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Stress-dependent macromolecular crowding in the mitochondrial matrix

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Koopman,

Thank you for submitting your study entitled "Viscosity and macromolecular crowding in the mitochondrial matrix: impact on protein diffusion and structure" [EMBOJ-2021-108533]. The manuscript has been assessed by three reviewers, whose reports are enclosed below.

As you will see, the referees find your study potentially interesting. However, they also raise some points that need to be addressed before they can support publication here. In particular, referee #3 expresses several methodological concerns and requests additional experiments and controls to further substantiate the main findings. Also, during cross-commenting, the referees pointed out that the absolute values for the viscosity of the matrix need to be validated in a system where the viscosity can be more directly controlled. In addition, the linear regression to calculate viscosity upon CAP treatment should be repeated using also the other two available constructs to get a more reliable/robust graph.

Given the interest in your study, I am pleased to invite submission of a manuscript revised as indicated in the reports attached herein. I should also add that it is our policy to allow only a single round of major revision. Therefore, acceptance of your manuscript will depend on the completeness of your responses in this revised version.

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content is published elsewhere.

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2) Please use scatter blots showing the individual datapoints when n=2. The use of statistical tests needs to be justified.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14602075/authorguide#dataavailability

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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11) Regarding data quantification
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Elisabetta Argenzio, PhD
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Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 20th Sep 2021:

Referee #1:

This is a highly innovative and interesting study describing one of the first attempts to quantify viscosity and molecular mobility within the mitochondrial matrix. The study is testing various ways to calculate changes in molecular mobility based on previously developed equations. The first part of the study looks at peptides of different molecular weights that were over-expressed in the cells and were fused to GFP. The study then employs chloramphenicol as a stress inducer to test the effect on these parameters.
There are a few ways this study can be improved both in the design as well as the presentation.

1. The manuscript is very lean on the explanation of the methodologies and on using illustrations to make these perceivable by biologists. The methods of each experiment should be described in detail enough so that others could imagine what exactly is done. The addition of illustrations can be very helpful.
2. Example of images used for the calculation of the different parameters highlighting the ROIs used the equation applied should be added to each experiment.
3. The limitation of each of the methods should have been studied and identified. There is only one criteria that is mentioned which are that mitochondria had to be in sufficient distance from others so that their borders can be clearly delineated. How was the expression level and the shape of the cell or mitochondria influencing each of the parameters?
4. The method should be validated against modulations of viscosity that can be calculated, such as changes in solutes in permeabilized cells and mitochondria.
5. Authors may want to consider the use of permeabilized isolated mitochondria to validate the approach.
6. GFP tends to aggregate and form dimers and oligomers. Has this been considered?
7. Chloramphenicol affects a large set of mitochondrial functions, including biogenesis and turnover, fuel preference, respiration, pH, and membrane potential. It also affects cristae density. Therefore, its use as a perturbation of cristae density is problematic. Perhaps the authors can use a more specific way to change cristae density such as the MICOS complex.

Referee #2:

The manuscript by Elianne P. Bulthuis et al addresses the interesting and timely question whether macromolecular crowding and/or viscosity plays a role in solute diffusion within the mitochondrial matrix. Applying expression of mitochondrially-targeted AcGFP1 concatemers in HeLA cells, they experimentally and computationally analyzed mobility of the fluorescence proteins and observed a decrease of mobility as a function of MW, which is typical for macromolecular crowding. Inhibition of respiration by chloramphenicol treatment resulted in increasing viscosity of the matrix, suggesting that impairment of mitochondrial function alters matrix viscosity and the degree of macromolecular crowding, which apparently can also affect molecular protein structure.

This is a very well written manuscript, the experiments and analysis are performed at high standards and are convincing. Overall, the conclusions are well supported by the data and report a previously unexplored aspect of mitochondrial biology, which likely has profound functional consequences. There are only a few minor points that should be addressed to improve the manuscript.

• Please highlight in the text the advantage of AcGFP1 compared to other GFP forms in respect to its absence of dimerization.
• In Figure 2 A, a combination of green and red lookup tables was used, which is unfavorable for colorblind people. The authors should consider using e.g., green and magenta to avoid this problem.
• In Figure 2 B, the higher molecular weight band of AcGFP14 seems to be present at a lower molecular weight (approx. 75 kDa) in the fluorescence analysis compared to the chemiluminescence detection with the anti-GFP antibody (approx. 100 kDa). This seems to be a result of incorrect alignment in Fig. 2 B when comparing the data with uncropped blots provided in the supplementary figure. Please adjust the alignment in Fig. 2 B accordingly.
• Representative micrographs for the CLSM and EM-based analysis of mitochondrial dimensions that were used for computation of Dsolvent should be provided in a supplementary figure.
• The authors should include a discussion of the dynamic ultrastructural changes in mitochondria and particularly the compaction of the matrix as observed previously, for example in classical work by C R Hackenbrock 1966 (PMID: 5968972). Discussing how structural changes could impact protein concentrations, viscosity and protein stability could add yet another twist to the manuscript.

Referee #3:

The manuscript by Bulthuis et al. performs measurements of mitochondrial matrix viscosity based on FRAP and FLIP using fluorescent proteins of increasing molecular size targeted to mitochondria. This is a very similar study to that published by the same group some years ago (Dieteren et al., 2011a) but using a different cell type (HeLa cells instead of HEK) and more proteins (concatemers of 1 to 4 GFP molecules, instead of only 1 and 2). This manuscript also contains the novelty of performing the measurements in cells treated with chloramphenicol (CAP) to inhibit mitochondrial translation and induce mitochondrial respiratory deficiency.
The findings are potentially interesting for the field. However, the study, in its current form, raises major methodological concerns. Also, the results shown do not support the major conclusion, which is that changes in matrix viscosity would affect enzymatic functions that take place inside mitochondria.

1. The results clearly show that the matrix viscosity increases with CAP treatment. However, the matrix viscosity measurements in this study in basal conditions, between 2.68 and 3.32 cP, are slightly higher but still in the same order of magnitude that those measured in Dieteren et al., 2011a, using exactly the same methodology. However, they are about 10- to 20-fold lower than other measurements performed by three different independent methods and research groups. The authors try to justify this discrepancy in page 13, but they claim that it is possibly due to BODIPY binding to mitochondrial membranes or because of different experimental settings. However, not all of these methods were based on BODIPY probes and they all seemed to have calculated more similar values to each other, which are significantly higher than in this manuscript or this group's previous work. This is a key point of this study, and it would be convenient to try to clarify this or reconcile these findings by using alternative measurement methods and/or mathematical models.

2. As pointed out by the authors, the expression levels of the different GFP concatemers vary greatly, but it is not exactly a function of their MW as AcAGFP3 levels are lower than AcGFP4 (Figure 2B). When looking at Figure 2A, the impression is that fewer cells actually express the AcGFP3 rather than the expression per cell being lower. And this is also indicated by Figure 2C when comparing the flow cytometry plot of AcGFP3 vs AcGFP4.

3. The appearance of the degradation product in the AcGFP4 is highly concerning because, as shown in the paper, it definitely affects the measurements. The authors were able to come up with a corrected Tmono coefficient (Figures 3A) by adjusting the data from the rest of the concatemers to a linear fit. However, in the CAP treatment experiments, they only use AcGFP1 and AcGFP4, for which they calculate the corrected coefficient with a linear regression using two points (the axis origins and the data for AcGF1) (Figure S2E), which one could say that it is not exactly accurate.

4. In line with the previous point, all the measurements in the CAP treated cells were done using AcGFP1 and AcGFP4. As mentioned, the measurements with AcGFP4 are not exactly reliable because of the coexistence of a lower MW species. In addition, many conclusions are based on linear regressions using two points (Figure 5C and 6A and B).

5. The fact that the GFP concatemers are in linear conformation in normal cells and in compact form in the CAP treated cells is only based on mathematical predictions and there are no actual measurements. Again, the fact that they theoretically diverge from the linear fit in Figure 5C (right panel) is based on a line that passes through two points, when one of them is not exactly reliable.

6. The conclusion of points 4 and 5 is that the authors should consider performing the measurements in CAP treated cells using AcGFP3 and AcGFP4 in order to obtain more reliable results and more robust conclusions.

7. It is logical to think that the consequence of CAP treatment is the increase of protein content in the matrix due to the nuclear-encoded components of the OXPHOS system not being able to assemble into the membrane when the mtDNA-encoded subunits are missing. However, this was not shown experimentally in a direct way. The changes observed in matrix 'darkness' by EM show great variability (Figure 4C) and do not seem to be of a magnitude that would explain the 20-fold increases in viscosity. Also, this parameter is already different, according to the statistical significance, between AcGFP1 and AcGFP4, both CAP treated and untreated (why?). The paper cited to explain this possible phenomenon (Bisht et al. 2016) is speculative and describes a completely different phenomenon. The authors show in Figures S6 and S7 that there is no UPRmt, so this would indicate that there are no unfolded proteins inside the mitochondrial matrix. Some of the proteins they tested for in these figures are actually mitochondrial matrix proteins and they did not show any increase. Also, one has to take into account that a decrease in OXPHOS activity and of membrane potential also affects mitochondrial protein import, so this could decrease the amount of protein that are reaching the mitochondria. Therefore, the authors should demonstrate that the mitochondrial protein content actually increases with the CAP treatment by performing cellular and submitochondrial fractionation and testing for OXPHOS related nuclear-encoded proteins.

8. The conclusion that macromolecular crowding increments the viscosity in the matrix and that this affects mitochondria bioreactions has not been substantiated in this study. CAP treatment logically decreases OXPHOS function, as shown in Figure S6A, B and C, because complexes I, III, IV and V cannot be formed. However, one could think of measuring the activity of TCA cycle or beta oxidation enzymes, for example, to determine whether these potential physicochemical changes in the mitochondrial matrix really affect the processes located in there.

REBUTTAL TO THE COMMENTS OF REFEREE 1

We thank the referee for her/his positive and constructive comments. We have performed additional experiments (FRAP) with AcGFP1, AcGFP1[2], AcGFP1[3] and AcGFP1[4] for CAP-treated cells and created a new computer model for data analysis from scratch to better reflect the cristae structure in HeLa cells (the model in the original version of the manuscript was developed for HEK293 cells). The latter has not impacted on the message of the manuscript. Moreover, we performed TMRM experiments in the context of the orthodox-condensed transition in CAP-treated cells (comment of referee 2). Textually, we added a substantial number of new references and have rewritten large parts of the manuscript (and changed figures: main, expanded view and appendix) to enhance clarity. All changes are highlighted (yellow) in the revised manuscript.

Comment 1: The manuscript is very lean on the explanation of the methodologies and on using illustrations to make these perceivable by biologists. The methods of each experiment should be described in detail enough so that others could imagine what exactly is done. The addition of illustrations can be very helpful.

Reply 1: We have extensively rewritten the main text of the manuscript, as well as the Appendix, to address this point. We now included/updated several explanatory cartoons to better explain our reasoning and experimental approach as follows: (**1**) Fig. 1 explains the overall strategy of the experimental approach with direct references to key figures (marked red), (**2**) Fig. 3C explains how we use the computer model to determine the diffusion constant from the experimental FRAP data, (**3**) Fig. 6A visualizes how our data can be interpreted in the context of macromolecular crowding, (**4**) Fig. 6B illustrates how mitochondrial macromolecular crowding fits in the broader concept of mitochondrial morphofunction and mitochondrial and cellular physiology, (**5**) Fig. EV1 explains the concept of "compact" and "extended" structure for the four fluorescent proteins used in this study, (**6**) Appendix Fig. S2A shows how the FRAP and FLIP regions were selected, (**7**) Appendix Fig. S2F shows how mitochondrial dimensions were calculated (for EM this is explained in the Appendix-Materials and Methods), and (**8**) Appendix Fig. S3A shows the structure and properties of the computer model. With respect to the latter, we developed a completely new computer model (Brownian Dynamics; BD) to be able to compute the solvent-dependent diffusion constants for the fluorescent proteins ($D_{solvent}$) in Hela mitochondria. This new BD model essentially gave the same results as the model used in the originally submitted manuscript (developed for HEK293 cells), but better reflected the cristae geometry in HeLa cells. This is explained in detail throughout the main text and in the Appendix (yellow).

Comment 2: Example of images used for the calculation of the different parameters highlighting the ROIs used the equation applied should be added to each experiment.

Reply 2: We used ROIs for analysis of the FRAP signal (these ROIs are shown in Fig. 3A and Appendix Fig. S2A), EM intensity (ROIs are shown in lower right panel of Fig. 5A) and TMRM intensity (new experimental data; ROIs depicted in Fig. EV2D (left panel)). To quantify mitochondrial diameter and length we performed a line-profile analysis (illustrated in Appendix Fig. S2F and also explained in the Appendix-Materials and Methods).

Comment 3: The limitation of each of the methods should have been studied and identified. There is only one criteria that is mentioned which are that mitochondria had to be in sufficient distance from others so that their borders can be clearly delineated. How was the expression level and the shape of the cell or mitochondria influencing each of the parameters?

Reply 3: Intense illumination of AcGFP1 is not phototoxic (info now added to the Appendix). Confocal microscopy was used for the FRAP experiments and mitochondria have a diameter < 1 µm in our studies (Table 1) No biasing selection was made based upon the initial fluorescence intensity of the mitochondria (signals were not saturated). As primary quality control criteria (Results section) we state that: "As a quality control measure this analysis included only individual mitochondria: (**i**) that were fully located within the focal plane (confirmed by an

axial scan) and, (**ii**) in which FRAP was paralleled by fluorescence loss in photobleaching (FLIP) in a part distal to the FRAP region (Appendix Fig. S2), indicating that these mitochondria possessed a continuous matrix." To reduce noise, multiple FRAP experiments were averaged (Fig. 3A). This yielded similar errors on the average T_{mono} values within the -CAP and + CAP conditions (Table 1). By rewriting large parts of the manuscript, we also aimed to better indicate the potentially weak points of the used strategies. For instance, the occurrence of a breakdown product in AcGFP1[4]-expressing cells (*e.g.* Fig. 2B) and the inability to use the mathematical model for $D_{solvent}$ quantification in CAP-treated AcGFP1[2]-, AcGFP1[3]- and AcGFP1[3]-expressing cells (Results section: "Chloramphenicol decreases the number of cristae, increases matrix electron density and partially immobilizes matrix-targeted FPs")

Comment 4: The method should be validated against modulations of viscosity that can be calculated, such as changes in solutes in permeabilized cells and mitochondria. Authors may want to consider the use of permeabilized isolated mitochondria to validate the approach.

Reply 4: We fully understand this comments and discussed with several of our expert colleagues in the field on how to technically perform such experiments. However, neither we nor our colleagues were able to come up with a reliable and easy to interpret experimental strategy to address this comment. The main issue is that information is required on the impact of the permeabilization procedure on the macromolecular crowding level and viscosity of the matrix fluid. Ideally, one would like to isolate the mitochondrial matrix "fluid" (solvent) and measure diffusion of solutes in this fluid by classical means. However, doing such an analysis would require huge amounts of mitochondrial matrix fluid, which is practically unfeasible. In addition, as far as we know, there is no quantitative strategy that allows a 100% pure isolation and/or proper normalization of the amount of isolated matrix fluid. Given the relevance of this comment, we contacted Dr. Michael J. Saxton (University of California, Davis, USA), which is an expert on diffusion tracer analysis, and included experimental data from his paper (Saxton, J. Phys. Chem. B., 2014) in the revised manuscript. This allowed us to compare our mitochondrial diffusion data with the behavior of a large set of biomolecules (with different structural conformations) in aqueous solution. Additional inclusion of further experimental studies, allowed comparison of our mitochondrial diffusion data with EYFP diffusion in the cytosol and nucleus of HeLa cells. This approach is now described in detail in the Results section under "The mitochondrial matrix solvent reduces FP mobility in a manner compatible with macromolecular crowding" and in Fig. 4A-B. Using this strategy also delivered results compatible with other experimental studies (now cited in the revised manuscript). Taken together, we feel that this strategy is a good compromise between the experimentally possible and theoretical analysis. We hope that the reviewer can agree on this.

Comment 5: GFP tends to aggregate and form dimers and oligomers. Has this been considered?

Reply 5: We apologize for this omission. AcGFP1 is monomeric and to the best of our knowledge has no known binding partners. This is now stated in the Materials and Methods and Appendix.

Comment 6: Chloramphenicol affects a large set of mitochondrial functions, including biogenesis and turnover, fuel preference, respiration, pH, and membrane potential. It also affects cristae density. Therefore, its use as a perturbation of cristae density is problematic. Perhaps the authors can use a more specific way to change cristae density such as the MICOS complex.

Reply 6: This is a good point. However, the primary aim of our study was to investigate whether the mitochondrial matrix fluid exhibits macromolecular crowding. This is now more clearly stated throughout the manuscript (also the manuscript title was changed). Related to our primary aim, we provide evidence that macromolecular crowding occurs in the absence of CAP (Fig. 4AB). Based upon our previous work, we applied CAP treatment as a means to increase the amount of protein (and thereby the macromolecular crowding level) in the mitochondrial matrix. It is difficult to predict whether CAP treatment would impact on matrix fluorescent protein diffusion by the abovementioned effects. For the sake of diffusion analysis, we demonstrated functional

mitochondrial impairment (Expanded view Figure EV2A-B-C-D), but no effect on mitochondrial dimensions or volume (Table 1). We also could not demonstrate effects of CAP on UPR^{mt}-markers, mtDNA-levels and mitochondrial fission/fusion protein levels (Expanded view Figure EV2E-F-G). In this sense, our results support the conclusion that macromolecular crowding is increased in CAP-treated cells. This is now more clearly stated and better explained in the revised manuscript. In the revised version of the manuscript we also now included new experiments for CAP-treated cells with $AcGFP^1$, $AcGFP^2$ and $AcGFP1^3$ and $AcGFP1^4$ (Table 1 and Fig. 5D-E-F). The experimental FRAP data for the CAP-treated cells displayed a slower recovery and an increasing immobile fraction as a function of MW of the proteins (Fig. 5D). Although the new BD model was unable to quantitatively reproduce this experimental CAP data (*i.e.* the simulated FRAP curves displayed no immobile fraction; Appendix Fig. S3), the enhanced immobile fraction supports the conclusion that macromolecular crowding is increased. Regarding the MICOS complex, this is an interesting idea. However, it would require transient transfection to target MICOS-complex proteins. Although this is technically feasible, one would need information on whether mitochondrial internal structure is changed (*i.e.* MICOS-targeting transfection was efficient) at the level of single mitochondria. This means that for each mitochondrion for which a FRAP curve is obtained, it is necessary to have information on its internal structure (by EM). The latter is essential for generating adequate computer model-mediated prediction of $D_{solvent}$. We feel that, although highly interesting, such an approach falls outside the scope of the current study.

Radboudumc
university medical center

REBUTTAL TO THE COMMENTS OF REFEREE 2

We thank the referee for her/his positive and constructive comments. We have performed additional experiments (FRAP) with AcGFP1, AcGFP1[2], AcGFP1[3] and AcGFP1[4] for CAP-treated cells and created a new computer model for data analysis from scratch to better reflect the cristae structure in HeLa cells (the model in the original version of the manuscript was developed for HEK293 cells). The latter has not impacted on the message of the manuscript. Moreover, we performed TMRM experiments in the context of the orthodox-condensed transition in CAP-treated cells (comment by this referee). Textually, we added a substantial number of new references and have rewritten large parts of the manuscript (and changed figures: main, expanded view and appendix) to enhance clarity. All changes are highlighted (yellow) in the revised manuscript.

Comment 1: Please highlight in the text the advantage of AcGFP1 compared to other GFP forms in respect to its absence of dimerization.

Reply 1: We apologize for this omission. AcGFP1 is monomeric and to the best of our knowledge has no known binding partners. This is now stated in the Materials and Methods and Appendix.

Comment 2: In Figure 2A, a combination of green and red lookup tables was used, which is unfavorable for colorblind people. The authors should consider using e.g., green and magenta to avoid this problem.

Reply 2: This error was corrected (Fig. 2A).

Comment 3: In Figure 2 B, the higher molecular weight band of AcGFP14 seems to be present at a lower molecular weight (approx. 75 kDa) in the fluorescence analysis compared to the chemiluminescence detection with the anti-GFP antibody (approx. 100 kDa). This seems to be a result of incorrect alignment in Fig. 2 B when comparing the data with uncropped blots provided in the supplementary figure. Please adjust the alignment in Fig. 2 B accordingly.

Reply 3: This is probably due to the gel-front not running perfectly horizontal (see Appendix Fig. S1). In this context, we now better discuss the apparent MW of the fluorescent proteins in the Discussion (first paragraph)

Comment 4: Representative micrographs for the CLSM and EM-based analysis of mitochondrial dimensions that were used for computation of Dsolvent should be provided in a supplementary figure.

Reply 4: This information is now included in Appendix Fig. S2A (CLSM) and in the Appendix-Materials and Methods (section: "Electron Microscopy").

Comment 5: The authors should include a discussion of the dynamic ultrastructural changes in mitochondria and particularly the compaction of the matrix as observed previously, for example in classical work by CR Hackenbrock 1966 (PMID: 5968972). Discussing how structural changes could impact protein concentrations, viscosity and protein stability could add yet another twist to the manuscript.

Reply 5: We now included this topic in the Discussion (section: "CAP treatment does not induce mitochondrial orthodox-to-condensed transition" and in this context also performed additional TMRM experiments (Expanded Fig. EV2).

Radboudumc
university medical center

REBUTTAL TO THE COMMENTS OF REFEREE 3

We thank the referee for her/his positive and constructive comments. We have performed additional experiments (FRAP) with AcGFP1, AcGFP1^2, AcGFP1^3 and AcGFP1^4 for CAP-treated cells and created a new computer model for data analysis from scratch to better reflect the cristae structure in HeLa cells (the model in the original version of the manuscript was developed for HEK293 cells). The latter has not impacted on the message of the manuscript. Moreover, we performed TMRM experiments in the context of the orthodox-condensed transition in CAP-treated cells (comment of referee 2). Textually, we added a substantial number of new references and have rewritten large parts of the manuscript (and changed figures: main, expanded view and appendix) to enhance clarity. All changes are highlighted (yellow) in the revised manuscript.

Comment 1: The results clearly show that the matrix viscosity increases with CAP treatment. However, the matrix viscosity measurements in this study in basal conditions, between 2.68 and 3.32 cP, are slightly higher but still in the same order of magnitude that those measured in Dieteren et al., 2011a, using exactly the same methodology. However, they are about 10- to 20-fold lower than other measurements performed by three different independent methods and research groups. The authors try to justify this discrepancy in page 13, but they claim that it is possibly due to BODIPY binding to mitochondrial membranes or because of different experimental settings. However, not all of these methods were based on BODIPY probes and they all seemed to have calculated more similar values to each other, which are significantly higher than in this manuscript or this group's previous work. This is a key point of this study, and it would be convenient to try to clarify this or reconcile these findings by using alternative measurement methods and/or mathematical models.

Reply 1: This is a very difficult issue to resolve. To the best of our knowledge, there are not many studies presenting quantitative information regarding mitochondrial matrix solvent viscosity (*i.e.* cP values). We further observed that the published papers (including recent reviews) do not discuss the discrepancy between the novel (rotor-based) studies and the earlier studies of ourselves and the Verkman group (using FRAP and fluorescence correlation spectroscopy experiments). To address this comment, we have totally rewritten and extended this section in the Discussion (section: "Viscosity of the mitochondrial matrix solvent in the absence of CAP"). In response to Comment 4 of Referee 1, we also investigated the possibilities for alternative measurements of mitochondrial matrix fluid (see our response there). With respect to the latter, we feel that the strategy presented is a good compromise between experimental and theoretical analysis (using our new, more HeLa-tailored, FRAP model) and hope that the reviewer can agree on this.

Comment 2: As pointed out by the authors, the expression levels of the different GFP concatemers vary greatly, but it is not exactly a function of their MW as AcAGFP3 levels are lower than AcGFP4 (Figure 2B). When looking at Figure 2A, the impression is that fewer cells actually express the AcGFP3 rather than the expression per cell being lower. And this is also indicated by Figure 2C when comparing the flow cytometry plot of AcGFP3 vs AcGFP4.

Reply 2: We fully agree with this remark and now have adapted the relevant part in the Results (section: "Expression, localization and integrity of mitochondrial matrix-targeted FPs"). Given the logarithmic scale in Fig. 2C (flow cytometry), the fluorescence of most cells (red, green, blue, purple) is above the lowest value (black dots in Fig. 2C: "parental"). There is also low expression of the concatemers in the absence of DOX (black dots in Fig. 2C for AcGFP1, AcGFP1^2, AcGFP1^3 and AcGFP1^4). It is always challenging to directly link microscopy images of individual cells (Fig. 2A) to data from large cell populations (Fig. 2C). Therefore we now more carefully state in the Results that: "Fluorescence analysis by flowcytometry (Fig. 2C) correlated well with in-gel fluorescence signals (Fig. 2D). This demonstrates that all inducible FP cell lines exhibit a low fluorescence signal in their non-induced state, and strongly suggests that the cellular fluorescence intensities reflect mitochondria-specific signals. The former observation is compatible with low-level induction ("leakage") of FP expression in the mitochondrial matrix occurring in the absence of added DOX, as observed previously in our DOX-inducible HEK293 cell models (Dieteren et al., 2011b)".

Comment 3: The appearance of the degradation product in the AcGFP4 is highly concerning because, as shown in the paper, it definitely affects the measurements. The authors were able to come up with a corrected Tmono coefficient (Figures 3A) by adjusting the data from the rest of the concatemers to a linear fit. However, in the CAP treatment experiments, they only use AcGFP1 and AcGFP4, for which they calculate the corrected coefficient with a linear regression using two points (the axis origins and the data for AcGF1) (Figure S2E), which one could say that it is not exactly accurate. In line with the previous point, all the measurements in the CAP treated cells were done using AcGFP1 and AcGFP4. As mentioned, the measurements with AcGFP4 are not exactly reliable because of the coexistence of a lower MW species. In addition, many conclusions are based on linear regressions using two points (Figure 5C and 6A and B). The fact that the GFP concatemers are in linear conformation in normal cells and in compact form in the CAP treated cells is only based on mathematical predictions and there are no actual measurements. Again, the fact that they theoretically diverge from the linear fit in Figure 5C (right panel) is based on a line that passes through two points, when one of them is not exactly reliable. The conclusion ….. is that the authors should consider performing the measurements in CAP treated cells using AcGFP3 and AcGFP4 in order to obtain more reliable results and more robust conclusions.

Reply 3: We fully agree with this comment and now included new experimental data for AcGFP1, AcGFP1^2, AcGFP1^3 and AcGFP1^4 in CAP-treated cells in the revised manuscript (Table 1 and Fig. 5D-E-F). Although the new (HeLa-specific) mathematical FRAP model was unable to quantitatively reproduce this experimental CAP data (*i.e.* the simulated FRAP curves displayed no immobile fraction; Appendix Fig. S3), the enhanced immobile fraction supports the conclusion that macromolecular crowding is increased in CAP-treated cells. Regarding the conformation of the fluorescent concatemers, we now more robustly investigated this in the absence of CAP (Fig. 4AB and Results section: "Quantifying the viscosity of the mitochondrial matrix solvent and predicting FP structural conformation"). In the presence of CAP, the viscosity was determined using the FRAP time constant of AcGFP1, which was still compatible with the mathematical FRAP model. This is also explained in the section: "Quantifying the viscosity of the mitochondrial matrix solvent and predicting FP structural conformation".

Comment 4: It is logical to think that the consequence of CAP treatment is the increase of protein content in the matrix due to the nuclear-encoded components of the OXPHOS system not being able to assemble into the membrane when the mtDNA-encoded subunits are missing. However, this was not shown experimentally in a direct way. The changes observed in matrix 'darkness' by EM show great variability (Figure 4C) and do not seem to be of a magnitude that would explain the 20-fold increases in viscosity. Also, this parameter is already different, according to the statistical significance, between AcGFP1 and AcGFP4, both CAP treated and untreated (why?). The paper cited to explain this possible phenomenon (Bisht et al. 2016) is speculative and describes a completely different phenomenon. The authors show in Figures S6 and S7 that there is no UPRmt, so this would indicate that there are no unfolded proteins inside the mitochondrial matrix. Some of the proteins they tested for in these figures are actually mitochondrial matrix proteins and they did not show any increase. Also, one has to take into account that a decrease in OXPHOS activity and of membrane potential also affects mitochondrial protein import, so this could decrease the amount of protein that are reaching the mitochondria. Therefore, the authors should demonstrate that the mitochondrial protein content actually increases with the CAP treatment by performing cellular and submitochondrial fractionation and testing for OXPHOS related nuclear-encoded proteins.

Reply 5: The primary aim of our study was to investigate whether the mitochondrial matrix fluid exhibits macromolecular crowding. This is now more clearly stated throughout the manuscript (also the manuscript title was changed). We provide evidence that macromolecular crowding occurs in the absence of CAP (Fig. 4AB; Results section: "The mitochondrial matrix solvent reduces FP mobility in a manner compatible with macromolecular crowding"). The darker matrix of AcGFP1 relative to AcGFP1^4 in the absence and presence of CAP (Fig. 5C), is compatible with the expression levels of AcGFP1 (higher) and AcGFP1^4 (lower) shown in Fig. 2B-C (absence of CAP) and Appendix Fig. 1A-B (absence and presence of CAP). To address Comment 4 (*i.e.* regarding macromolecular crowding in CAP-treated cells, EM data and UPRmt) we now have rewritten and more carefully phrased the appropriate sections in the Results ("Chloramphenicol decreases the number of cristae, increases matrix electron density and partially immobilizes matrix-targeted FPs") and Discussion ("CAP treatment

slows solute diffusion and increases mitochondrial matrix viscosity"). We now also performed TMRM measurements for semi-quantitative analysis of mitochondrial deltaPSI and demonstrate a potential hyperpolarization in CAP-treated cells (Expanded view Fig. EV2D). Regarding experimental strategies to demonstrate the "increase in protein content in a more direct way" please see our reply to Comment 4 of Referee 1.

Comment 7: The conclusion that macromolecular crowding increments the viscosity in the matrix and that this affects mitochondria bioreactions has not been substantiated in this study. CAP treatment logically decreases OXPHOS function, as shown in Figure S6A, B and C, because complexes I, III, IV and V cannot be formed. However, one could think of measuring the activity of TCA cycle or beta oxidation enzymes, for example, to determine whether these potential physicochemical changes in the mitochondrial matrix really affect the processes located in there.

Reply 7: Indeed we did not directly demonstrate that macromolecular crowding and viscosity in the mitochondrial matrix alter mitochondrial reactions. However, the aim of the study was to investigate whether macromolecular crowding was occurring in the mitochondrial matrix fluid. In our view, we have provided evidence that this crowding occurs (albeit at low levels) in the absence of CAP, and (likely) in the presence of CAP. We agree with the Referee that understanding the effect of this crowding on the activity of mitochondrial enzymes is highly relevant. However, this would require an experimentally-accessible in-situ readout of this activity at the level of individual mitochondrial within living cells. If at all possible, this would require a complete new experimental strategy/study, which we feel falls outside the scope of the current study. To reflect this comment, we have rewritten and more carefully phrased the last section of the Discussion ("Summary and conclusion").

Dear Dr Koopman,

Thank you for submitting your revised manuscript (EMBOJ-2021-108553R) to The EMBO Journal. Your amended study was sent back to the three referees for their re-evaluation, and we have received comments from two of them, which I enclose below. Please note that while referee #3 was at this time not able to reassess the work, we have considered your response to this expert's critique carefully in the editorial team and found all issues to be satisfactorily addressed. As you will see, the other reviewers stated that the work has been substantially improved by the revisions and that they are now broadly in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider referee #1's additional comments regarding the abstract wording by adjusting it to a broader audience.

We also now need you to take care of a number of minor issues related to formatting and data annotation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

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Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
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Formatting changes required for the revised version of the manuscript:

>> Please change the Reference List format from numbered to alphabetical only.

>> Change the title of the current 'Conflict of Interest' statement to 'Disclosure and Competing Interests Statement'.

>>Introduce a separate 'Statistical Analysis' section in the Material and Methods part, detailing the algorithms applied.

>> Author Contributions: Please remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution.
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>> Please move the 'Dataset availability section' to the end of the Material & Methods part.

>> Callouts: recheck and adjust order of callouts for figures 5A and 4 in the main text.

>> Appendix file: please amend the ToC on the first page with page numbers.

>>As to our journal policies, we kindly ask you to provide uncropped, unassembled source data files for the western blots shown

in Appendix Figures S4 and S5.

>> Consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (26th Mar 2023).

Referee #1:

The revised manuscript represents a significant improvement over the original version. I only have minor points of major importance to make this future paper read by the EMBO readership. The authors need to have a biologist outside their field review the abstract and approve that it is clear and interesting. As it is now, the abstract is only clear after one reads the paper, which is not what the abstract intended. Currently, the paper is fascinating, but the abstract reads as if it was copied from the methods section and is not reflecting the excitement of the findings in the paper.

Assume that the readers have never heard about Macromolecular crowding. Most scientists did not, and all of the mitochondria scientists did not. I suggest you explain in a couple of words in the opening of the abstract what it is and why this is important.

You can remove the first sentence of the abstract as it is not informative to EMBO readers.

Referee #2:

The authors have addressed all the points we raised on the initial version in a satisfactory manner. I think this is a very interesting study that shed new lights onto previously overlooked aspects of mitochondrial biology.

The authors addressed the minor editorial issues.

2nd Revision - Editorial Decision **19th Jan 2023**

Dear Dr Koopman,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Please consider us again in the future for your most exciting work.

Best regards,

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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Results, Appendix (Quality control: regarding the selection of mitochondria to be included or excluded from the FRAP analysis)
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