

The loss of OPA1 accelerates intervertebral disc degeneration and osteoarthritis in aged mice

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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)

This manuscript focuses on the role of OPA1 in disc and cartilage. OPA1 is a GTPase mediating IMM fusion, which plays an important role in mitochondrial dynamics. In the study “The loss of OPA1 accelerates intervertebral disc degeneration and osteoarthritis in aged mice”, Vedavathi Madhu et al. showed that OPA1 knockdown impaired the morphology of mitochondria and other organelles in the NP cells in vitro, while tissue-specific OPA1-KO mice had age-dependent disc degeneration, osteoarthritis, and vertebral osteopenia in vivo. The age-dependent phenotype of OPA1-KO mice is interesting and striking to me, which is connected to the mechanism of the in vitro studies. However, lacking analysis of the metabolic role of OPA1 in the in vivo model significantly reduced the impact of this study.

Major comments:

For the in vitro studies, shOPA1 #2 is missed in many studies, such as Fig. 1E and Fig. 3 et al. It is essential to include both shRNA for all the studies, to avoid the off-target effect of shRNA.

NP cells were isolated from rats for the in vitro metabolic studies. Similar metabolic analysis should be done with isolated NP cells from the OPA1-KO mice ex vivo. Some assays should be tested in vivo.

It is unclear why adding glucose reduces OCR in both control and OPA1-KD cells, although this is consistent with their previous publication. This should be further examined.

Both cells show higher basal OCR. What substrates were used by these cells in the Seahorse assay? Cells were cultured under hypoxia for 24 hours before the experiment. Were the cells under hypoxia or normoxia during the experiment?

The increased OCR in OPA1-KD cells is not in line with the reduced PDH activity (5D).

“Hydroxyproline and hydroxylysine were raised” is not consistent with the data in Fig 4F and 4G, which showed lower levels in KO cells. hydroxyproline and hydroxylysine are not listed in Fig. 4C. Thus, it is unclear if Fig. 4D-Q are the graphs of the same data from the metabolomic assay in Fig. 4C. It is common to include 6-10 repeats in each group for the metabolomic assay. N=4 is very low for this assay, and Fig. 4L-O showed a big variation in the KO cells, which may result in misleading conclusions.

“Sigma mean” is not a common way to show isotope tracing data, which is very confusing. MID should be used to show fractional enrichment. M+0 should be included as well. It is also important to show the total abundance of all the metabolites in the isotope tracing assay. The current data can't support all the conclusions.

The method of isotope tracing is unclear, such as “50% of DMEM media contained 13C-labeled glucose.” “The enrichment was calculated after subtraction from the background of the non-labeled treatment samples.”, which is not the correct way for data analysis.

The calculation for the fluxes in 5B-G should be clearly described in this manuscript.

The lengthy discussion seems to repeatedly cover some contents in the result section.

Minor points:

The use of A' for the figure panel is not common.

A typo in line 82.

“.” is missing in line 339.

DAPI-only images should be in Fig. 7.

Reviewer #2

(Remarks to the Author)

Madhu and colleagues have undertaken a study examining the role of OPA1 in intervertebral disc degeneration and osteoarthritis. Their research sheds light on OPA1's critical involvement in regulating mitochondrial function and metabolism of NP cells in vitro. Furthermore, they provide evidence that deletion of OPA1 accelerates the degenerative process in disc, osteoarthritis, and vertebral osteopenia in mice. However, the authors emphasize examining the effects of OPA1 mainly at the morphological and multicellular levels, which restricts the wider significance of the study. Additionally, a notable absence of comprehensive investigation into the upstream or downstream mechanisms underscores the need for further cooperation or rescue experiments. Addressing these shortcomings would strengthen the study's novelty and align it more closely with the standards of Nature Communications.

The Major comments are as follows:

1. The authors have extensively explored OPA1's role in regulating mitochondrial function and cell metabolism, suggesting that NP cell dysfunction results from OPA1 loss. To strengthen this claim, the authors should elucidate the process of selecting OPA1 as the target using unbiased approaches, such as scRNA-seq.
2. OPA1 deletion was observed to affect NP cell phenotype and alter the IVD matrix composition in mice. The authors should investigate whether OPA1 deletion changes NP's cellular heterogeneity or notochord cells' cell identity. Mechanisms underlying OPA1-induced cell fate switch should be explored further.
3. While this article identifies OPA1-induced changes in the intervertebral disc, cartilage, and vertebrae, rather than discussing them broadly, it would be more beneficial to enhance the depth of in vivo study in the intervertebral disc. It would align more closely with the results of the in vitro study. It is not advisable to include data on cartilage and vertebrae in this study.
4. Considering the importance of OPA1 in disc and cartilage, the authors should profile OPA1 expression during the degenerative process of disc and cartilage in humans or mice.
5. Disrupting autophagy and dysregulating metabolism impaired OPA1-deficient NP cells in vitro. Validation of these results in OPA1-cKO mice, rather than solely focusing on degeneration evaluation, is recommended.
6. The authors discussed age-related degenerative phenotypes associated with OPA1 absence in mouse discs. However, they did not observe a decrease in aggrecan or col2 in the nucleus pulposus or an increase in COL1 in the annulus fibrosus, traditional markers of degeneration according to their previous findings. Addressing these inconsistencies and contradictions is essential.
7. In investigating age-dependent disc degeneration and OA accelerated by OPA1-KO, did the authors explore the relationship between OPA1 loss and inflammation, a key mediator of age-related disorders?

These considerations will enrich the study's depth and contribute to a more comprehensive understanding of OPA1's role in skeletal health and disease.

Reviewer #3

(Remarks to the Author)

In this work, the authors generated OPA1 loss-of-function in cultured NP cells and mice for detailed phenotypic and metabolic analyses. Notably, they found that OPA1 knockdown in the NP cells changed the morphology of mitochondria and other organelles. And that the dKO mice show degeneration in IVD and joints. Thus, this work provides new information to the field. However, some discrepancies or issues need to be addressed for improving the manuscript.

Major issues or questions:

In their plots showing fold changes, the authors depicted the values of the control samples always as '1.0' (for example, Fig.1A and 1D, etc.), which may not reflect the variation between different control samples and may have skewed the statistical analysis.

Figure S1C: The decrease of BNIP3 in ShOpa is more obvious than that of CHOP, while the manuscript text and the statistical plot indicate the reverse.

Figure 2D: LC3II in cells without Baf A1 has no significant changes when there's ShOpa1. This is a discrepancy with Figure 2B and the manuscript text.

Figure 3: For ECAR, the line of shOpa1 is above the control line in Figure 3A, but the authors states "Under basal conditions, ECAR remained unchanged". If the authors did certain calculations to get this conclusion, please describe them in the methods. Also, the authors need to provide details about how to compute other parameters in the plots. In addition, it would be more informative if the authors provide the value of ATP concentration directly for JATP, but not some numbers in the unit of pH changes.

Also in Figure 3: the text and legend miss the description of some figures.

Figure 4: there are discrepancies between what the figure shows and what the manuscript text states: "hydroxyproline, and hydroxylysine were raised" and "s-adenosyl methionine were significantly lower in OPA1 knockdown cells." Also please check if the enrichment analysis is based on this.

Line 254: "caudal discs of Opa1cKO mice showed changes in NP cell morphology", please elaborate the changes.

The authors may consider an immunohistochemistry assay of Opa1 in the cKO mice. In addition, examination of a notochordal marker in the cKO mice would provide necessary information of the phenotype.

Line 310: "a substantial increase in mean total and the cross-sectional thickness (Cs.Th.)". Figure 8K seems to show no significant changes.

Regarding the bone parameters in Fig. 8, the authors should provide more details about their measurement, and provide interpretation and discussion why Opa1 cKO caused these changes in trabecular and cortical bones and how they affect vertebral bone health.

Figure 9: the authors need to provide details for calculating toluidine blue scores and osteophyte scores.

Figure 9G: does the asterisk in the lateral side denote osteophyte or subchondral bone? A cartilage staining but not H&E would help to clarify this.

Since Figure 9 and 10 are based on the same histology images, they should be consolidated into a figure.

Minor issues:

Figure 1: the authors may consider including bright field microscopic images of the Opa1-depleted NP cells, to show the morphology of the cells.

Figure 1E: the fluorescence intensity could be varied between ctrl and ShOpa1, as in shCtrl, PMP70 is too weak compared to Mito, and GM130/Mito is showing yellow, while ShOpa1 showed stronger PMP70 and GM130, which overshadowed the Mito fluorescence. Does this reflect that these organelles have higher levels in ShOpa1, or it is just due to some technical issues?

Figure 2A: Green fluorescence (LC3B) has variations among different cells in shCtrl. Thus, the authors may need to include a statistical analysis here. In addition, the red and green don't have to be merged, which interferes with the observation of green fluorescence, if Mito and LC3B don't necessarily colocalize.

Figure 7E: the AF should be demarcated like in other panels.

Figure 8: it would be helpful if the authors denote the VL and DH in the CT images for better appreciation.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

It is good to see the new Figure 9 showing the metabolic alteration in the OPA1 mice model. However, most of the previous concerns are partially addressed. Thus, the metabolic analysis remains weak in this revision.

First, the metabolomics experiment is updated to n=6 (4 is listed in the legend) for one shRNA. It is unclear why the authors didn't include the second shRNA in this assay. The rebuttal to the previous Comment 1 seems to be logical, but not reasonable, since the similar result in some assays can't predict the other assays.

Second, related to previous Comment 3, the author only provided some rebuttal in the responding letters, without any new data or new discussion in the manuscript. The previous concern about the OCR is not addressed at all in the revised manuscript.

Third, related to previous Comment 5. The authors provided references to justify their method for isotope tracing assay. However, there are many other references showing widely accepted approaches in isotope tracing assay, which are ignored by the authors. The authors stated to correct the "50% of DMEM media contained ¹³C-labeled glucose.", which is not clarified in the revised manuscript. Thus, these data remain questionable.

Reviewer #2

(Remarks to the Author)

Although the authors made extensive revisions in the updated manuscript, most concerns raised by the reviewer were not directly addressed. Notably, NP cells have been shown to be heterogeneous in both humans and mice (DOI:

10.1038/s41467-023-44313-9 and 10.1096/fj.202301217R). Performing single-cell RNA sequencing on NP cells from OPA1-KO mice, rather than bulk RNA-seq, could elucidate the specific target cell populations affected by OPA1, as mentioned in Comments 1 and 2.

Reviewer #3

(Remarks to the Author)

In this revision the authors have done a lot of work, in order to address the issues raised in last review. For the revised manuscript, I have a couple of questions as listed below:

Fig. 1D was not changed. Please explain.

Fig. 2: The statistical analysis of p62 western looks very different from that in the last version. It is also very different from what the western blot shows, at least for shCtrl and #2. Was the quantification accurately performed? If yes, the authors may show the more representative western results, to avoid this confusion.

Similar issue for Figure S1C: the 3 data points shown here for BNIP3 did not show high variance, at least for shCtrl and #2. Thus, does the western blot figure show the highest 3 for ctrl and the lowest 3 for shOpa1 #2?

Line 144: "we treated cells with bafilomycin A1 for 2 hours and noted that autophagic flux was unaffected (Fig. 2D, D'). In Figure 2D, there's obvious difference between Bafilomycin treatment and no treatment (as well as between ShC and ShOpa1), why did the authors state "autophagic flux was unaffected"? If "autophagic flux was unaffected" as claimed, how the conclusion "These data indicate dysfunction in the initiation of the autophagic pathway in OPA1-KD NP cells" were drawn? In the last version, this same conclusion was drawn based on a different observation, which was "failure to enhance LC3II accumulation". Please clarify.

For measuring ATP concentration, the authors responded, "The methods used to calculate the ATP yield or glycolytic capacity/reserve have been extensively described and well established by Mookerjee et al., (PLoS One 2016; J Biol Chem 2017". As Mookerjee et al., measured ATP concentration in pmol/min/ug protein, it is not clear why the authors were measuring pH changes instead of directly measuring ATP concentration, different from what Mookerjee et al did.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this round of revision, the author remains relying on rebuttal, instead of providing new experimental evidence. The rebuttal of using only one shRNA is not convincing and, thus not acceptable to me. The rest of the previous points are particularly addressed, which is Ok with me.

Reviewer #2

(Remarks to the Author)

I acknowledge the author's dedication to addressing the feedback provided. The examination of OPA1 expression in NP across species indicates its essential role, but its ubiquitous expression in other IVD components limits its SPECIFICITY for NP. Hence, I remain concerned about the unresolved issue(s) mentioned in my previous comments.

Reviewer #3

(Remarks to the Author)

In this revision, the authors changed western images, added discussions, and corrected errors, which have addressed most issues, except the following one:

For "Fig. 1D was not changed. ", I meant in the first round of review "In their plots showing fold changes, the authors depicted the values of the control samples always as '1.0' (for example, Fig.1A and 1D, etc.), which may not reflect the variation between different control samples and may have skewed the statistical analysis.". So this comment was to ask if the three identical data points are due to the authors' data processing or their real-world findings (though it is rare to have 3 identical data points in biological studies).

A new question for the new Fig. S15A: it is very informative that the authors provided analyses of mouse, rat, and human NP tissues. Since the authors mentioned "Bonafide markers Acan and Brachyury/T, Keratin 18 and 19 (T, Krt18, and Krt19, respectively) (not shown)", it would be interesting for the readers to appreciate the expression of these NP-specific markers, such as T, Krt18, and Krt19, in mouse, rat and human NP tissues.

A minor issue for Fig. S15A: why did non-NP populations disappear in the plots for Acan and Opa1?

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

My concerns remain the same as in the previous round of revision. First, I don't agree with the author's rebuttal "Duplicate metabolic experiments using shOpa1 clone # 2 will not provide any more insights than those already provided in the manuscript." The authors can't use other data to assume the two shRNA would have similar metabolic effects. Second, while I understand the author's current funding situation, these assays were required from the very first round of review in February 2024. Third, I acknowledge that metabolic experiments could be time and resource-consuming. However, I need to point out that all metabolic experiments for the 2nd shRNA are in vitro assays. Since the authors failed to provide additional experimental evidence during THREE rounds of revisions OVER a year, I wonder if 2 shRNA may not share the same metabolic alterations. Thus, lots of metabolic conclusions of this manuscript are not fully supported by the data, which is not acceptable to me.

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Authors response:

We thank the Editor and the reviewers for their valuable suggestions to improve the manuscript and for recognizing the importance of our findings. We have now provided substantial new data including RNASeq analyses of NP tissues from *Opa1cKO* mice (Fig. 9, Suppl. Figs. 7-14), comparative transcriptomic analyses of *Opa1cKO* tissues with human degenerated NP tissues (Suppl. Fig. S13, S14), additional replicates for metabolomics with a better coverage of metabolites (Fig. 4), IHC staining for OPA1 LC3 (Fig. 6D', H, H'), KRT19 and MMP13 (Fig. S6) on *Opa1cKO* discs. These new findings further support our conclusions that broader metabolic dysregulation and mitochondrial defects promote disc degeneration in *Opa1cKO* mice.

Reviewer's comments

Reviewer #1 (Remarks to the Author):

This manuscript focuses on the role of OPA1 in disc and cartilage. OPA1 is a GTPase mediating IMM fusion, which plays an important role in mitochondrial dynamics. In the study "The loss of OPA1 accelerates intervertebral disc degeneration and osteoarthritis in aged mice", Vedavathi Madhu et al. showed that OPA1 knockdown impaired the morphology of mitochondria and other organelles in the NP cells in vitro, while tissue-specific OPA1-KO mice had age-dependent disc degeneration, osteoarthritis, and vertebral osteopenia in vivo. The age-dependent phenotype of OPA1-KO mice is interesting and striking to me, which is connected to the mechanism of the in vitro studies. However, lacking analysis of the metabolic role of OPA1 in the in vivo model significantly reduced the impact of this study.

We thank the reviewer for his laudatory comments and for recognizing the importance of our findings. We agree with the reviewer and appreciate the insightful suggestion on determining metabolic function of OPA1 in the in vivo model. To address this suggestion, we have performed a RNAseq analyses of NP tissues from 12-month-old *Opa1cKO* mice. We chose this age for the analyses since at this time point phenotypic degeneration in disc is not evident thereby providing us with a better understanding of the underlying metabolic changes and NP cellular heterogeneity that drive degeneration with aging in *Opa1cKO* mice. Our new results clearly demonstrate that OPA1-deletion causes a significant metabolic dysregulation in disc affecting several major pathways including glycolysis, TCA cycle and 1-carbon metabolism along with a prominent deleterious effect on mitochondrial homeostasis. These new results are presented in Figures (Fig. 9; Suppl. Fig. 7, 8, 10) and discussed in the Results and Discussion sections (page #6, #9, #13-16).

Major comments:

1. For the in vitro studies, shOPA1 #2 is missed in many studies, such as Fig. 1E and Fig. 3 et al. It is essential to include both shRNA for all the studies, to avoid the off-target effect of shRNA.

We thank the reviewer for this comment and agree with the importance of using 2 separate ShRNA-clones to avoid any off-target effects. As discussed in the manuscript, the experiments pertaining to mitochondrial morphology, number, mtDNA content, cristae morphology, mitophagy, autophagy, autophagic flux, mitochondrial protein quantification, and ubiquitination are all done with both ShRNA clones. These studies clearly showed that both the ShRNA clones exert a similar effect on the measured parameters and thus the observed changes are specific. Thus, after conclusively establishing the targeting specificity of both clones, we

chose one of the clones for subsequent studies such as seahorse and metabolomics. Importantly, the new LC3B staining on disc sections (Fig.6H, H') and *in vivo* metabolic analyses (Fig. 9) clearly shows that Opa1-deletion both *in vitro* and *in vivo* affect the same biological processes and metabolic pathways, providing a strong credence to specificity of our *in vitro* findings. We have included a note explaining this rationale in the methods and Results section.

2. NP cells were isolated from rats for the in vitro metabolic studies. Similar metabolic analysis should be done with isolated NP cells from the OPA1-KO mice ex vivo. Some assays should be tested in vivo.

We thank the reviewer for this comment. Unfortunately, despite several attempts we could not successfully grow NP cells from OPA1-KO mice. However, we have now performed RNAseq data analyses of NP tissues from 12-month-old *Opa1*cKO mice to understand broader metabolic changes due to OPA1 depletion. We observed significant downregulation of important metabolic pathways involved in hypoxia adaptation, glycolysis, one carbon metabolism, and cholesterol metabolism. Furthermore, autophagy/mitophagy-related DEGs are downregulated in *Opa1*cKO mice (Fig. 9; Suppl. Fig. 7B, 8, 10). These new findings are thus in strong agreement with our *in vitro* findings and highlights a significant metabolic dysregulation in discs of *Opa1*cKO mice.

3. It is unclear why adding glucose reduces OCR in both control and OPA1-KD cells, although this is consistent with their previous publication. This should be further examined. Both cells show higher basal OCR. What substrates were used by these cells in the Seahorse assay? Cells were cultured under hypoxia for 24 hours before the experiment. Were the cells under hypoxia or normoxia during the experiment? The increased OCR in OPA1-KD cells are not in line with the reduced PDH activity (5D).

NP cells reside in hypoxic niche and these cells receive oxygen and nutrients (glucose) by diffusion across the cartilaginous endplates and predominantly use glycolysis for energy production. Several studies have shown that glucose is essential for NP cellular activities such glycolytic flux, protein and proteoglycan production, and cell survival (Autophagy 2020; JCI Insight 2023). Under basal condition, cells were in Krebs Ringer Phosphate HEPES/HRPH (2 mM HEPES, 136 mM NaCl, 2 mM NaH₂PO₄, 3.7 mM KCl, 1mM MgCl₂, 1.5mM CaCl₂, 0.1% [w:v] fatty acid free BSA. In the absence of glucose, cells produce energy using endogenous substrates (glycogen), and when glucose is introduced, the cells switch to glycolysis. During Seahorse assay cells were in Normoxia. We agree with the reviewer that increased OCR does not correlate with lower PDH activity. This non-ATP linked oxygen consumption in OPA1 knockdown cells suggests mitochondrial dysfunction.

4. "Hydroxyproline and hydroxylysine were raised" is not consistent with the data in Fig 4F and 4G, which showed lower levels in KO cells. hydroxyproline and hydroxylysine are not listed in Fig. 4C. Thus, it is unclear if Fig. 4D-Q are the graphs of the same data from the metabolomic assay in Fig. 4C. It is common to include 6-10 repeats in each group for the metabolomic assay. N=4 is very low for this assay, and Fig. 4L-O showed a big variation in the KO cells, which may result in misleading conclusions.

We thank the reviewer for pointing out this oversight. We have now reperformed the metabolic experiments with 6 repeats in each group. Moreover, to extract additional metabolites and increased the coverage of our analysis, we now utilized a higher number of starting cells plated in a 10 cm² plate. With increased starting material, we have identified 416 total metabolites of which 165 were differentially present between the groups. This new metabolic analysis is now presented in Figure 4.

5. “Sigma mean” is not a common way to show isotope tracing data, which is very confusing. MID should be used to show fractional enrichment. M+o should be included as well. It is also important to show the total abundance of all the metabolites in the isotope tracing assay. The current data can’t support all the conclusions.

We appreciate the reviewer’s suggestion. We have now included the M+o data in the graphs and the fraction of all other detected topoisomers is shown (Fig. 5). Since “Sigma mean” is one of the ways of presenting the overall abundance of labelled topoisomers we have retained this parameter in the graphical presentation of the data in addition to M+o data (Kurland et al., J. Biol Chem 2000;275(47):36787-36793; Verma et al., Metabolomics 2015;11:529-544).

The method of isotope tracing is unclear, such as “50% of DMEM media contained ¹³C-labeled glucose.”

We regret the confusion. We used DMEM supplemented with 2.5mM 1,2-¹³C glucose and 2.5mM unlabeled glucose for a total of 5 mM glucose with a 50% label. We have clarified this in the Methods section.

“The enrichment was calculated after subtraction from the background of the non-labeled treatment samples.”, which is not the correct way for data analysis.

We have followed the methodology reported by Jennings and Matthews (Anal Chem 2005;77(19):6435-44) wherein the enrichment in experimental labeled samples is calculated after subtracting the background of the non-labeled treatment samples.

The calculation for the fluxes in 5B-G should be clearly described in this manuscript.

The authors regret the brevity of the description due to the word limit; we have now provided additional descriptions for the flux calculations. These methods have been described in detail in previous publications, citations which are now cited in the revised text (Madhu et al., J. Bone Miner Res 2020;35(8):1504-1524; Autophagy 2023;19(6):1821-1843; Qiu et al., Anal Chim Acta 2007;583(2):277-283).

The lengthy discussion seems to repeatedly cover some contents in the result section.

We have addressed this point in the revised manuscript to avoid repetition of the results in the discussion section.

Minor points:

A typo in line 82.

“.” is missing in line 339.

We have made these corrections.

DAPI-only images should be in Fig. 7.

Due to limitation on the figure size, we have not included separate images for the DAPI channel. We have ensured that the representative merged images clearly show the DAPI staining.

Reviewer #2:

Madhu and colleagues have undertaken a study examining the role of OPA1 in intervertebral disc degeneration and osteoarthritis. Their research sheds light on OPA1's critical involvement in regulating mitochondrial function and metabolism of NP cells in vitro. Furthermore, they provide evidence that deletion of OPA1 accelerates the degenerative process in disc, osteoarthritis, and vertebral osteopenia in mice. However, the authors emphasize examining the effects of OPA1 mainly at the morphological and multicellular levels, which restricts the wider significance of the study. Additionally, a notable absence of comprehensive investigation into the upstream or downstream mechanisms underscores the need for further cooperation or rescue experiments. Addressing these shortcomings would strengthen the study's novelty and align it more closely with the standards of Nature Communications.

The authors thank the reviewer for their valuable feedback and for recognizing the novelty of our findings. To further investigate the mechanisms, we have now performed RNAseq analyses of NP tissues from 12-month-old *Opa1*CKO mice. We chose this time point for the analyses since at this age phenotypic degeneration of discs is not evident thereby providing a better understanding of the underlying mechanisms that drive degeneration with aging in *Opa1*CKO mice. Our new results demonstrate that OPA1-deletion causes a significant cellular dysfunction and alters major metabolic pathways including glycolysis, TCA cycle, and 1-carbon metabolism along with a prominent effect on the integrity of mitochondrial ETC complexes (Fig. 9; Suppl. Fig. 7B, 8, 10). Additionally, critical recycling processes such as autophagy are affected in *Opa1*CKO mice (Fig. 6H, H', Fig. 9K). Importantly, the newly performed comparative analyses of transcriptomic data of *Opa1*CKO mice with patient NP tissue samples clearly show strong thematic similarities between the two data sets with metabolic dysregulation at its core. These additional studies further strengthen our findings and highlight the metabolic dysregulation in *Opa1*CKO mice as the driver of degenerative disc phenotype. It is also important to state that since the noted phenotype is aging-dependent and evident around 20 months of age, it is not feasible to perform rescue experiments given the time and resource constraints.

Major comments:

1. The authors have extensively explored OPA1's role in regulating mitochondrial function and cell metabolism, suggesting that NP cell dysfunction results from OPA1 loss. To strengthen this claim, the authors should elucidate the process of selecting OPA1 as the target using unbiased approaches, such as scRNA-seq.

We thank the reviewer for constructive feedback. We have now included new RNA sequencing results on NP tissue from *Opa1*CKO mice. These new results clearly demonstrate the alterations in major metabolic pathways including glycolysis, TCA cycle, and 1-carbon metabolism along with a prominent effect on the integrity of mitochondrial ETC complexes (Fig. 9; Suppl. Fig. 7B, 8, 10)

2. OPA1 deletion was observed to affect NP cell phenotype and alter the IVD matrix composition in mice. The authors should investigate whether OPA1 deletion changes NP's cellular heterogeneity or notochord cells' cell identity. Mechanisms underlying OPA1-induced cell fate switch should be explored further.

We have now performed RNAseq on 12-month-old mice, when the average modified Thompson grades are comparable across the genotypes. The findings showed DEGs associated with cartilage/chondrocytes, Smad/TGF- β signalling, and CTGF signalling were substantially

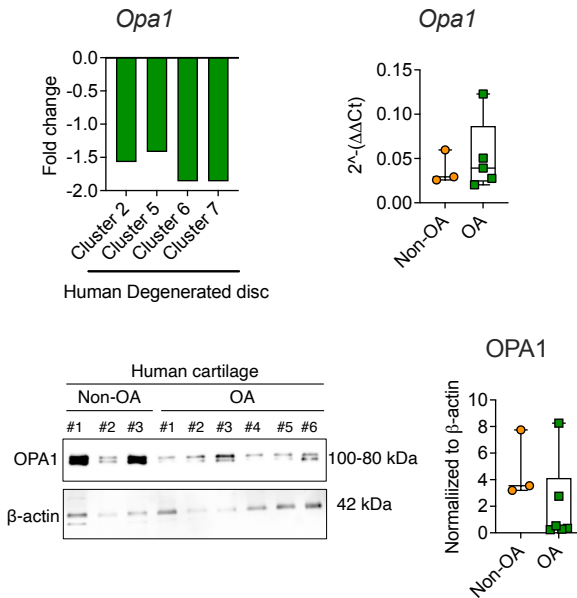
elevated in *Opa1*KO mice. We also performed immunohistochemistry (IHC) on 20-month-old mice for Cytokeratin 19 (Krt19), a marker for the notochord and the NP progenitors (J Cell Physiol 2020;235(1):128-140). *Opa1*KO mice showed a remarkable decrease in Krt19-expressing cells. These findings clearly demonstrate that in the absence of OPA1, NP cells alter their phenotype and contribute to disc degeneration.

3. While this article identifies OPA1-induced changes in the intervertebral disc, cartilage, and vertebrae, rather than discussing them broadly, it would be more beneficial to enhance the depth of in vivo study in the intervertebral disc. It would align more closely with the results of the in vitro study. It is not advisable to include data on cartilage and vertebrae in this study.

As stated above, we have now added RNAseq data from NP tissues of *Opa1*KO mice to investigate in depth the mechanisms of disc degeneration in the absence of OPA1. Additionally, transcriptomic comparison of our findings with the human patient samples shows many similarities and provide strong credence to our findings that dysregulated metabolism and autophagy contribute to disc degeneration. Remarkably, in these mice, we also noted a significant impact on the vertebrae, an integral constituent of the spinal motion segment and articular cartilage of the knee. Since vertebral health is intricately linked to that of the disc and since articular cartilage shares many similarities with disc tissues, it is of high interest to note that these tissues are similarly affected by the loss of OPA1. Moreover, since phenotypes in both the vertebrae and knee joint are novel and have never been reported, we choose to continue including these results in the manuscript.

4. Considering the importance of OPA1 in disc and cartilage, the authors should profile OPA1 expression during the degenerative process of disc and cartilage in humans or mice.

We thank the reviewer for this comment. The analysis of multiple clusters of degenerated human disc NP tissues (each cluster contains several patient samples) shows a significant downregulation of *Opa1* when compared to cluster of healthy NP tissue samples (GSE70362) (Kazezian et al., Sci Rep 2015;5(1):15662; Novais et al., Aging Cell 2020;19(5):e13148). We also include Western blot and gene expression data from human non-OA and OA for the reviewer's perusal. However, due to variability of the disease presentation and underlying phenotypes and endotypes of human OA, we believe that any definitive profiling will require a large number of highly characterized tissue samples. We are also not able to collect the requisite number of healthy control samples and therefore do not believe that these preliminary findings should be included in the manuscript. We are therefore only including these findings for the reviewer's perusal.



5. Disrupting autophagy and dysregulating metabolism impaired OPA1-deficient NP cells in vitro. Validation of these results in OPA1-cKO mice, rather than solely focusing on degeneration evaluation, is recommended.

We agree with the review and have provided additional in vivo results showing metabolic dysregulation and altered autophagy in *Opa1*cKO mice (Fig. 6H, H', Fig. 9, Fig. S7-S14). See our detailed opening response to the general comment above.

6. The authors discussed age-related degenerative phenotypes associated with OPA1 absence in mouse discs. However, they did not observe a decrease in aggrecan or col2 in the nucleus pulposus or an increase in COL1 in the annulus fibrosus, traditional markers of degeneration according to their previous findings. Addressing these inconsistencies and contradictions is essential.

Disc degeneration is a heterogeneous pathology with multiple underlying causes and has differing presentations (Novais et al., *Aging Cell* 2020;19(5):e13148). Accordingly, degeneration seen in *Opa1*cKO mice is qualitatively different from classical age-related disc degeneration noted in wild-type mice. For example, we have previously demonstrated that *MCT4*KO mice show disc degeneration without an appreciable decrease in aggrecan or an increase in chondroitin sulfate or collagen I, however, there was a significant increase in aggrecan-cleavage (Silagi et al., *JBMR* 2020;35(2):550-570). We have added a note in the revised manuscript to provide clarity on the phenotype.

7. In investigating age-dependent disc degeneration and OA accelerated by OPA1-KO, did the authors explore the relationship between OPA1 loss and inflammation, a key mediator of age-related disorders?

The reviewer makes an interesting suggestion. The newly preformed RNAseq of NP tissue of *Opa1*cKO mice showed upregulation of DEGs involved in signaling through TNFR2, TRAF3, TGF- β and IL-11 binding (a member of IL-6 family cytokines). All these signaling pathways are

known to be involved in tissue fibrosis and inflammation. We have discussed these findings in the revised manuscript (Fig. S9, 11, 12A) (page #10,16)

Reviewer #3:

In this work, the authors generated OPA1 loss-of-function in cultured NP cells and mice for detailed phenotypic and metabolic analyses. Notably, they found that OPA1 knockdown in the NP cells changed the morphology of mitochondria and other organelles. And that the cKO mice show degeneration in IVD and joints. Thus, this work provides new information to the field. However, some discrepancies or issues need to be addressed for improving the manuscript.

We thank the reviewer for encouraging comments and recognition of the novelty of our findings.

Major comments:

In their plots showing fold changes, the authors depicted the values of the control samples always as '1.0' (for example, Fig.1A and 1D, etc.), which may not reflect the variation between different control samples and may have skewed the statistical analysis.

We agree with the reviewer we now have addressed this point and now show the actual values of all the control samples reflecting variation between different samples and rerun the statistics.

Figure S1C: The decrease of BNIP3 in ShOpa1 is more obvious than that of CHOP, while the manuscript text and the statistical plot indicate the reverse.

All 6 data points for CHOP show little variance, however there is higher variance in BNIP3 levels (n=6), consequently we do not observe statistical significance for BNIP3.

Figure 2D: LC3II in cells without Baf A1 has no significant changes when there's ShOpa1. This is a discrepancy with Figure 2B and the manuscript text.

We thank the reviewer for pointing this out. We have now performed additional experiments to address this discrepancy where we have simultaneously probed the same blot for both OPA1 and LC3 thus eliminating a possibility of insufficient OPA1 knockdown. Our results are in agreement and clearly show a decrease in LC3II levels in NP cells transduced with ShOpa1.

Figure 3: For ECAR, the line of ShOpa1 is above the control line in Figure 3A, but the authors states "Under basal conditions, ECAR remained unchanged". If the authors did certain calculations to get this conclusion, please describe them in the methods. Also, the authors need to provide details about how to compute other parameters in the plots. In addition, it would be more informative if the authors provide the value of ATP concentration directly for JATP, but not some numbers in the unit of pH changes.

We appreciate the point the reviewer is raising and agree that ShOpa1 line is above the ShCtrl line in Figure 3A, however, there is no statistical difference between the groups. The methods used to calculate the ATP yield or glycolytic capacity/reserve have been extensively described and well established by Mookerjee et al., (PLoS One 2016; J Biol Chem 2017; J Vis Exp 2015; Biochem Biophys Acta 2015) and have been reported by us in relationship to NP cells (Madhu et al., Autophagy 2023;19(6):1821-1843; Johnston et al. JCI Insight 2023;8(8):e164883). We have cited this literature in the revised manuscript but could not describe it in detail due to strict limits on the word count and manuscript length.

Also in Figure 3: the text and legend miss the description of some figures.

We now have rectified these issues.

Figure 4: there are discrepancies between what the figure shows and what the manuscript text states: "hydroxyproline, and hydroxylysine were raised" and "s-adenosyl methionine were significantly lower in OPA1 knockdown cells." Also please check If the enrichment analysis is based on this.

We thank the reviewer for pointing out these errors. We have now addressed these issues.

Line 254: "caudal discs of Opa1cKO mice showed changes in NP cell morphology", please elaborate the changes.

We have elaborated this point in the revised text (page #7).

The authors may consider an immunohistochemistry assay of Opa1 in the cKO mice. In addition, examination of a notochordal marker in the cKO mice would provide necessary information of the phenotype.

We thank the reviewer for this suggestion. We have now included IHC on OPA1 and notochordal marker KRT19 (Fig. 5D', Fig. S6).

Line 310: "a substantial increase in mean total and the cross-sectional thickness (Cs.Th.)". Figure 8K seems to show no significant changes.

Regarding the bone parameters in Fig. 8, the authors should provide more details about their measurement and provide interpretation and discussion why Opa1 cKO caused these changes in trabecular and cortical bones and how they affect vertebral bone health.

We appreciate the suggestions. We have clarified the discrepancy in the text and provided additional discussion on changes in the vertebral bone in the revised manuscript (Page #9).

Figure 9: the authors need to provide details for calculating toluidine blue scores and osteophyte scores.

Hindlimbs were decalcified (EDTA, 19%, 21 days), processed, paraffin embedded, and sectioned along the coronal plane, as we have described (Collins et al., Ann Rheum Dis 2023;82:1464-1473). Midcoronal sections were chosen, and OA severity was analyzed by histology and histomorphometry. For histological analysis, sections were stained with hematoxylin and eosin (H&E) or toluidine blue. The Articular Cartilage Structure (ACS) score (0-12 scale) and toluidine blue score (0-12 scale) were independently analyzed on medial tibial plateaus, lateral tibial plateaus, medial femoral condyles, and lateral femoral condyles, as we and others have previously described (Collins et al., Ann Rheum Dis 2023;82:1464-1473; McNulty et al., Cartilage. 2011;2(4):354-63; Armstrong et al., Osteoarthritis Cartilage. 2021;29(4):536-46). Briefly, the ACS and toluidine blue scores for OA damage are calculated using a 0-12 scale where: 0=normal/no damage; 1-3=minimal damage; 4-6= mild damage; 7-9=moderate damage; and 10-12=severe damage. Osteophytes (0-3 scale) were scored on the medial and lateral femurs and tibias of all mice using H&E stained sections, as we have described (Collins et al., Ann Rheum Dis 2023;82:1464-1473; McNulty et al., Cartilage. 2011;2(4):354-63; Armstrong et al., Osteoarthritis Cartilage. 2021;29(4):536-46), were: 0=no osteophyte; 1=small osteophyte

(same as cartilage thickness), 2=medium osteophyte (up to 3 times the thickness of cartilage); 3=large osteophyte (greater than 3 times the thickness of cartilage). All sections were scored by a blinded individual that has experience of the grading schemes. Synovial hyperplasia was assessed using a 0-3 scale as described in (McNulty et al., Cartilage. 2011;2(4):354-63; Armstrong et al., Osteoarthritis Cartilage. 2021;29(4):536-46). In joints that presented with synovial ossification, a maximal synovial hyperplasia score was given. Analysis was performed by a blinded scorer with experience of the OA scoring techniques. Detailed histomorphometric analysis of articular cartilage thickness and area, calcified cartilage thickness and area, and subchondral bone thickness and area were analyzed on the MTP and LTP using ImageJ software as we have previously described (Collins et al., Ann Rheum Dis 2023;82:1464-1473). We have included this expanded description in Method section.

Figure 9G: does the asterisk in the lateral side denote osteophyte or subchondral bone? A cartilage staining but not H&E would help to clarify this.

The asterisk represents osteophyte. We now have replaced H&E staining with toluidine blue to provide a better clarity.

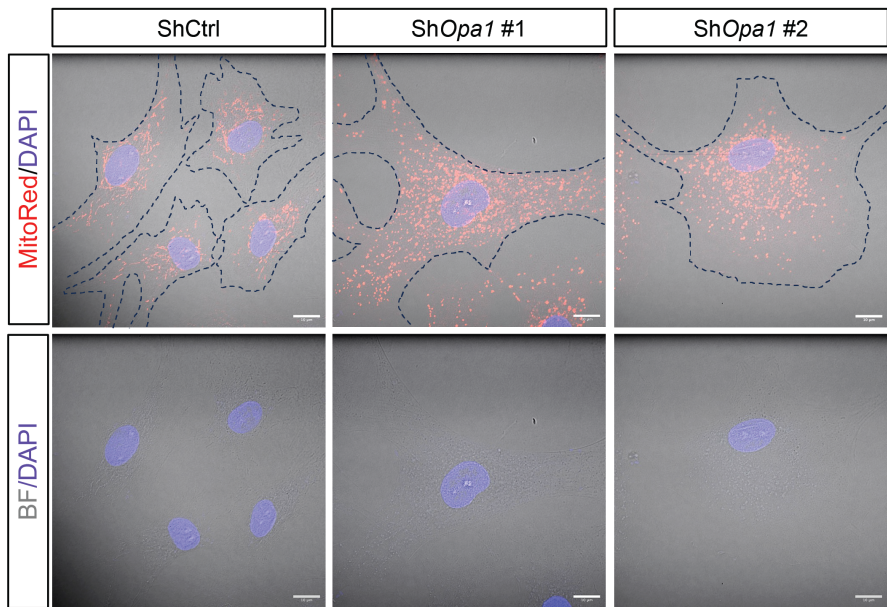
Since Figure 9 and 10 are based on the same histology images, they should be consolidated into a figure.

We have consolidated the data and have moved Figure 9 to supplementary material.

Minor issues:

Figure 1: the authors may consider including bright field microscopic images of the Opa1-depleted NP cells, to show the morphology of the cells.

We did not include the brightfield images due to space limitations within the figure but have included an image here for the reviewer’s perusal.



The fluorescence intensity could be varied between ctrl and ShOpa1, as in shCtrl, PMP70 is too weak compared to Mito, and GM130/Mito is showing yellow, while ShOpa1 showed stronger PMP70 and GM130, which overshadowed the Mito fluorescence. Does this reflect that these organelles have higher levels in ShOpa1, or it is just due to some technical issues?

Confocal images for a specific marker were acquired at the identical setting for ShCtrl and ShOpa1 cells. Moreover, we did not quantify the fluorescence intensity for the individual markers but instead used them to identify the specific organelles to observe their morphology. We have included an image here for the reviewer's perusal.

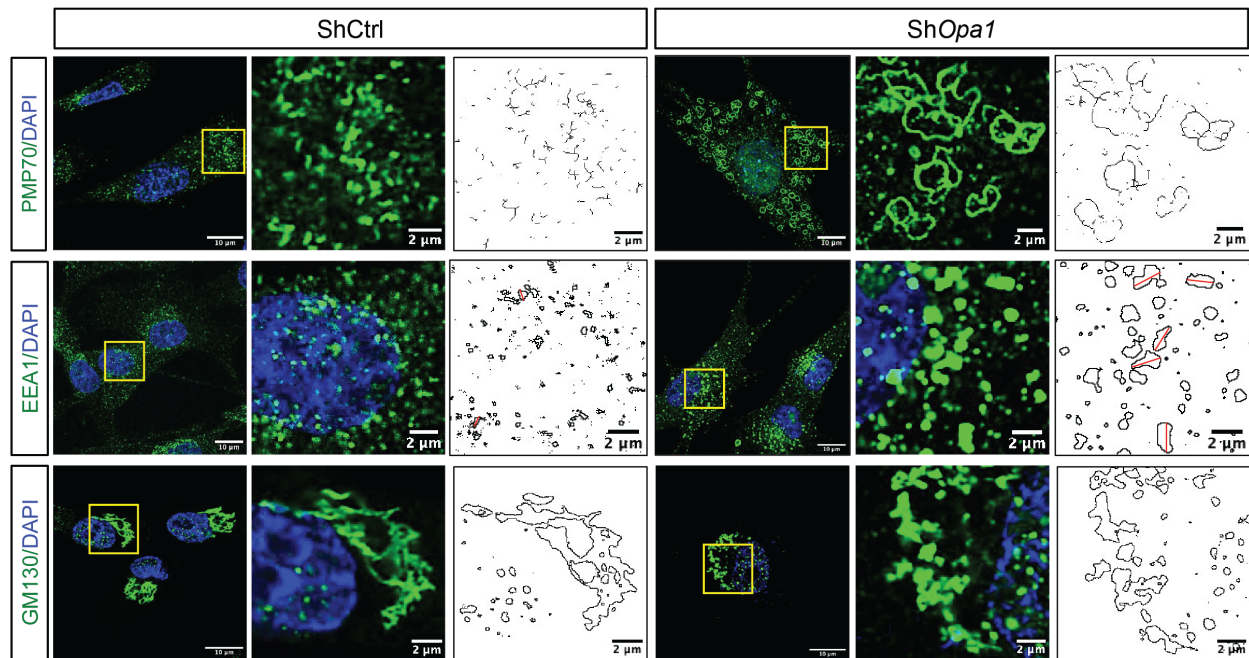


Figure 2A: Green fluorescence (LC3B) has variations among different cells in shCtrl. Thus, the authors may need to include a statistical analysis here. In addition, the red and green don't have to be merged, which interferes with the observation of green fluorescence, if Mito and LC3B don't necessarily colocalize.

We have shown here a representative images of individual fluorescence channels for the LC3 and mitochondrial staining experiment for the reviewer's perusal. However, due to limited space within each figure and the limitation on the number of figures, we chose to show the merged channel where the marker staining can be observed in conjunction with mitochondria. As far as variability in staining between individual cells in the ShCtrl group, this is to be expected for the primary cells and therefore multiple cells from different staining experiments were used for the quantification of LC3 puncta as is standard practice. We have included the quantitative data with appropriate statistical analysis for LC3 staining in the manuscript which clearly shows a significant decrease in LC3 puncta number in cells transduced with both ShOpa1 clones (Fig. 2A, A').

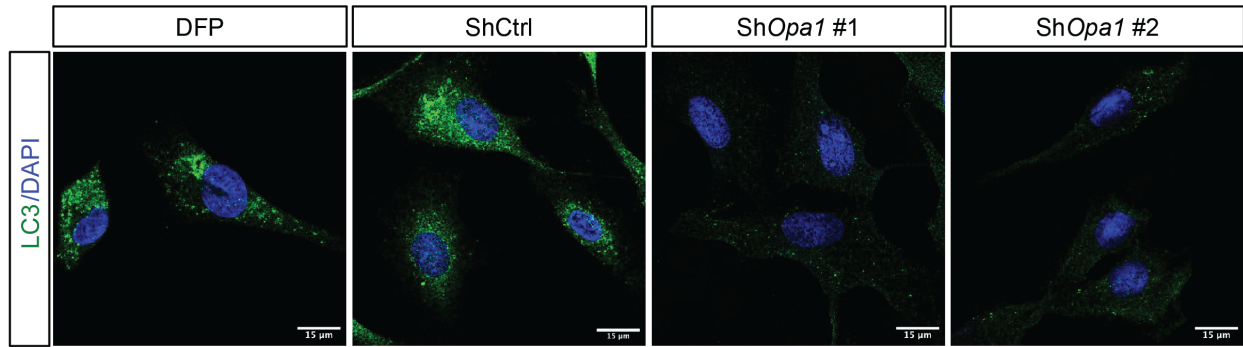


Figure 7E: the AF should be demarcated like in other panels.

We have now demarcated the AF compartment as suggested.

Figure 8: it would be helpful if the authors denote the VL and DH in the CT images for better appreciation.

We have denoted the VL and DH on the CT images.

Authors Response

We thank the Editor and the reviewers for their valuable suggestions to improve the manuscript and for recognizing the importance of our findings. Now we have addressed the reviewers' concerns and provide additional analyses of deposited scRNAseq data from NP tissue of the mouse, rat and human to demonstrate specificity of Opa1 deletion in NP tissue (Supple Figure S15).

Reviewer #1:

It is good to see the new Figure 9 showing the metabolic alteration in the OPA1 mice model. However, most of the previous concerns are partially addressed.

First, the metabolomics experiment is updated to n=6 (4 is listed in the legend) for one shRNA. It is unclear why the authors didn't include the second shRNA in this assay. The rebuttal to the previous Comment 1 seems to be logical, but not reasonable, since the similar result in some assays can't predict the other assays.

We thank the reviewer for pointing out the oversight. We have amended the manuscript to indicate that the widely targeted metabolomics was performed on 6 independent samples (n = 6). We understand the reviewer's point about the use of a single clone in metabolic studies; however, as we indicated earlier, both the *Opa1* shRNA clones showed a very similar *Opa1* knockdown efficiency in NP cells. Importantly, knockdown by both clones demonstrated a similar effect on mitochondrial morphology, numbers, and mitophagy. Noteworthy, and most importantly, our in vitro knockdown experiments showing metabolic dysregulation in NP cells are validated and supported by robust in vivo data, wherein similar processes and metabolic pathways are affected in the NP tissue of *Opa1*cKO mice underscoring mitochondrial dysfunction (Figure 9; Figures S8; S10; S12). This strong congruence between in vitro (using shRNA) and in vivo results highlights a high phenotype specificity and addresses the biological role of *Opa1* in the NP cells. We, therefore, opine that while a single clone was chosen for the in vitro metabolic studies, our findings are valid and highly relevant to the in vivo function of *Opa1* and to the study's overall conclusions. It is also important to note that our in vitro knockdown studies are conducted using primary NP cells, which are difficult to isolate and expand, and therefore, obtaining enough cells for metabolic assays requires the use of a large number of animals. For this and the reasons stated above, we chose to use a single clone for select metabolic studies.

Second, related to previous Comment 3, the author only provided some rebuttal in the responding letters, without any new data or new discussion in the manuscript. The previous concern about the OCR is not addressed at all in the revised manuscript.

We appreciate the reviewer pointing this out and regret the confusion. Concerning the decrease in OCR, since NP cells rely primarily on glycolysis for energy production, when glucose is available, cells "switch" to more glycolytic metabolism, which lowers oxidative metabolism, thereby decreasing oxygen consumption/OCR. We have now explained this

observation in the revised manuscript (page 6, line 171-174).

Third, related to previous Comment 5. The authors provided references to justify their method for isotope tracing assay. However, there are many other references showing widely accepted approaches in isotope tracing assay, which are ignored by the authors.

Indeed, we agree and acknowledge that researchers use many methods for isotope tracing assays. However, we chose to use the sigma mean approach that was reported by Kurland et al. (*J. Biol Chem* 2000;275(47):36787-36793) and Verma et al. (*Metabolomics* 2015;11:529-544) because of our familiarity with this method, and its prior use when performing metabolic studies of the NP cells (Madhu et al. *Autophagy* 2023).

The authors stated to correct the “50% of DMEM media contained ¹³C-labeled glucose.”, which is not clarified in the revised manuscript. Thus, these data remain questionable.

We regret the confusion, and we have now clarified this in the Methods section as “DMEM was supplemented with 2.5mM 1,2-¹³C glucose and 2.5mM unlabeled glucose for a total of 5 mM glucose with a 50% label” (Page 20, line 599-600).

Reviewer #2:

Although the authors made extensive revisions in the updated manuscript, most concerns raised by the reviewer were not directly addressed. Notably, NP cells have been shown to be heterogeneous in both humans and mice (DOI: 10.1038/s41467-023-44313-9 and 10.1096/fj.202301217R). Performing single-cell RNA sequencing on NP cells from OPA1-KO mice, rather than bulk RNA-seq, could elucidate the specific target cell populations affected by OPA1, as mentioned in Comments 1 and 2.

The authors appreciate the reviewer's comment. We now provide analyses of deposited scRNAseq data from NP tissue of the mouse (P30, Zhang et al. *FASEB J.* 2024, GSM749668 and 8-week-old, Tan et al. *Cell Rep.* 2024, GSE186549), rat (Rohanifar et al. *Appl Sci* (Basel) 2022 GSE211407) and human (Chen et al. *Nat Commun* 2024, GSM7831813) (Suppl. Fig.S15). This analysis clearly shows that NP cells and all the constituent subpopulations, regardless of the species, express Aggrecan (*Acan*) as well as *Opa1*, and will therefore exhibit similar phenotypic changes following *Opa1* deletion in *Acan*-expressing cells (*Opa1* deletion in our *Opa1cKO* mice is driven by *Acan*-CreERT2 driver). We have included a comment in the revised manuscript to discuss this point (page 10 and 17, line 310-316 and 503-506; page 23, line 706-715).

It is important to note that the published studies show that NP tissues in mice, rats, and human are largely homogenous in their cell composition and are derived from the embryonic notochord. Particularly, in the mouse, 2 major cell populations within the NP

(mature and progenitor cells) have been identified that show a significant overlap in their transcriptome with only a small number of differentially expressed genes. The authors of these aforementioned studies have concluded that the NP cell populations in mice represent different states of cellular differentiation: differentiated/mature and undifferentiated/immature. More importantly relevant to this study, as indicated above, all these NP cell populations express *Opa1* and high levels of Aggrecan. In contrast to mice, in the human NP, while several constituent cell clusters/populations, including those with fibrotic and chondrocytic properties, have been proposed, a careful review of the unsupervised clustering of scRNAseq data clearly shows a more homogenous NP cell cluster with a significantly overlapping transcriptome. Notably, the gene signature definitions used to define the constituent NP cell subclusters in human discs are not widely adopted, and consequently, in healthier human intervertebral discs, different studies have reported cell clusters with differing characters (Chen et al. *Nat Commun* 2024, Wang et al. *iScience* 2023). Likewise, there is a lack of extensive functional validation concerning the role of these subpopulations within the human NP. Importantly, based on the pseudotime trajectory analyses, these subclusters represent differing states of differentiation within NP tissue (Chen et al. *Nat Commun* 2024, Wang et al. *iScience* 2023), and they all express high levels of Aggrecan and show a uniform *OPA1* expression (Suppl. Fig. S15). We, therefore, opine that, like mice, in humans, loss of *OPA1* in aggrecan-expressing NP cells will affect all the constituent cell subpopulations regardless of their function or the state of differentiation.

We would also like to highlight that it is not feasible for us to generate scRNAseq on the *Opa1*CKO mice since it will require more than a year to generate an adequate number of mice for this experiment. We have already provided the bulk-RNAseq data and extensive characterization of the transcriptomic changes from the NP tissue of *Opa1*CKO mice in the manuscript, which clearly underscore important degenerative and metabolic changes in the tissue.

Reviewer #3:

In this revision the authors have done a lot of work, in order to address the issues raised in last review. For the revised manuscript, I have a couple of questions as listed below:

Fig. 1D was not changed. Please explain.

Two independent studies from Tazze et al., (*Cell Metabolism* 2017; 25:1374–1389) and Cogliati et al., (*Cell* 2013;155:160–171) have shown that chronic or prolonged inhibition of *OPA1* leads to mitoDNA depletion, whereas changes in the cristae shape, mitochondrial morphology and function are an early consequence of *OPA1* deletion. Our results corroborate the two studies mentioned above, and we have, therefore, retained our earlier findings from Fig. 1D.

Fig. 2: The statistical analysis of p62 western looks very different from that in the last version. It is also very different from what the western blot shows, at least for ShCtrl

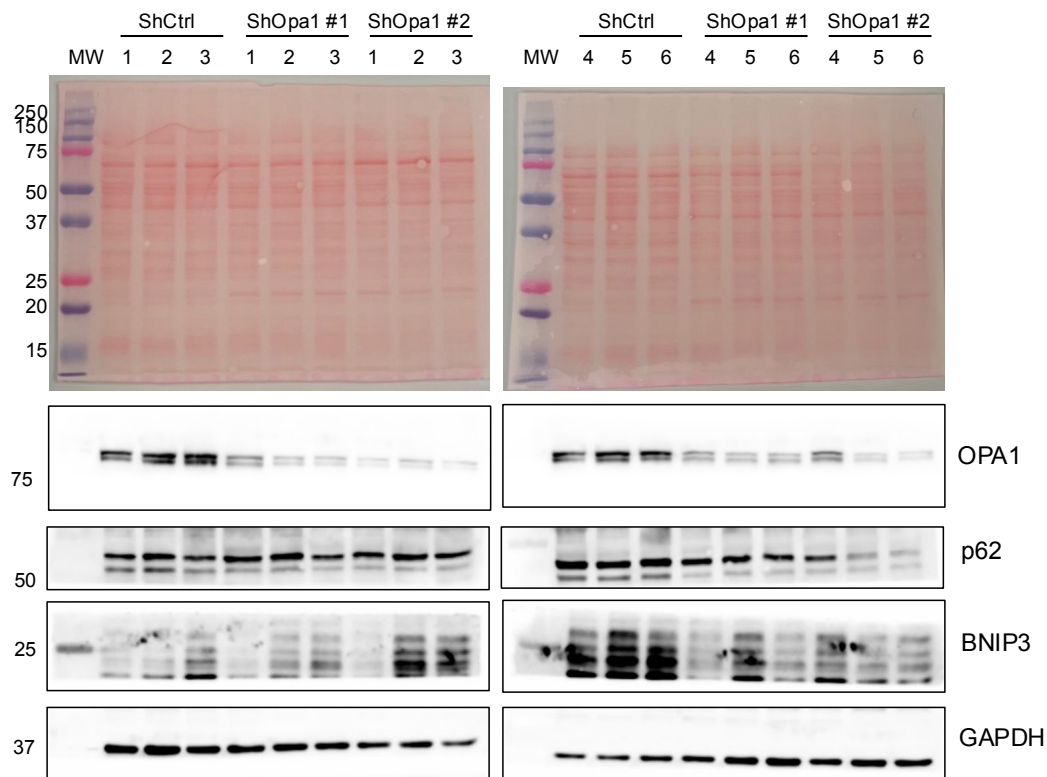
and #2. Was the quantification accurately performed? If yes, the authors may show the more representative western results, to avoid this confusion.

The authors regret the confusion. We have confirmed that the statistical analysis of p62 results is accurate and now include a more representative Western blot image.

Similar issue for Figure S1C: the 3 data points shown here for BNIP3 did not show high variance, at least for ShCtrl and #2. Thus, does the western blot figure show the highest 3 for ctrl and the lowest 3 for shOpa1 #2?

We regret the confusion; we have confirmed the quantification and statistical analysis of Western blot results showing BNIP3 levels shown in Suppl. Figure 1C and have included a more representative image.

For the reviewer's perusal, we show below all six independent Western blot samples for OPA1, p62, BNIP3, and GAPDH with corresponding ponceau red stained membranes.



Line 144: "we treated cells with bafilomycin A1 for 2 hours and noted that autophagic flux was unaffected (Fig. 2D, D'). In Figure 2D, there's obvious difference between Bafilomycin treatment and no treatment (as well as between ShC and ShOpa1), why did the authors state "autophagic flux was unaffected"? If "autophagic flux was unaffected" as claimed, how the conclusion "These data indicate dysfunction in the initiation of the autophagic pathway in OPA1-KD NP cells" were drawn? In the last

version, this same conclusion was drawn based on a different observation, which was “failure to enhance LC3II accumulation”. Please clarify.

We regret the confusion and now clarify this point in the revised manuscript. We observed that *Opa1*-knockdown in NP cells resulted in decreased LC3II levels compared to cells transduced with ShC, indicating that there was either an overall decrease in the autophagy or autophagy happening at a much faster rate. To ascertain this, we treated cells with Bafilomycin A1, an inhibitor of autophagic degradation. This short-term treatment caused robust accumulation of LC3II in Sh*Opa1* cells, although the accumulated levels did not exceed levels seen in BafA1 treated control group (ShC), suggesting that a) decreased LC3II under basal conditions were not due to increased autophagic flux, has it been the case, BafA1 treatment would have resulted in significantly higher levels compared to control b) *Opa1*-knockdown did not inhibit the acidification of autolysosomes or autophagosome-lysosome fusion and thus autophagic degradation of the cargo, implying that the *Opa1* knockdown caused an overall decreased in autophagy. Moreover, a slightly less accumulation of LC3II in BafA1 treated control cells also suggest autophagic flux at a steady state, wherein fewer faulty organelles and proteins require degradation. Accordingly, we conclude that "while autophagic flux was unaffected," there was an overall decrease in autophagy". We have reworded the statements in the result section to reflect this explanation (Page 5, line 134-135 and 145-155).

For measuring ATP concentration, the authors responded, "The methods used to calculate the ATP yield or glycolytic capacity/reserve have been extensively described and well established by Mookerjee et al., (PLoS One 2016; J Biol Chem 2017". As Mookerjee et al., measured ATP concentration in pmol/min/ug protein, it is not clear why the authors were measuring pH changes instead of directly measuring ATP concentration, different from what Mookerjee et al did.

We have employed this method in our earlier research on NP cells (Madhu et al. *Autophagy* 2023) and sincerely regret the typographical error in labeling the ATP measurement units and the confusion it caused. We confirm that, as reported by Mookerjee et al. (*PLoS One* 2016; *J Biol Chem* 2017) the unit for ATP measurement is pmol/min/ug protein (Figure 3D) and mpH/min/ug protein for the glycolytic capacity (Figure 3G).

AUTHOR'S RESPONSE

Reviewer #1:

In this round of revision, the author remains relying on rebuttal, instead of providing new experimental evidence. The rebuttal of using only one shRNA is not convincing and, thus not acceptable to me. The rest of the previous points are particularly addressed, which is Ok with me.

The authors thank the reviewer for appreciating the extensive revisions and additional data we have provided during the first two rounds of revision. To address the reviewer's remaining comment, we now provide a TEM image showing alteration in Golgi morphology for the second shRNA clone (Fig. S2A). Additionally, we include confocal images showing altered morphology of cis-Golgi (GM130), peroxisomes (PMP70) and early endosomes (EEA1) following transduction of NP cells with sh*Opa1* clone #2. These new results are in agreement with our earlier observations with sh*Opa1* clone #1 and further support the specificity of the knockdown experiments and the biological consequences of *Opa1* loss in NP cells.

We would also like to draw the reviewer's attention to several experiments included in Figure 1, where both the shRNA clones were used and clearly demonstrated very similar knockdown efficiency and responses of NP cells in terms of changes in mitochondrial ultrastructure, morphology, mitochondrial numbers, and their effect on mitochondrial DNA copy numbers. Furthermore, in Figure 2, we show that both the shRNA clones again have identical effects on multiple parameters affecting autophagy and mitophagy in NP cells, using immunofluorescence imaging and Western blot analyses. Most importantly, our extensive *in vivo* studies, generating and characterizing disc-specific-*Opa1* knockout mice (we aged and characterized these mice until 2 years), strongly support our *in vitro* loss-of-function experiments and demonstrate a similar effect on autophagic pathways and cell metabolism. Thus, a strong congruence between *in vitro* and *in vivo* studies further addresses any concerns about the non-specificity of a few knockdown studies performed with a single shRNA clone. Notably, a very similar degenerative phenotype seen in knee articular cartilage where knockout mice develop osteoarthritis further validates the central conclusion of the study that dysregulation of mitochondrial fusion through deletion of *Opa1* is deleterious to the health of cartilaginous tissues.

We believe that at this juncture of the review, with newly included organelle morphology data and the additional metabolic experiments that we had included during the first cycles of the review, duplicate metabolic experiments using sh*Opa1* clone # 2 will not provide any more insights than those already provided in the manuscript. We want to highlight that these metabolic experiments are time and resource-consuming, and with our current funding status having two of our NIH grants supporting this work ending, we are not in a position to perform them.

Reviewer #2:

I acknowledge the author's dedication to addressing the feedback provided. The examination of OPA1 expression in NP across species indicates its essential role, but its ubiquitous

expression in other IVD components limits its SPECIFICITY for NP. Hence, I remain concerned about the unresolved issue(s) mentioned in my previous comments.

The authors appreciate the reviewer's laudatory comment on our proactive approach to addressing their comments. It is important to note that all mammalian cell types and tissues have mitochondria, except for the red blood cells. Importantly, OPA1 is the master regulator of inner mitochondrial membrane fusion regardless of the cell or tissue type. We concur that targeting *Opa1* in the disc compartment with Acan-CreERT2 has an impact on the IVD compartment as a whole; nevertheless, bulk RNAseq performed on NP tissues from *Opa1*-cKO mice revealed that the transcripts most impacted were those linked to metabolism and autophagy/mitophagy (Figure 9). Moreover, we also believe that AF cells and articular chondrocytes, which, similar to NP cells, exist in an avascular environment, and evidence a degenerative phenotype in *Opa1*cKO mice will have more or less similar cellular response following *Opa1* deletion. Our study highlights that altered tissue metabolism and mitochondrial quality control are the main causes of the disc and cartilage phenotype seen in our mouse model, and thus, NP cells are representative of above-mentioned cell types in our in vitro experiments.

Reviewer #3:

In this revision, the authors changed western images, added discussions, and corrected errors, which have addressed most issues, except the following one:

For "Fig. 1D was not changed. ", I meant in the first round of review "In their plots showing fold changes, the authors depicted the values of the control samples always as '1.0' (for example, Fig.1A and 1D, etc.), which may not reflect the variation between different control samples and may have skewed the statistical analysis.". So this comment was to ask if the three identical data points are due to the authors' data processing or their real-world findings (though it is rare to have 3 identical data points in biological studies).

We apologize for overlooking the mtDNA/nuDNA graph in Figure 1D. In place of the fold change, we have now provided the actual data points of control.

A new question for the new Fig. S15A: it is very informative that the authors provided analyses of mouse, rat, and human NP tissues. Since the authors mentioned "Bonafide markers Acan and Brachyury/T, Keratin 18 and 19 (T, Krt18, and Krt19, respectively) (not shown)", it would be interesting for the readers to appreciate the expression of these NP-specific markers, such as T, Krt18, and Krt19, in mouse, rat and human NP tissues. A minor issue for Fig. S15A: why did non-NP populations disappear in the plots for Acan and Opa1?

We have now presented the expressions of the markers Brachyury/T, Keratin 18 and 19 (T, Krt18, and Krt19, respectively) in NP tissue from mice, rats, and humans (Supple Figure 15). Additionally, we have incorporated non-NP populations into the Acan and *Opa1* plots (Fig. S16).