

Mitochondria-derived nuclear ATP surge protects against confinement-induced proliferation defects

Corresponding Author: Dr Sara Sdelci

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript Ghose et al. has greatly improved after its first submission to the Nature Cell Biology Journal. A wealth of new experimental data that include necessary controls, and detailed point-to-point answers greatly strengthen the new interesting concept of a metabolic response after mechanical confinement to secure cell fitness. After agreeing to most of the authors responses, this reviewer (#1) has only a number of minor points left.

1. In your response to this reviewer you state: '..., the lack of a nuclear ATP surge during mechanical confinement significantly reduced the number of nucleoli'. However, nucleoli were not cleanly detected (Fig. 5D). This becomes especially apparent in the oligomycin A-treated conditions, where it seems that more regions, in addition to the annotated regions, are free of chromatin. Was there a thresholding approach being used? Connected to this point, the concern of previous reviewer #4 (point 2d) is not fully answered. A nucleolus marker will clearly help to generate a clean analysis.
2. In your response to this reviewer you state: 'Our analysis was further supported by Western blot, revealing a moderate increase in H3K9me3 and a significant increase in H3K27me3 in conditions of confinement in the absence of nuclear ATP compared to control confined conditions (Figure 5 A), ...'. To draw such conclusions (Fig. 5A; but also S7D), western blot data need to be generated from 3 independent experiments and quantified.
3. The solid white line in Fig. 2E 'conf' does not seem to depict the line profiles quantified in panel F (the strong mitotracker-positive peripheral signal as shown in F does not seem to reflect the weak perinuclear signal as shown in E). Please double-check. – Also, to make your analysis approach as shown in Fig. 2 E - H more coherent, I suggest to measure in panels E and F only from the center of the nucleus up to the 10% enlarged ROI as shown in panel G.
4. In the new Fig. S4B, I suggest to turn around the left and right panels as this will make the measurement approach more intuitive, namely that you have measured ER movement across and beyond the cell nucleus. In addition, I suggest to draw an outline around the nucleus in the suspension cell (Fig. S4B; top right image).
5. Even though I understand your argumentation on the data depiction in Fig. 2H panel including the graph that you presented in your response letter, it would be a much cleaner data set if you presented the NAM mean pixel intensity signal as a percentage of all mitochondria in a cell throughout the manuscript. This was also an issue for reviewer #4 (his/ her point 5). I suggest that, if reviewer #4 keeps raising this issue you adapt your NAM graphs. If not, I will not insist on this issue further.
6. In your response to this reviewer you state: 'We have now addressed this concern by replacing all single channel pseudo-colour images with grey images wherever nuclear staining was shown, which we agree have greatly improved the visualization.' However, I only see this change in Fig. 2. I suggest you apply these changes for every single channel everywhere (thus, i.e. also Figs. 3B, I; 5D; also in the supplement). Instead of 'merge' (i.e. Fig. 2A), the terms Hoechst (font in blue) and Mitotracker (font in magenta) might be indicated. Another advice is to generally use cyan or yellow instead of blue for merged images, to increase visibility.
7. In your response letter you further state: 'Second, we have added data related to perinuclear actin ...'. It is not clear whether this actin analysis equals the analysis location for NAMs as indicated in Fig. 2G? Please specify.

Reviewer #3

(Remarks to the Author)

I am satisfied.

Reviewer #4

(Remarks to the Author)

The authors did a good job at answering my concerns. There are only a few clarifications that I think should be made to the text.

1. I appreciated the way the authors describe the relationship between the ER, mitochondria and actin in their response to my previous comments and I agree with their view. However, the way the ER data is described in the result section does not quite reflect this. The authors should thus modify the way this section is described.

2. The authors really expanded their ATP analysis and made it much clearer. However, it is still not always clear which experiments are done in the presence or the absence of glucose. This should minimally be indicated in the figure legends to make it clear for the readers.

3. I have an issue with Figure 4B and S5J. They seem to contain the same control data. I imagine that the experiments presented in these two graphs were done together, so they should be presented together rather than duplicating the same data.

4. The statement "MFN1 and FIS1 are integral membrane proteins that participate in the recruitment of DRP1, and the tethering of ER and mitochondria, thereby directly contributing to MERCs formation" (lines 246-248) is somewhat misleading as the role of Fis1 in fission is still controversial and it is MFN2, not MFN1 that has been shown to participate in MERCS.

Reviewer #5

(Remarks to the Author)

This study investigates the response of the cells when a sudden large compressive strain is applied to cancer cell line cells and finds that mitochondria accumulate outside the nuclear periphery. The authors "propose a mechanism by which actin dynamics, the ER and the physical shape of mitochondria contribute to NAM formation, driving a mechano-metabolic response of cells via an increase in nuclear ATP levels under mechanical confinement stress." (quote from the authors' responses to reviewers).

1. The authors did not differentiate the fundamental differences between two totally different processes: a) active invasion and squeezing through narrow ECM pathways by cancer cells or normal cells during invasion in vivo that require endogenous contractile forces and the resultant compressive strains in some directions and tensile strains in other directions that are gradual and time-dependent; b) the applied sudden constant large compressive strain in the current study. The physiological and/or pathological relevance of the current methodology is questionable.

2. The huge sudden compressive strain (a step function) on the nucleus is estimated to be ~80% (Fig 2C,D). It is not clear this would ever happen in vivo. In cell culture, this would certainly cause cell and nuclear damage and DNA damage. Published reports already show that normal cells at this huge nuclear strain can be turned into cancerous cells. Therefore this protocol may not apply to normal cell invasion during development. The authors used only cancer cells in their study and found blebb formation after the mechanical perturbation in these cells. Whether this sudden constant compressive strain triggers extensive apoptosis or other forms of cell deaths is not clear.

3. The authors focused on the F-actin role in the accumulation of mitochondria around the nuclear periphery. However, if the mitochondria were actively translocated by the cell, as the authors suggested in their responses to the reviewers, then microtubules and dynein motors must play critical roles in this translocation. However, no experimental evidence or live cell imaging is shown to support the active dynamic translocation of the mitochondria before, during, and after the compressive strain.

4. If the authors' hypothesis were correct, one would assume that the DNA damage in the nucleus and the energy required to repair the DNA damage for ATP would generate a sink to attract the mitochondria to the nucleus. This might explain why the mitochondria accumulated around the nucleus but not other organelles or cytoplasmic structures. But this hypothesis was not tested in the current study. One critical control experiment would be to use a chemical to generate DNA damage and examine whether the resultant DNA repair and ATP usage would also cause mitochondria to move/translocate toward the nucleus.

5. The authors did many experiments and accumulated extensive data but most of them are provisionally relevant and downstream changes. They used these data to support their general idea, which is phenomenological, but the more interesting and fundamental question of how low ATP in the nucleus (as a result of DNA repair) is sensed by the cell and whether this sensing of ATP triggers the mitochondrial directional movement or translocation to the nuclear periphery is not addressed and not answered.

Reviewer comments:

Reviewer #1

(Remarks to the Author)

All of my concerns have been satisfactorily addressed. The study has improved considerably through the review rounds, and I commend the authors for their efforts.

Reviewer #4

(Remarks to the Author)

The authors satisfactorily addressed my comments. I only have one small issue related to the ATP experiments (again). I thought that the pyruvate experiments were done in the absence of glucose, but the graph indicates that this is in the presence of glucose. I also realised that there is clearly has more cells that what is indicated under the graph for the pyruvate data. The authors should verify that the data is OK and that the stats reflect the data that is shown in the graph (I sthere was an addition of data points for the revision). They also need to clearly state in the text whether glucose was used or not. Otherwise, the manuscript is good for publication.

Reviewer #5

(Remarks to the Author)

This reviewer appreciates the authors for their detailed responses and their revision to the manuscript. The manuscript in its current form is much improved. However, there are several issues that need to be addressed.

1. The reviewer is surprised that the authored used nocodazole instead of colchicine to disrupt microtubules (MTs). Nocodazole is known to cause actomyosin contraction in addition to depolymerize MTs. Colchicine treatment does not induce actomyosin contraction and is a more specific molecule for disrupting MTs. Nevertheless, the results from the authors after Nocodazole is surprising: NAMs accumulation around the nucleus increased after the drug. This begs the question what molecular motors move MTs to the nuclear periphery on what filament systems after MT tracks are removed. The authors should at least discuss this issue in Discussion, although additional experiments would be preferable.
2. The authors performed chemically-induced DNA damage experiments, as suggested by this reviewer. They showed that this drug did not cause mitchondrial relocalization around the nucleus, different from external compression induced DNA damage. While this new result is interesting and suggests that there is a major difference in cellular responses to chemically-induced vs mechanically-induced DNA damage. This begs a new question: how did the mitochondria accumulate around the nucleus? What is the mechanosensing pathway that leads to preferential accumulation of NAMs around the nucleus? This is a fundamental question that requires carefully designed experiments to address. According to the working model that the authors proposed (see below), it is not the DNA damage or lack of ATP in the nucleus that attracts mitochondria to the nucleus. If the authors' model were true, what is the initial mechanotransduction cascade that leads to this NAM formation? Because of this reasoning, this reviewer stated in the previous communication (comment #5) that the study was phenomenological and not mechanistic. The authors should at least acknowledge this issue in revised Discussion.
3. In response to the reviewer's concern on the large compressive strain (~80%), the authors replied that their compression would not cause apoptosis and the cells still proliferate. Even if the cells still proliferated and there was no cell apoptosis, there would still be a potential for DNA damage and/or nuclear envelope damage, as published works have demonstrated for large strains >50%. In addition, the authors stated in their response to this reviewer's comment that "we demonstrate a causal and mechanistically dissected pathway from mechanical stress → mitochondrial re-localisation (NAM formation) → nuclear ATP surge → chromatin remodelling and DNA damage repair → cell cycle progression." In this statement, the authors have the model of DNA damage repair and nuclear ATP surge as the functional role of NAM formation. It appears that the authors would like to argue it both ways: 1) there is no DNA/nuclear damage after compression; 2) There is NAM formation after compression to supply ATP for DNA damage repair. How could the authors reconcile these two conflicting arguments? The authors are suggested to discuss this important issue.
4. The authors applied a certain compressive strain to the cells, not compressive stress. But the authors often use the term mechanical stress. What is the magnitude of the mechanical stress applied to the cell? The authors should estimate it and compare it with physiologically relevant stress magnitudes in vivo. This issue should also be discussed in Discussion.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Response to Reviewers

Reviewer #1 (Remarks to the Author):

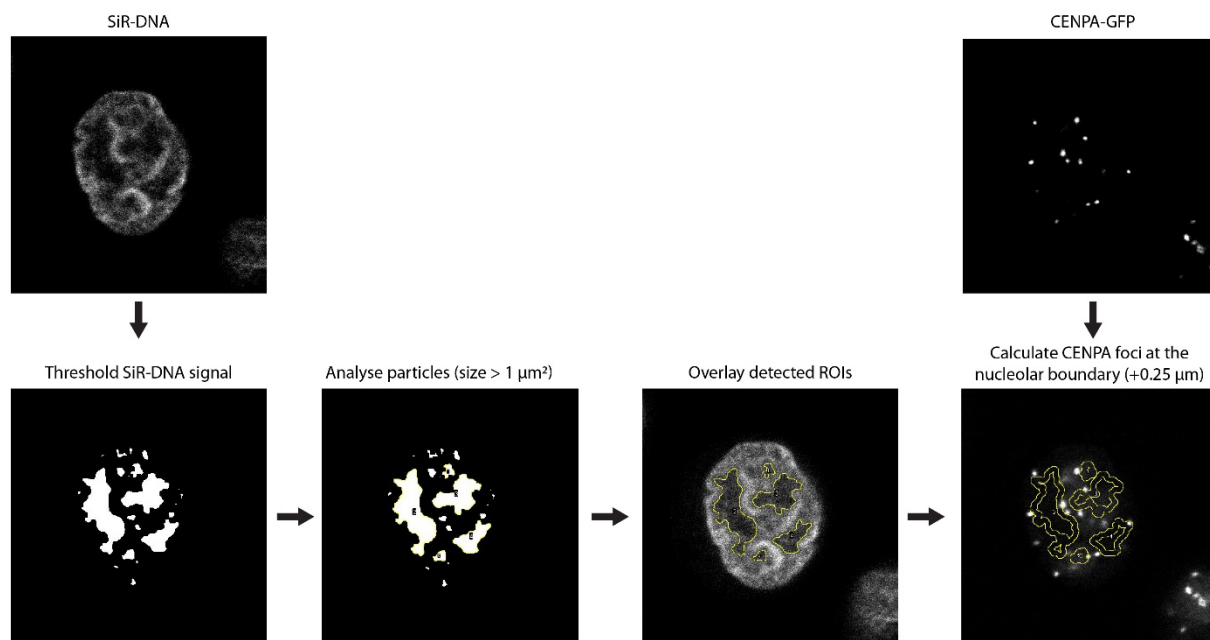
The manuscript Ghose et al. has greatly improved after its first submission to the Nature Cell Biology Journal. A wealth of new experimental data that include necessary controls, and detailed point-to-point answers greatly strengthen the new interesting concept of a metabolic response after mechanical confinement to secure cell fitness. After agreeing to most of the authors responses, this reviewer (#1) has only a number of minor points left.

We are very glad to hear that the Reviewer appreciates our changes and the additional data that were included compared to the previous submission. We also thank the Reviewer for their critical and constructive feedback and we have now addressed all these comments. We hope that the Reviewer will agree with our responses to the specific points that were raised as outlined below.

1. In your response to this reviewer you state: ‘..., the lack of a nuclear ATP surge during mechanical confinement significantly reduced the number of nucleoli’. However, nucleoli were not clearly detected (Fig. 5D). This becomes especially apparent in the oligomycin A-treated conditions, where it seems that more regions, in addition to the annotated regions, are free of chromatin. Was there a thresholding approach being used? Connected to this point, the concern of previous reviewer #4 (point 2d) is not fully answered. A nucleolus marker will clearly help to generate a clean analysis.

We appreciate the reviewer’s comment and acknowledge the limitations associated with nucleoli detection in confined cells. Under mechanical confinement (as described in our study), fixation is both inefficient and unreliable, compromising overall image quality and the reproducibility of immunofluorescence staining. As a result, nucleolar staining cannot be consistently performed in these conditions. Moreover, live-cell imaging options for nucleoli detection are limited, and most available dyes produce high background signals that would still necessitate thresholding—similar to the approach we used based on SiR-DNA signal depletion. Addressing this challenge would require generating a stable cell line expressing a fluorescently labelled nucleolar marker, which is beyond the scope of this study. Importantly, our work does not aim to characterize nucleolar structure. Instead, we detected nucleoli as SiR-DNA-depleted nuclear regions (as has been previously suggested <https://doi.org/10.1016/j.celrep.2016.07.016>, <https://doi.org/10.1101/gad.283838.116>). Importantly these regions serve as a proxy to assess macroscopic chromatin reorganization under mechanical confinement in the absence of ATP. We consider this indirect approach appropriate for the study’s objectives and have updated the *Methods* section and figure descriptions to clearly explain how we performed it.

Briefly, to detect nucleoli, the signal acquired from SiR-DNA was inverted, and the ‘stain-free’ nucleolar regions (<https://doi.org/10.1016/j.celrep.2016.07.016>, <https://doi.org/10.1101/gad.283838.116>) were detected using thresholding (Reviewer Response Figure 1). Following thresholding, particles were detected and an Area filter of $>1\mu\text{m}^2$ was applied, based on our observations in control suspension cells and informed by previously described nucleoli sizes ([https://doi.org/10.1016/s0960-9822\(00\)00455-3](https://doi.org/10.1016/s0960-9822(00)00455-3)). Our results in control conditions align with previous observations on the number (an average of 3-5 nucleoli per cell), verifying the validity of our approach. Following nucleoli detection, CENPA foci were quantified to be at the nucleolar boundary ($\pm 0.25\mu\text{m}$) or not (Reviewer Response Figure 1).



Reviewer Response Figure 1. Analysis workflow for nucleoli and CENPA quantification The SiR-DNA image was inverted, and a threshold was applied. Nucleoli were detected and segmented depending on their size ($> 1\mu\text{m}^2$). For each nucleolus, a boundary region of interest (ROI) was generated by computing the XOR between its boundary and the dilated ($+0.25\mu\text{m}$) and eroded ($-0.25\mu\text{m}$) versions. These ROIs were used to identify nucleoli-associated CENPA foci.

2. In your response to this reviewer you state: ‘Our analysis was further supported by Western blot, revealing a moderate increase in H3K9me3 and a significant increase in H3K27me3 in conditions of confinement in the absence of nuclear ATP compared to control confined conditions (Figure 5 A), ...’. To draw such conclusions (Fig. 5A; but also S7D), western blot data need to be generated from 3 independent experiments and quantified.

We appreciate the reviewer's comment. Since Western Blot is a semi-quantitative approach, we did not quantify the replicates in our original submission. Nevertheless, following the reviewer's concern, we have now included a quantification and statistics for both H3K9me3 and H3K27me3 marks in Figure 5 A. Our quantification of 3 biological replicates confirmed that H3K9me3 was upregulated, albeit not significantly in confinement, compared to suspension cells regardless of oligomycin A treatment. The facultative heterochromatin mark, H3K27me3 was also upregulated in both confinement conditions, however it was significantly higher in oligomycin confined compared to control confined cells.

Given the acute nature of our confinement, we believe that while trends are clear in our analysis, more stable changes would require longer confinement durations. We have also ensured that the *Results* section in the manuscript reflects the conclusions from our analysis (Lines 416-421).

3. The solid white line in Fig. 2E 'conf' does not seem to depict the line profiles quantified in panel F (the strong mitotracker-positive peripheral signal as shown in F does not seem to reflect the weak perinuclear signal as shown in E). Please double-check. – Also, to make your analysis approach as shown in Fig. 2 E - H more coherent, I suggest to measure in panels E and F only from the center of the nucleus up to the 10% enlarged ROI as shown in panel G.

We thank the reviewer for pointing this out. Indeed, we agree that presenting this analysis with the same approach as the NAM quantification, i.e. using the 10% expanded ROI, is more consistent. We have now modified the white lines in Figure 2 E (Figure 2 G in the revised manuscript), and the plots in Figure 2 F (Figure 2 H in the revised manuscript) to reflect MitoTracker and Hoechst levels from the centre of the nucleus to the expanded ROI.

To further improve the presented of our results, we have now also changed the order of results and first introduced the quantification of NAM formation in the main text, followed by the line profile analysis to better justify the use of the 10% ROI.

4. In the new Fig. S4B, I suggest to turn around the left and right panels as this will make the measurement approach more intuitive, namely that you have measured ER movement across and beyond the cell nucleus. In addition, I suggest to draw an outline around the nucleus in the suspension cell (Fig. S4B; top right image).

We have now made these changes, and thank the reviewer for these constructive suggestions.

5. Even though I understand your argumentation on the data depiction in Fig. 2H panel including the graph that you presented in your response letter, it would be a much cleaner data set if you

presented the NAM mean pixel intensity signal as a percentage of all mitochondria in a cell throughout the manuscript. This was also an issue for reviewer #4 (his/ her point 5). I suggest that, if reviewer #4 keeps raising this issue you adapt your NAM graphs. If not, I will not insist on this issue further.

We acknowledge the reviewer's suggestions for the alternate presentation of our NAM analysis and understand their perspective. In accordance with this, we have now included an additional panel Figure S2 C showing the mitochondria at the nuclear periphery as a percentage of the total mitochondria in the cell. We agree that this orthogonal analysis approach additionally validates our findings. We have also added the following statement in the text (Line 152-156): "We also used an orthogonal analysis approach to quantify NAM, where the mitochondria at the nuclear periphery was quantified as a percentage of the total mitochondria in the cell. As before, we observed a significant increase in the fraction of mitochondria at the nuclear periphery in cells under confinement, validating our findings (Figure S2 C)."

We would however like to emphasize that we are confident that our current format of presenting NAM formation is representative of the observations under the various conditions. Based on the reviewer's comment above, and given that Reviewer #4 did not raise this concern, we have maintained our original format of analysis through the rest of the manuscript.

6. In your response to this reviewer you state: 'We have now addressed this concern by replacing all single channel pseudo-colour images with grey images wherever nuclear staining was shown, which we agree have greatly improved the visualization.' However, I only see this change in Fig. 2. I suggest you apply these changes for every single channel everywhere (thus, i.e. also Figs. 3B, I; 5D; also in the supplement). Instead of 'merge' (i.e. Fig. 2A), the terms Hoechst (font in blue) and Mitotracker (font in magenta) might be indicated. Another advice is to generally use cyan or yellow instead of blue for merged images, to increase visibility.

We thank the reviewer for their suggestions, and apologize for any misunderstanding on our part during the previous submission. We have now changed all relevant images to be in grayscale if single channel, and in combinations of cyan, magenta, grey and/or green when merged. Wherever relevant, we have renamed 'merge' to the respective channel in the respective colours.

7. In your response letter you further state: 'Second, we have added data related to perinuclear actin ...'. It is not clear whether this actin analysis equals the analysis location for NAMs as indicated in Fig. 2G? Please specify.

We thank the reviewer for pointing out this limitation in our data presentation. In fact, the analysis for the perinuclear actin was performed by a similar approach as used for NAM formation, by measuring actin signal intensity within a defined ROI (ring region) around the nuclear membrane.

In our previous submission, we had only mentioned this analysis in the *Methods* section, however, we have now also mentioned this in the main text: “Given this direct role of actin in NAM accumulation, we next quantified perinuclear actin levels by measuring actin signal intensity within a defined boundary region along the nucleus.” [Line: 23].

Reviewer #3 (Remarks to the Author):

I am satisfied.

We are very pleased to have been able to address all of this Reviewer’s concerns and thank them very much for their constructive suggestions, which we believe have helped to improve the quality of our presentation and strengthened the conclusions in our manuscript.

Reviewer #4 (Remarks to the Author):

The authors did a good job at answering my concerns. There are only a few clarifications that I think should be made to the text.

We thank the reviewer for their previous suggestions, and are glad to have been able to address the vast majority of them. We hope that through our responses below, we will be able to address all the remaining clarifications raised by the reviewer.

1. I appreciated the way the authors describe the relationship between the ER, mitochondria and actin in their response to my previous comments and I agree with their view. However, the way the ER data is described in the result section does not quite reflect this. The authors should thus modify the way this section is described.

We appreciate that the reviewer shares our point of view with regards to the ER, mitochondria and actin analysis. We have now revisited the manuscript text to improve the *Results* section to reflect our previous response to the reviewer. To briefly summarise:

- We have changed the title to reflect the notion that through a combined mechanism, the ER, mitochondria and actin altogether modulate the formation of NAMs. [Line 239]

- We have restructured the *Results* section by first introducing the different possibilities of NAM regulation, either through mitochondrial morphology or through the ER dynamics [Lines 241-254]. As a result we do not suggest a causal relationship between the two modes of regulation. We have then presented our findings describing the relationship between mitochondrial morphology and NAMs [Lines 272-284], followed by the relationship between the ER and NAMs [Lines 285-301].
- Finally, we have explicitly mentioned that, despite the vital role of each structure, it is the coordinated interaction between mitochondrial dynamics, ER and NAM, that drives NAM formation under mechanical confinement. [Lines 310-313]

2. The authors really expanded their ATP analysis and made it much clearer. However, it is still not always clear which experiments are done in the presence or the absence of glucose. This should minimally be indicated in the figure legends to make it clear for the readers.

We apologise that this was not clearly presented in our data plots. We have now added this information to the figure legends wherever relevant. In plots where only some of the samples were in glucose-free DMEM, we have also indicated this with shaded boxplots (Figure 4 B).

In addition, in response to the Reviewer's next point (Point 3), we have combined Figures 4 B and S5 J (into the new Figure 4 B), which offers a clearer overview of the distinction between glucose-free and glucose-containing media, without the need to refer between a main and a supplementary figure separately.

3. I have an issue with Figure 4B and S5J. They seem to contain the same control data. I imagine that the experiments presented in these two graphs were done together, so they should be presented together rather than duplicating the same data.

We appreciate the reviewer's concern and agree that combining these two graphs offers a better presentation and clarity of interpretation. We have now combined these data plots, as previously presented in Figure 4 B and S5 J, into a new figure panel (Figure 4 B).

4. The statement "MFN1 and FIS1 are integral membrane proteins that participate in the recruitment of DRP1, and the tethering of ER and mitochondria, thereby directly contributing to MERCs formation" (lines 246-248) is somewhat misleading as the role of Fis1 in fission is still controversial and it is MFN2, not MFN1 that has been shown to participate in MERCs.

We appreciate the reviewer's concern. It is indeed true that the vast majority of literature regarding MERCs have found that MFN2 is the primary mediator of MERCs, not MFN1. However, recent literature has shown that both MFN1 and MFN2 localise to MERCs (<https://doi.org/10.1083/jcb.201911122>, <https://doi.org/10.1002/JPER.23-0072>) and that their interaction with ER-bound proteins may regulate the ER-mitochondria contact sites (<https://doi.org/10.1083/jcb.202304031>; <https://doi.org/10.1016/j.biocel.2021.106101>). In addition, FIS1, has also been shown to regulate mitochondria-ER contact sites in various contexts (<https://doi.org/10.1038/emboj.2010.346>, <https://doi.org/10.1016/j.biocel.2021.106101>).

Nevertheless, we do understand the concern of the reviewer and have rephrased our hypotheses. As discussed in response to this reviewer's point 1, we have now restructured the *Results* section and introduced our hypotheses on mitochondrial morphology and on ER dynamics in parallel. More specifically, we have restructured the results section by first introducing the different possibilities of NAM regulation, either through mitochondrial morphology or through the ER dynamics [Lines 241-254]. As a result we do not discuss a direct relationship between regulators of mitochondrial morphology (i.e. FIS1, MFN1 and DRP1) and mitochondria-ER contact sites. We have then presented our findings describing the relationship between mitochondrial morphology and NAMs [Lines 272-284], followed by the relationship between the ER and NAMs [Lines 285-301].

We hope that the reviewer will find these edits make the narrative more convincing.

Reviewer #5 (Remarks to the Author):

This study investigates the response of the cells when a sudden large compressive strain is applied to cancer cell line cells and finds that mitochondria accumulate outside the nuclear periphery. The authors "propose a mechanism by which actin dynamics, the ER and the physical shape of mitochondria contribute to NAM formation, driving a mechano-metabolic response of cells via an increase in nuclear ATP levels under mechanical confinement stress." (quote from the authors' responses to reviewers).

1. The authors did not differentiate the fundamental differences between two totally different processes: a) active invasion and squeezing through narrow ECM pathways by cancer cells or normal cells during invasion in vivo that require endogenous contractile forces and the resultant compressive strains in some directions and tensile strains in other directions that are gradual and time-dependent; b) the applied sudden constant large compressive strain in the current study. The physiological and/or pathological relevance of the current methodology is questionable.

We thank the reviewer for the opportunity to clarify the scope and physiological relevance of our approach. We completely agree that cell invasion *in vivo* is a complex, multi-step process involving dynamic interactions with the extracellular matrix (ECM), cell-generated forces, and temporally varying mechanical cues. Importantly, studying this complex process within a single project or experiment is rather difficult if not impossible to achieve, and hence various previous studies (<https://doi.org/10.1016/j.tcb.2011.09.006>; <https://doi.org/10.1016/j.cell.2011.11.016>; <https://doi.org/10.1038/s41580-019-0172-9>), and now us, are investigating one specific aspect of this multi-step process.

Within this context, our study does not aim to reproduce the full sequence of events involved in the physiological process of invasion or metastasis. Rather, we focus on a defined and experimentally tractable aspect: how cells can respond to acute mechanical stress—particularly the nucleus and associated organelles—and we investigate this response both on short (minutes) and on long time scales (hours). In our study, we have applied an acute compressive force that has previously been published to be relevant, by us (<https://doi.org/10.1126/science.aba2644>) and other labs (<https://doi.org/10.1126/science.aba2894>). This rapid application of compressive stress is used as a proxy to investigate how cells react to sudden spatial constraints that lead to acute mechanical cell deformation, which can occur locally and transiently *in vivo* (<https://doi.org/10.1073/pnas.2414009121>, <https://doi.org/10.1002/adv.202414024>, <https://doi.org/10.1016/j.isci.2022.105330>, <https://doi.org/10.1091/mbc.E16-05-0286>, <https://doi.org/10.1101/2025.03.03.641275>) such as when a cell abruptly encounters a constriction within the ECM or during invasive processes. In our study we assessed both immediate and long-term adaptive cellular responses to acute mechanical stress, and identified a novel organelle-specific response that maintains cell fitness, a finding that we believe adds as a significant and novel contribution to the field. Particularly, the mechano-metabolic crosstalk between the nucleus and mitochondria we identified in our study is a new mechanism to ensure cellular resistance to mechanical stress.

Nevertheless, to more tangibly address the reviewers concern about the physiological relevance of our findings, we analysed patient-derived tumour microarrays for the presence of nuclear-associated mitochondria (NAMs) across tumours from 17 different individuals, distinguishing between the tumour core and the invasive front (Figure S2 N). We found clear evidence of the presence of NAM-positive cells containing both nuclear shape changes and indentations, and the mitochondria located within them and at the nuclear periphery (Figure 2I-J, Figure S2 N). Cells present in the tumour core exhibited a NAM-high penetrance of 1.8%, while cells in the invasive front showed a significantly elevated penetrance of 5.4% (Figure 2K).

Metastasis and migration are inherently heterogeneous and involve both active and passive mechanical cues. While the mechanical strains experienced *in vivo* may often develop gradually, there are contexts—such as during immune cell trafficking or extravasation—where cells experience relatively rapid mechanical shifts. As observed with our results, cells at the tumour

invasive front exhibit a higher penetrance of the NAM phenotype (Figure 2K). We opted for a simple and elegant reconstruction of this confinement in vitro, applying an acute uniaxial confinement stress, which allowed us to isolate and dissect the immediate subcellular responses to such mechanical challenges, including changes in actin dynamics, organelle positioning, and altered nuclear energetics. Other studies have highlighted relatively longer duration adaptations using similar systems (<https://doi.org/10.1126/science.aba2894>).

In addition to the experimental evaluation of the patient-derived tumour microarrays, we have also briefly introduced acute confinement and our relevant in vivo findings in the *Introduction* [Lines 52-55; 70-72] as well as in *Discussion* sections in our revised manuscript [Lines 554-558].

We hope that through these additional experiments and discussions, and through our response to the reviewer's concern, we have been able to convincingly convey the rationale and relevance of our work.

2. The huge sudden compressive strain (a step function) on the nucleus is estimated to be ~80% (Fig 2C,D). It is not clear this would ever happen in vivo. In cell culture, this would certainly cause cell and nuclear damage and DNA damage. Published reports already show that normal cells at this huge nuclear strain can be turned into cancerous cells. Therefore this protocol may not apply to normal cell invasion during development. The authors used only cancer cells in their study and found blebb formation after the mechanical perturbation in these cells. Whether this sudden constant compressive strain triggers extensive apoptosis or other forms of cell deaths is not clear.

We appreciate the reviewer's critical assessment and hope they will find our detailed response satisfactory in contextualising our results, relevant literature, and addressing their concerns.

We would like to emphasise that the confinement approach used in our study has been widely applied in various studies over the recent years (<https://doi.org/10.1242/jcs.263704>, <https://doi.org/10.1083/jcb.202205051>, <https://doi.org/10.7554/eLife.61037>, [10.1038/s41467-024-47227-2](https://doi.org/10.1038/s41467-024-47227-2), [10.1126/science.aba2644](https://doi.org/10.1126/science.aba2644), [10.1126/science.aba2894](https://doi.org/10.1126/science.aba2894)). The novelty of our work lies in combining subcellular fractionation proteomics with confinement, which led to the discovery that acute confinement induces a specific mitochondria–nucleus association (NAM), which alters nuclear energetics in the short term and contributes to long-term cellular fitness.

Cells migrating in vivo often encounter complex three-dimensional environments with variable pore sizes and mechanical constraints. While the precise magnitude and kinetics of nuclear deformation in vivo are difficult to quantify, numerous studies have shown that both normal and transformed cells—including epithelial, mesenchymal, and immune cell types—can withstand substantial nuclear deformation without immediate deleterious effects. For example, Liu et al. (Cell, 2015; <https://doi.org/10.1016/j.cell.2015.01.007>), and more recently Lomakin et al. 2020 (<https://doi.org/10.1126/science.aba2894>) demonstrated that various cell types remain viable when

confined to vertical heights as low as 3 μm , with deformation levels comparable to those observed in our study, yet without signs of apoptosis. Furthermore, our prior work using zebrafish progenitor stem cells (<https://doi.org/10.1016/j.cell.2015.01.008>; <https://doi.org/10.1126/science.aba2644>) confirms that even non-mammalian cells can undergo large nuclear deformations in confined environments while remaining functional and motile.

In the current study, the compressive strain applied is acute but not abrupt. As detailed in our *Methods* section, we used a pressurised dynamic confiner (4DCell) equipped with a pressure regulator, which allows for a precise and reproducible control over the confinement process. As shown in Supplementary Movie_S1, full confinement is achieved over approximately 90 seconds, providing a tightly controlled and experimentally tractable model of acute mechanical compression.

Importantly, we found that U2OS cells subjected to acute confinement and then released into standard culture conditions proliferated at the same rate as unconfined controls (Figure S7 J), indicating that the confinement protocol does not compromise cell viability or proliferation. Furthermore, and as discussed in response to Point 1 raised by this reviewer, our newly added analysis of patient-derived tumour microarrays (TMAs) revealed NAM-positive cells exhibiting nuclear shape changes and mitochondria localised at the nuclear periphery (Figure 2 I-K, Figure S2 N). Notably, NAM penetrance increased from 1.8% in tumour core regions to 5.4% in the invasive front (Figure 2 K), supporting the physiological relevance of our findings in a native tissue context.

Lastly, with respect to blebbing, although we used transformed cell lines in this study, the blebbing response observed is a well-documented mechanical adaptation to increased intracellular pressure and confinement, rather than a sign of injury (<https://doi.org/10.1038/nature03550>; <https://doi.org/10.1016/j.ceb.2013.05.005>). Bleb-based migration is especially prevalent in low-adhesion environments and is not intrinsically associated with oncogenic transformation.

3. The authors focused on the F-actin role in the accumulation of mitochondria around the nuclear periphery. However, if the mitochondria were actively translocated by the cell, as the authors suggested in their responses to the reviewers, then microtubules and dynein motors must play critical roles in this translocation. However, no experimental evidence or live cell imaging is shown to support the active dynamic translocation of the mitochondria before, during, and after the compressive strain.

We thank the reviewer for raising this important point. Indeed, mitochondrial translocation has been previously associated with microtubule networks and motor proteins such as dynein. To address this, we have now evaluated NAM formation in cells treated with Nocodazole, which effectively depolymerizes microtubules (Figure S3 E). As shown in (Figure 3 C-D), Nocodazole

treatment did not abolish NAM formation. On the contrary, we observed a significant increase in NAM levels.

This result suggests a more nuanced interplay between the microtubule and actin cytoskeletons in regulating NAM formation. As shown in Figure 3 A-B, disruption of actin polymerisation led to a loss of NAM, whereas microtubule depolymerisation increased it (Figure 3 C-D). These opposing effects indicate that while F-actin is essential for NAM formation, microtubules may act in a modulatory or antagonistic capacity for NAM formation under confinement.

Additionally, we have shown that promoting mitochondrial fusion enhances NAM formation, whereas promoting fission reduces it (Figure 3 J-K). Prior studies have shown that microtubules facilitate mitochondrial shape dynamics (<https://doi.org/10.1016/j.cellsig.2017.07.020>; <https://doi.org/10.1038/srep13924>; <https://doi.org/10.1038/s41598-023-31121-w>). Therefore, our observation that Nocodazole—which inhibits microtubule-mediated fission—leads to increased NAM formation also serves as an orthogonal validation of our findings in FIS1 knockout cells.

Together, these data support the idea that NAM formation is not solely dependent on any one specific mechanism, but rather involves a coordinated regulation of mitochondrial morphology and cytoskeletal dynamics, particularly under mechanical stress. We have now revisited the respective *Results* and *Discussion* sections to ensure that the correct message of a coordinated regulation of NAMs is put forth.

4. If the authors' hypothesis were correct, one would assume that the DNA damage in the nucleus and the energy required to repair the DNA damage for ATP would generate a sink to attract the mitochondria to the nucleus. This might explain why the mitochondria accumulated around the nucleus but not other organelles or cytoplasmic structures. But this hypothesis was not tested in the current study. One critical control experiment would be to use a chemical to generate DNA damage and examine whether the resultant DNA repair and ATP usage would also cause mitochondria to move/translocate toward the nucleus.

We thank the reviewer for pointing out this important control. We would like to start by highlighting that we hypothesised that the accumulation of mitochondria to the nucleus is a specific response to safeguard the genome in response to physical confinement stress.

However, we do agree that to make that claim, the suggested experiment by the reviewer is important. To that end, we have now treated adherent HeLa cells with Etoposide and measured the levels of γ H2AX and two mitochondrial markers, SHMT2 and MTHFD2. We observed that Etoposide treatment significantly increased the levels of γ H2AX compared to control conditions as expected (Figure S7 G-I). However, it did not alter the levels of either SHMT2 or MTHFD2

within the nuclear periphery (Figure S7 G-I), supporting that DNA damage alone is not sufficient to drive NAM formation. This confirms that NAM formation is not driven by DNA damage but is a direct consequence of cell confinement. We have reflected this in our *Results* and *Discussion* sections.

5. The authors did many experiments and accumulated extensive data but most of them are provisionally relevant and downstream changes. They used these data to support their general idea, which is phenomenological, but the more interesting and fundamental question of how low ATP in the nucleus (as a result of DNA repair) is sensed by the cell and whether this sensing of ATP triggers the mitochondrial directional movement or translocation to the nuclear periphery is not addressed and not answered.

We thank the reviewer for this thoughtful and stimulating comment. We respectfully disagree with the notion that our findings are merely phenomenological or provisionally relevant. Our study directly identifies a mechanical confinement-triggered, actin- and ER-dependent redistribution of mitochondria to the nuclear periphery as the initiating event, leading to a surge in nuclear ATP of mitochondrial origin. Through genetic (MFN1, FIS1, DRP1 KOs), pharmacological (Oligomycin A, BAM15, Latrunculin A, SMIFH2), and functional (FRET-ATP sensors, ATAC-seq, DNA repair, cell cycle) assays, we demonstrate a causal and mechanistically dissected pathway from *mechanical stress* → *mitochondrial re-localisation (NAM formation)* → *nuclear ATP surge* → *chromatin remodelling and DNA damage repair* → *cell cycle progression*.

Importantly, we show that:

- The nuclear ATP surge requires actin-driven mitochondrial proximity (NAM accumulation), but not an increase in mitochondrial energy production per se (Figure 3, Figure 4, Figure S5).
- This proximity can be genetically enhanced (FIS1 KO) or disrupted (MFN1 KO, actin inhibitors), leading to corresponding gains or losses in nuclear ATP.
- Blocking mitochondrial ATP synthesis or its spatial delivery (Oligomycin A, BAM15, Latrunculin A) abolishes the nuclear ATP increase and delays DNA damage repair and S-phase progression (Figures 4–6).

As such, our study explicitly demonstrates that the cell senses mechanical confinement via cytoskeletal remodelling, leading to mitochondrial repositioning that enables nuclear metabolic adaptation, rather than responding to a pre-existing drop in nuclear ATP. Indeed, we did not observe decreased nuclear ATP as a precondition. Rather, the surge is an active adaptive response to mechanical stress, not a passive readout of depletion.

While further work could explore the detailed ATP sensing mechanism in play, our focus here was to uncover and functionally validate how nuclear energy levels are acutely modulated in response to mechanical stress, a biologically and physiologically relevant stimulus. We believe this represents a fundamental insight into mechano-metabolic adaptation.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

All of my concerns have been satisfactorily addressed. The study has improved considerably through the review rounds, and I commend the authors for their efforts.

We are very happy to have been able to address all of this reviewer's comments, and thank them for a constructive revision process.

Reviewer #4 (Remarks to the Author):

The authors satisfactorily addressed my comments. I only have one small issue related to the ATP experiments (again). I thought that the pyruvate experiments were done in the absence of glucose, but the graph indicates that this is in the presence of glucose. I also realised that there is clearly has more cells that what is indicated under the graph for the pyruvate data. The authors should verify that the data is OK and that the stats reflect the data that is shown in the graph (I sthere was an addition of data points for the revision). They also need to clearly state in the text whether glucose was used or not. Otherwise, the manuscript is good for publication.

We thank the reviewer for their constructive suggestions through the revision process and are happy that they feel the manuscript is ready for publication. In our final submission, we have explicitly mentioned the presence or absence of glucose in the context of Oligomycin A or pyruvate treatment (wherever relevant), as follows:

[Line 341] "Additionally, in the presence of glucose, cells were supplemented with 3 mM pyruvate for 30 minutes prior to confinement".

[Line 369] "To verify whether confinement induces a nuclear ATP surge in a cell line-independent manner, we measured confinement-dependent nuclear ATP levels in U2OS cells in glucose-free conditions."

Finally, we thank the reviewer for pointing out the disparity between the data points and indicated n. Indeed while preparing the figure, the wrong n was indicated, which has now been correctly represented. This does not, in any way, impact the statistical results or conclusions from the data.

Reviewer #5 (Remarks to the Author):

This reviewer appreciates the authors for their detailed responses and their revision to the manuscript.

The manuscript in its current form is much improved. However, there are several issues that need to be addressed.

1. The reviewer is surprised that the authors used nocodazole instead of colchicine to disrupt microtubules (MTs). Nocodazole is known to cause actomyosin contraction in addition to depolymerize MTs. Colchicine treatment does not induce actomyosin contraction and is a more specific molecule for disrupting MTs. Nevertheless, the results from the authors after Nocodazole is surprising: NAMs accumulation around the nucleus increased after the drug. This begs the question what molecular motors move MTs to the nuclear periphery on what filament systems after MT tracks are removed. The authors should at least discuss this issue in Discussion, although additional experiments would be preferable.

Nocodazole is a well-known microtubule-depolymerizing agent. While it may have indirect effects on actin cytoskeleton, its primary target is microtubules. Importantly, we would like to point out that, given the extensive perturbations we have used to show that NAM is reliant on the actin cytoskeleton, our results from Nocodazole treatment, which shows an opposite effect to actin downregulation, are specific for the effects of microtubule depolymerisation.

To emphasize this point, we have now included a statement about this in the Discussion section:

[Line 577] “While Nocodazole may indirectly impact actin, given its contrasting effect to actin perturbations in our study, the increase in NAM is most likely a result of microtubule depolymerisation, and not indirect effects on actin.”

2. The authors performed chemically-induced DNA damage experiments, as suggested by this reviewer. They showed that this drug did not cause mitochondrial relocalization around the nucleus, different from external compression induced DNA damage. While this new result is interesting and suggests that there is a major difference in cellular responses to chemically-induced vs mechanically-induced DNA damage. This begs a new question: how did the mitochondria accumulate around the nucleus? What is the mechanosensing pathway that leads to preferential accumulation of NAMs around the nucleus? This is a fundamental question that requires carefully designed experiments to address. According to the working model that the authors proposed (see below), it is not the DNA damage or lack of ATP in the nucleus that attracts mitochondria to the nucleus. If the authors' model were true, what is the initial mechanotransduction cascade that leads to this NAM formation? Because of this reasoning, this reviewer stated in the previous communication (comment #5) that the study was phenomenological and not mechanistic. The authors should at least acknowledge this issue in revised Discussion.

Our model proposes a rapid relocalisation of the mitochondria to the nuclear periphery. We have shown that this is dependent on a coordinated network of the actin cytoskeleton and the ER, together with mitochondrial morphological changes. Importantly, we have also shown that an active actin polymerisation is required for the rapid (in seconds) nature of this relocalisation. Our findings suggest a structurally adaptive ecosystem within the cell, which enables the immediate nucleus-mitochondria association. While this is our hypothesis based on our findings, we agree that future studies to further evaluate this mechanism would indeed help paint a more nuanced understanding of the processes leading to the functional readout in our study. We have mentioned this in the Discussion section:

[Line 589] While our findings suggest a structurally adaptive mechanism involving ER and actin cytoskeletal networks facilitates rapid mitochondrial relocalization, our study leaves scope for future work to delineate the upstream mechanosensing pathways initiating this response.

3. In response to the reviewer's concern on the large compressive strain (~80%), the authors replied that their compression would not cause apoptosis and the cells still proliferate. Even if the cells still proliferated and there was no cell apoptosis, there would still be a potential for DNA damage and/or nuclear envelope damage, as published works have demonstrated for large strains >50%. In addition, the authors stated in their response to this reviewer's comment that "we demonstrate a causal and mechanistically dissected pathway from mechanical stress → mitochondrial re-localisation (NAM formation) → nuclear ATP surge → chromatin remodelling and DNA damage repair → cell cycle progression." In this statement, the authors have the model of DNA damage repair and nuclear ATP surge as the functional role of NAM formation. It appears that the authors would like to argue it both ways: 1) there is no DNA/nuclear damage after compression; 2) There is NAM formation after compression to supply ATP for DNA damage repair. How could the authors reconcile these two conflicting arguments? The authors are suggested to discuss this important issue.

We apologise if there has been a misunderstanding or miscommunication. The fundamental basis of the functional part of our study shows that acute confinement induces DNA damage. In the presence of ATP, this DNA damage is correctly sensed, repaired, and cells are able to continue proliferating as in control unconfined conditions. In the absence of ATP (as with Oligomycin A or Latrunculin A treatment), there is a lack of DNA damage sensing, which results in inefficient delayed repair, and loss in cell proliferation.

In the above Reviewer comment "Even if the cells still proliferated and there was no cell apoptosis, there would still be a potential for DNA damage and/or nuclear envelope damage, as published works have demonstrated for large strains >50%."

We do/did not disagree with this at all. In fact, our response in the previous revision round specifically discusses that there is no effect on cell apoptosis or proliferation. The main take home message of our study is that, this is due to efficient ATP-dependent DNA damage repair, for which mitochondrial relocalisation to the nuclear periphery is required.

4. The authors applied a certain compressive strain to the cells, not compressive stress. But the authors often use the term mechanical stress. What is the magnitude of the mechanical stress applied to the cell? The authors should estimate it and compare it with physiologically relevant stress magnitudes in vivo. This issue should also be discussed in Discussion.

We thank the reviewer for highlighting the distinction between mechanical stress and strain. In our study, the confinement device imposes a well-defined geometric constraint on the cells (through a change in cell height), and as such, we are indeed applying a compressive strain by physically limiting the space available for the cells. While we refer broadly to "mechanical stress" in parts of the manuscript to describe the biological consequences of mechanical perturbation, we agree that the applied input in our system is a compressive strain. We did not directly measure stress (i.e., force per area). Indeed, the measurement of force responses at the cellular have been previously addressed and involve complex mechano-signalling pathways that can alter cell surface tension (Lomakin et al. Science 2020). However, the stress–strain relationship was not the focus of our study.

Instead, we investigated a novel mechanobiological response—mitochondrial aggregation around the nucleus and an associated increase in nuclear ATP levels —that facilitates DNA damage repair under physical confinement. We emphasize that our confinement model represents a physiologically relevant mechanical challenge to cells, and our interest in the current study lies in the downstream biological effects of such confinement, rather than in quantifying mechanical inputs per se.

