA002866), anti-TOP1MT (Sigma, HPA001915), anti-HSPD1 (Sigma, HPA001523), anti-LONP1 (Proteintech, 154401-A), anti-CLPP (Thermo Fisher Scientific, PA5-52722), anti-HSP10/EPF (RND Systems, MAB3298), anti-mtHSP70 (Thermo Fisher Scientific, MA3-028); WB: Lamin A/C (Santa Cruz, 376248), β-actin (Sigma, A2228), TFAM (Sigma, HPA063684), HSPD1 (Sigma, HPA001523), and ATF5 (Abcam, ab60126)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Primary human dermal fibroblast cell lines were obtained from the Progeria Research Foundation (PRF) (HGMDFN090, HGFDFN168, HGADFN003, HGADFN127, HGADFN178, HGADFN167). HeLa cells were from ATCC. All cell lines were negative for mycoplasma.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA