nature portfolio

Peer Review File

Compensatory activity of the PC-ME1 metabolic axis underlies differential sensitivity to mitochondrial complex I inhibition.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, del Prado and colleagues propose metabolic reprogramming by pyruvate carboxylase (PC) and malic enzyme 1 (ME1) as the adaptive mechanism underlying the differential sensitivity of brain cell types (astrocytes and neurons) towards mitochondrial dysfunction. By using pharmacological, viral as well as CRISPR-based approaches in cell lines to manipulate metabolic pathways, they provide evidence that OXPHOS-deficient cells are capable to maintain NADPH levels by leveraging PPP, and that increasing expression of ME1 alleviates the lethality observed when PPP is also hampered. By both reanalyzing available proteomics datasets of cancer cell lines as well as performing new experiments in vitro and in vivo, they suggest that this mechanism is conserved across cell types and could explain the selective vulnerability of some cancer cells towards OXPHOS inhibitors. Lastly, they propose that this mechanism is behind the selective vulnerability of neurons in the context of OXPHOS dysfunction, based on the different protein expression levels of these enzymes in primary cortical neurons versus astrocytes, as well as in vivo evidence of reduced neuroinflammation upon overexpression of ME1 in Ndufs4 KO mice.
The topic addressed in this study is extremely interesting for the field of mitochondrial disease, as the molecular mechanisms underlying the differential sensitivity of tissues or cells (and especially brain tissue) towards mitochondrial dysfunction are still poorly understood. In particular, the authors have performed quite some experiments to extend the previous yet uncomplete knowledge on the synthetic lethality between ETC and PPP, and I am positive the results will be of relevance for the field. The in vivo data on Ndufs4KO mice are quite interesting but still preliminary.
Major concerns:
- The original focus of the study proposed by the authors both in the title and in the abstract - the PC-ME1 axis in brain cells - is not well represented in the manuscript. In fact, the data provided are rather spanning from cell lines to cancer, with a minor part of the figures addressing these questions in the brain. Along the same line, I have found the part on the cancer a bit disconnected from the rest of the data. Perhaps the author should consider whether keeping these different aspects in the manuscript, but rephrasing the title and abstract. Also, if the focus is on brain cells, the experimental part on this should be strengthened (see points below).
- The authors claim they revealed that PC and ME1 expression patterns are different between astrocytes and neurons (lines 386-387, and 486-488). While the metabolic rewiring exerted by these two enzymes is very interesting, their differential protein expression between cultured astrocytes and neurons (and to some extent in situ) is known since decades (https://doi.org/10.1007/s11064-010-0257-5). Similarly, astrocytes are well known to possess a heightened metabolic flexibility well beyond their glycolytic capacity mentioned by the authors (lines 375-276) The authors should rephrase these statements in the light of the existing literature on the subject.
- The authors show that CI inhibition does not lead to death of neurons, but only to morphological changes (lines 366-367). However, cell viability of neurons is shown only after 24h of treatment, whereas the corresponding quantification is performed up to 72h in astrocytes. The morphological changes of neurons treated with piericidin as those shown in Fig. S5B are quite compatible of those marking stages of neuronal degeneration preceding cell death. The authors should also include quantification of cell viability of neurons for both 48h and 72h.
- Based on the representative image shown in Fig. S5B it is not clear how the authors could quantify axons or perform FRET measurements in axons at this time-point (Fig. S5I), considering the near

complete loss of neuronal processes at this stage. Also, axon morphology analysis by integrated density of images using beta3-tubulin labeling is not appropriate, as this antibody uniformly labels neuronal processes without distinction amid dendrites and axons. Markers such as SMI-312 would be more appropriate.
- The in vivo data on Ndufs4KO mice lack a few controls. The authors should at least a) validate the differential expression of either PC or ME1 in vivo between astrocytes and neurons in vivo, in control mice ; b) showing evidence of increased ME1 expression in tissue upon AAV9 infection, since presence of the GFP reporter alone is not sufficient
- Alleviation of neuroinflammation in Fig. 6D is quantified by the area positive for Iba1, a well established microglia marker. While expression of Iba1 is undoubtedly increased in reactive microglia, Iba1 should also label resting microglia, whereas AAV9_ME1 samples seem devoid of any signal. Can the authors explain this? I would also suggest to provide a zoom in for the Iba1/GFP channels shown in Fig. 6D, to disclose cellular morphological details and immunoreactivity of resting microglia.
- While ME1 overexpression has beneficial effects on motor coordination Ndufs4KO mice, there is no indication that the mechanism suggested in cultured neurons is truly applicable in neurons in vivo. The authors acknowledge that AAV9 delivery is not specific for neurons, however immunohistochemistry and /or TEM analysis of AAV9_veh and AAV9_ME1 slices should be performed to analyze morphological and ultrastructural features of infected neurons to provide some insight if boosting ME1 function is indeed beneficial for neurons. Likewise, the same metabolomic analysis shown for Ndufs4 brain samples in Fig S1E should be repeated in these settings to show if boosting ME1 function is restoring the levels of PPP-associated metabolites.
Minor points:
- The panel order is difficult to follow in Fig. 2, S2 and S5. There is no panel E in Fig. S3
- The text contains some typos: line 176 “lactated”, line 216 “focued”, line 434 “Figure C”

Reviewer #2 (Remarks to the Author):

In this manuscript, Prado, et al identify that inhibition of ETC complexes induces a dependency on the pentose phosphate pathway (PPP) for NADPH production. Upon dual inhibition of the PPP and ETC, malic enzyme 1 functions as an alternative source of NADPH. The expression of malic enzyme 1 can predict sensitivity of cells to ETC inhibition, including astrocytes and neurons in the brain. In a preclinical mouse model of complex I deficiency, overexpression of malic enzyme 1 mitigates neuroinflammation and motor function, establishing the physiological relevance of this metabolic adaptation to ETC inhibition.

This manuscript is both exciting and novel and warrants publication in Nature Communications after revision. Among the most exciting aspects of the paper include the fundamental connection between complex I activity and NADPH pools in mammalian metabolism. Moreover, complex I inhibition causing increased pyruvate carboxylase activity is quite interesting. Finally, the authors propose an interesting diversion of glutamine-derived carbon through the reductive carboxylation pathway and subsequently malic enzyme 1 as a means to generate NADPH upon complex I inhibition.

Below are suggested experiments to strengthen the claims made by the authors:

• In figure 1 the authors demonstrate a synthetic lethality of inhibition of the pentose phosphate pathway (PPP) with complex I inhibition. They show elevated PPP intermediates upon ETC inhibition, which could mean increased PPP activity but could also mean inhibition of downstream reactions such as nucleotide synthesis. To strengthen the claim that ETC inhibition increases the PPP the authors should perform 13C6-glucose tracing into the PPP intermediates in vehicle, complex I, and complex III inhibited cells +/- G6PD depletion. Additionally, 13C6-glucose tracing should be done in GFPT1, PHGDH, and MPC KO cells +/- complex I and III inhibition to demonstrate no difference in PPP incorporation, as these pathways were not synthetic lethal with ETC inhibition.

• 13C6-glucose tracing into the PPP intermediates should be done in astrocytes versus neurons to demonstrate this difference in the two cell types.

• The authors suggest that ME1 activity increases in cells with dual PPP and complex 1 inhibition. It would strengthen the manuscript to directly test ME1 activity in their cells treated with vehicle, PPP inhibition, complex I inhibition, and the combination. One assay the authors could use to test ME1 activity is 13C4-aspartate tracing, which will label the 13C4-oxaloacetate, 13C4-malate, and 13C3-pyruvate. An alternative ME1 activity assay would also be acceptable.

• The authors should perform metabolomics on the AAV-induced ME1 overexpression mouse model to measure NADPH and malate levels in the brain samples.

• The authors provide a lot of experimental evidence that the pentose phosphate pathway and malic enzyme are regulators of NADPH upon complex I inhibition. Another possible way to link complex I inhibition to NADPH pools is the NAD kinase reaction, whereby complex I inhibition decreases NAD+ levels, decreasing the amount of NADP generated via NAD kinase. It would be great if the authors could either experimentally test this potential connection between complex I and NADPH pools or add some text about it to the discussion.

• Throughout the manuscript, the authors sometimes report NADPH levels and other times report the ratio of NADPH:NADP. It would be ideal if both the relative levels of NADPH and NADP, and their ratio, is reported for each experiment

• Throughout the manuscript the authors refer to their perturbations as “ETC inhibition.” Since most of the assays only test complex I inhibition, it would be better for the authors to specify which complexes are being inhibited for each experiment, instead of broadly calling it ETC inhibition.

• The authors show the expression of ME1 in a panel of cell lines can predict their ETC dependence. Where do 143B cells, which were used in the initial experiments, fall on this ME1 expression scale? What about neurons and astrocytes?

• In Figure 3A there is a small error in the tracing schematic, where malate and aspartate should have 3 of their dots filled in to represent heavy carbons.

• In Figure 2J it would be great if the authors could present the impact of the combination treatment in the EV control as well

• The title of this paper “Compensatory activity of the PC-ME1 metabolic axis underlies differential

sensitivity to mitochondrial dysfunction between brain cell types" does not do justice to many of the novel aspects of the paper, especially because much of the work in astrocytes and neurons only begins in figure 4. It would be better if the authors altered the title to highlight their discovery that a novel function of the ETC is to maintain cellular NADPH homeostasis.

Reviewer #3 (Remarks to the Author):

The study from Prado and colleagues provide evidence for cytosolic NADP-reducing activity as required to sustain viability in respiration-deficient cells. Their study focuses initially on the NADP-reducing activity of the oxidative arm of the pentose phosphate pathway (oxPPP), but also investigate the cytosolic forms of malic enzyme (ME1) and isocitrate dehydrogenase (IDH1). The authors show that in cells with deficient mitochondrial respiration, rather than ATP deficiency, it is maintenance of the cellular antioxidant pool that determines whether the cell can survive or not. They show first that this is through activity of the oxidative PPP.

There is a substantial literature regarding the increase in mitochondrial ROS production in respiration deficient cells, which in turn put cells under significant oxidative stress burden, with increased reliance on the oxidative PPP – shown in multiple organisms. Indeed, it was already shown that ox PPP deficiency led to collapse of NADPH pool in the presence of oxidants (Castagna et al., 2011, Pandolfi et al., 1995 and Filosa et al., 2003. Critically for this manuscript, there is a previous publication in 2020 in Nature Communications from the last author of this manuscript that identified malic enzyme as the critical means by which cells avoid death from oxidative stress in respiration deficient cells (Balsa et al., 2020). They also showed that oxPPP isn't sufficient to respond, and that the phenotype of respiration deficiency is not through ATP generation problems. This suggests that the novelty of the current manuscript lies not in the novelty of the biology, but of the difference between cell types. However, this is a somewhat minor aspect of the manuscript, and overshadowed by the initial oxPPP findings, which could be argued as more important.

After showing initially that respiration deficient cells are highly dependent on oxPPP activity for NADPH regeneration, the authors then look for the next pathways whose activity might be required when both oxPPP and respiration are compromised – the relevance of this question being unclear. They show that under these specific conditions, ME1 becomes a critical pathway for cell survival. They show that in complex I deficient circumstances, reductive carboxylation of glutamine becomes the major pathway to generate citrate (published over 10 years ago), and broaden this to suggest that all respiration deficiencies might work in this way – something that requires more nuance given previous studies (Chen et al. Cell Metabolism, 2018; Mullen et al. Nature 2011).

Around Figure 2 and 3, the authors transition from an interest in oxPPP to ME1, the rationale for which is not clear. If oxPPP is the major means of compensation, the later focus on ME1 is a little unexpected given that it has little physiological relevant (cancer cell sensitivity was under conditions where both respiration and oxPPP were inhibited).

The later parts of the manuscript, comparing the expression of ME1 and PC between astrocytes and neurons is interesting, but ignores the first part that shows the oxPPP is the most important in the systems that were studied. While of interest, these results do not follow from the rest of the manuscript.

Overall, the manuscript narrative is not currently presented in a logical format, and the first part at least lacks significant novelty (see references cited above).

Minor comments:
1. The presence of pyruvate (1mM) in their media might mask changes in lactate that are only due to changes in glucose metabolism.
2. G6PDi being selective – a lot of the data is requiring this to be the fact, yet they don't show that their results can be phenocopied through genetic knockdown – this would be important. However, in addition to this, they only see a 50% reduction in apparent pathway activity upon inhibition, suggesting that they are not achieving high levels of inhibition (Extended Data Figure 2B) – is this enough? Indeed, the combination of this and the piericidin in a double pharmacological hit being used in many of the experiments needs additional genetic proofs where results are key.
3. Metabolomic analysis – is sedoheptulose wrongly assigned or a spelling error – sedoheptulose should be 7-phosphate, not bis-phosphate as currently. (Extended Data Figure 2C)

Reviewer #4 (Remarks to the Author):

In this manuscript Prado et al follow up on their initial observation that respiration deficient cells are highly sensitive to glucose withdrawal. They show that loss of the pentose phosphate pathway (PPP) inhibits proliferation in respiration deficient cell lines and primary neurons. They go on to show that combined complex I (CI) and PPP inhibition results in decreased NADPH levels in 143B cells, while malic enzyme (ME1) overexpression, which regenerates cytosolic NADPH, rescues the cell proliferation phenotype caused by combined CI/PPP inhibition. Stable isotope labelling experiments indicate that during complex I inhibition the TCA cycle is reconfigured to ensure a continued supply of malate. They then use a panel of cancer cell lines, combined with loss and gain of ME1 expression to demonstrate that reduced cell proliferation caused by combined CI/PPP inhibition is dependent on ME1 in vitro and in vivo. Finally, the authors focus on neurons and astrocytes, which have dramatically different ME1 expression levels, and show that CI inhibition does not alter ATP levels but reduces NADPH levels in neurons. Overexpression of ME1 in neurons rescued the decrease in NADPH and improved capacitance of neurons in vitro. Overexpression of ME1 in Ndufs4 KO mice did not improve the lifespan, bodyweight or locomotion phenotypes but gave some improvement in coordination/balance and reduced neuroinflammation.

This is a very interesting study with an impressive range of in vitro and in vivo techniques that are used to dissect the metabolic rewiring of cells during CI inhibition. The ability of ME1 overexpression to restore proliferation of cells with combined CI/PPP inhibition is convincing and the cancer cell panel experiments are a very nice demonstration of the wide applicability of these findings. The translational potential of ME1 overexpression to rescue CI inhibition phenotypes in the nervous system is less convincing. I have the following comments:

1. The authors frequently describe 'cell viability' phenotypes (line 132, 292, 306 etc.) when they are measuring cell number not specifically viability. Also, on line 219 the authors state 'cell survival' in the text describing Fig 2H, but the graph reads cell number. These should be corrected.
2. The methods state that all western blots were repeated at least 3 times. These repeats should be used for quantification and statistical analyses of the western blot data and quantifications included. Also, the molecular weight ladder or another indication of the MW of the bands for the western

blots should be included.
3. The Drosophila experiments in Figure 1E are very poorly explained and the model is not validated. It is not explained in which tissues ND49 and ND51 are knocked down. The rn-Gal4 driver expresses in certain regions in some imaginal discs but there is no justification for why this driver was used, nor is there any validation that ND49 and ND51 expression is reduced by RNAi expression. The methods state that the flies were raised at 17 degrees but Drosophila experiments are usually performed at the optimum temperature of 25 degrees. There is no explanation for why 17 degrees was used. Gal4 activity is temperature sensitive and so knockdown would be much weaker at 17 degrees than 25 degrees. It's also not explained whether the Pgd and Zw mutations were heterozygous or homozygous and there is no validation that the PPP is affected by these mutations. It's also hard to fully understand the viability data. 'Phenotype aggressiveness' is also the wrong term to use, phenotype severity would be more appropriate. It would be much better to use one of the several existing Drosophila CI deficiency models that are published and validated. I would recommend using one of these models or simply removing the Drosophila data completely from the manuscript.
4. The KO cell lines are validated by western blot but there is no validation that ME1 is overexpressed in the CNS in the Ndufs4 KO mice. Data showing ME1 overexpression in the Ndufs4 KO mice expressing AAV9-ME1 using e.g. western blot or immunostaining should be included.
5. The implication of the study, as mentioned in the discussion and illustrated in the model in Figure 7, is that when CI/PPP are inhibited in cancer cells reduced NADPH levels cause increased oxidative stress which leads to reduced cell proliferation. In neurons, CI deficiency alone leads to decreased NADPH and causes reduced capacitance and excitability through the same mechanism. One prediction of the model is that reducing oxidative stress should have similar beneficial effects to overexpression of ME1. The authors should test their model by analysing whether treatment with antioxidants improves the phenotypes in cancer cells and neurons, similar to ME1 overexpression.
6. The title of the manuscript and the abstract are misrepresentative. Nearly all the data in figures 1-4 are from non-neural proliferating cells and cancer cells, while the title and abstract give the reader the impression that the paper is focused on the brain. I recommend removing the word brain from the title and modifying the abstract so that it is more representative of the data and that most of the experiments are performed in non-neural cells. Moreover, the in vivo Ndufs4 KO ME1 overexpression data are the least convincing part of the story but are described as 'striking' in the abstract, while the data in the cancer cell lines is much more compelling but is not mentioned in the abstract. I would recommend re-writing the abstract to address these issues.

Minor points
1. On line 180/181 the authors suggest that neurons are highly dependent on ATP levels. This is inconsistent with the latter findings and the points made in the discussion and so should be clarified.
2. There are lots of typos throughout the manuscript including typos between lines 208 and 230 in the supplemental figure names. Also on line 320. Inmunoblot spelling should be corrected.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts."

Point-by-point response to the reviewers' critiques

We would like to thank you and the reviewers for the opportunity to revise our manuscript. We appreciate the detailed and constructive feedback provided.

We have carefully considered all the concerns raised by the referees and have undertaken comprehensive revisions to address each of them. We have performed all the additional experiments requested, including further validation of our in vivo experiments and extensive revisions to the manuscript's title, narrative, and flow to better reflect our data. Below, we provide a point-by-point response to each of the reviewers' comments, and we have thoroughly revised our manuscript to address all concerns raised.

We believe that these revisions have significantly strengthened our study and we hope that the reviewers find our responses satisfactory.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, del Prado and colleagues propose metabolic reprogramming by pyruvate carboxylase (PC) and malic enzyme 1 (ME1) as the adaptive mechanism underlying the differential sensitivity of brain cell types (astrocytes and neurons) towards mitochondrial dysfunction. By using pharmacological, viral as well as CRISPR-based approaches in cell lines to manipulate metabolic pathways, they provide evidence that OXPHOS-deficient cells are capable to maintain NADPH levels by leveraging PPP, and that increasing expression of ME1 alleviates the lethality observed when PPP is also hampered. By both reanalyzing available proteomics datasets of cancer cell lines as well as performing new experiments in vitro and in vivo, they suggest that this mechanism is conserved across cell types and could explain the selective vulnerability of some cancer cells towards OXPHOS inhibitors. Lastly, they propose that this mechanism is behind the selective vulnerability of neurons in the context of OXPHOS dysfunction, based on the different protein expression levels of these enzymes in primary cortical neurons versus astrocytes, as well as in vivo evidence of reduced neuroinflammation upon overexpression of ME1 in Ndufs4 KO mice.
The topic addressed in this study is extremely interesting for the field of mitochondrial disease, as the molecular mechanisms underlying the differential sensitivity of tissues or cells (and especially brain tissue) towards mitochondrial dysfunction are still poorly understood. In particular, the authors have performed quite some experiments to extend the previous yet uncomplete knowledge on the synthetic lethality between ETC and PPP, and I am positive the results will be of relevance for the field. The in vivo data on Ndufs4KO mice are quite interesting but still preliminary.
Major concerns:
- The original focus of the study proposed by the authors both in the title and in the abstract - the PC-ME1 axis in brain cells - is not well represented in the manuscript. In fact, the data provided

are rather spanning from cell lines to cancer, with a minor part of the figures addressing these questions in the brain. Along the same line, I have found the part on the cancer a bit disconnected from the rest of the data. Perhaps the author should consider whether keeping these different aspects in the manuscript, but rephrasing the title and abstract. Also, if the focus is on brain cells, the experimental part on this should be strengthened (see points below).

We appreciate the referee's insightful comments regarding the section dedicated to brain cell types in comparison to other facets explored in our manuscript.

We acknowledge that the portion of the manuscript specifically addressing the effect on different brain cell types constitutes only approximately 30% of the total content. However, we consider this aspect to be a pivotal discovery, particularly in elucidating the role of the PC-ME1 axis within the framework of mitochondrial dysfunction in different brain cell types.

In response to the referee's suggestion, we have carefully reconsidered the title and abstract to better reflect the scope of the manuscript and while also emphasize the significance of the findings related to brain cells.

- The authors claim they revealed that PC and ME1 expression patterns are different between astrocytes and neurons (lines 386-387, and 486-488). While the metabolic rewiring exerted by these two enzymes is very interesting, their differential protein expression between cultured astrocytes and neurons (and to some extent in situ) is known since decades (https://doi.org/10.1007/s11064-010-0257-5). Similarly, astrocytes are well known to possess a heightened metabolic flexibility well beyond their glycolytic capacity mentioned by the authors (lines 375-276) The authors should rephrase these statements in the light of the existing literature on the subject.

We appreciate the literature cited by the referee, which has been incorporated into the revised version of the manuscript. It is indeed recognized that the literature has already documented the differential protein expression of PC and ME1 between astrocytes and neurons, as well as the metabolic flexibility inherent to astrocytes.

However, we would like to emphasize that while the role of PC in anaplerosis in astrocytes has been extensively explored, the specific role of PC, and to a lesser extent ME1, within the context of mitochondrial dysfunction has remained largely unaddressed. Our study delves into this underexplored aspect, shedding light on the intricate interplay between these enzymes and mitochondrial function in brain cells.

- The authors show that CI inhibition does not lead to death of neurons, but only to morphological changes (lines 366-367). However, cell viability of neurons is shown only after 24h of treatment, whereas the corresponding quantification is performed up to 72h in astrocytes. The morphological changes of neurons treated with piericidin as those shown in Fig. S5B are quite compatible of those marking stages of neuronal degeneration preceding cell death. The authors should also include quantification of cell viability of neurons for both 48h and 72h.

We acknowledge the importance of comprehensive analysis in both cell types. In response to this concern, we have included additional data (S5E) showing the cell viability of neurons for both 48 hours and 72 hours, aligning with the duration of assessment conducted for astrocytes.

- Based on the representative image shown in Fig. S5B it is not clear how the authors could quantify axons or perform FRET measurements in axons at this time-point (Fig. S5I), considering the near complete loss of neuronal processes at this stage. Also, axon morphology analysis by integrated density of images using beta3-tubulin labeling is not appropriate, as this antibody uniformly labels neuronal processes without distinction amid dendrites and axons. Markers such as SMI-312 would be more appropriate.

In the rat cortical cultures with 24h of Piericidin A treatment we observed an almost complete degeneration of the projection of the neurons. Nevertheless, still a small subset of neurons was not completely degenerated and we could measure the ATP levels in the remaining neurites of these cells. For clarity and a more precise description of the analysis, we modified in the text and the figure caption, mentioning the measurements were done in proximal neurites and not axons.

We acknowledge the referee's concerns regarding the quantification of axons and the appropriateness of the methods used for analysis. In our rat cortical cultures treated with Piericidin A for 24 hours, we indeed observed a significant degeneration of neuronal projections. However, it's important to note that a subset of neurons retained some degree of structural integrity, allowing for the measurement of ATP levels in the remaining neurites of these cells.

To address this, we have revised the text and figure caption to clarify that the measurements were conducted in proximal neurites rather than axons. This adjustment ensures clarity and accuracy in describing the methodology used for analysis.

Regarding Fig S5B (where we provide new images of the neurons), following the referee's suggestion, we now include quantifications using the SMI-312 marker in addition to TUJ1 (beta3-

tubulin). Note that a significant reduction is observed again when neurons were incubated with piericidin for 24 hours (Figure 1).

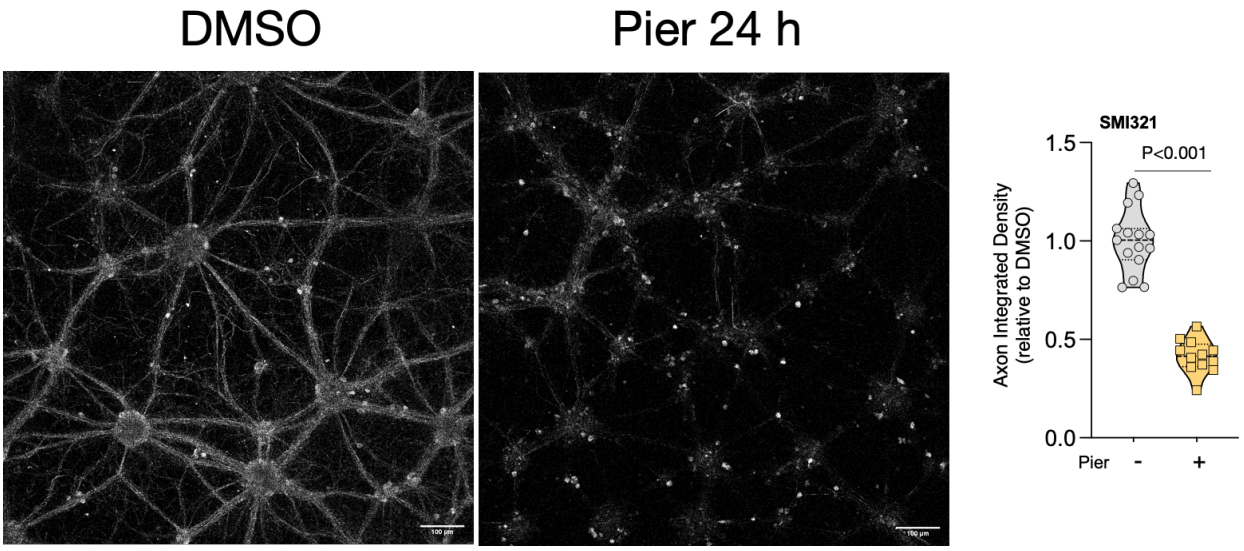

Figure 1. Representative images and quantification of cultured neurons treated with Piericidin for 24h and labeled with an antibody against SMI-312.

- The in vivo data on Ndufs4KO mice lack a few controls. The authors should at least a) validate the differential expression of either PC or ME1 in vivo between astrocytes and neurons in vivo, in control mice; b) showing evidence of increased ME1 expression in tissue upon AAV9 infection, since presence of the GFP reporter alone is not sufficient

We agree with the reviewer that the manuscript lacks these important controls, and we are now including both of them:

a) We isolated astrocytes and neurons from adult mice using a validated magnetic labeling protocol from Miltenyi. Briefly, the brain was dissociated with a gentleMACS Dissociator, and the resulting cell suspension was incubated with specific antibody-coupled beads (Anti-ACSA-2 MicroBeads or the Adult Neuron Isolation Kit). Figure 2 below clearly confirms that adult astrocytes possess increased levels of both PC and ME1 compared to adult neurons.

b) In the new Figure 6SA, we now show an immunoblot and its quantification, confirming increased ME1 protein expression in the vestibular nuclei (VN) of mice injected with AAV9-ME1.

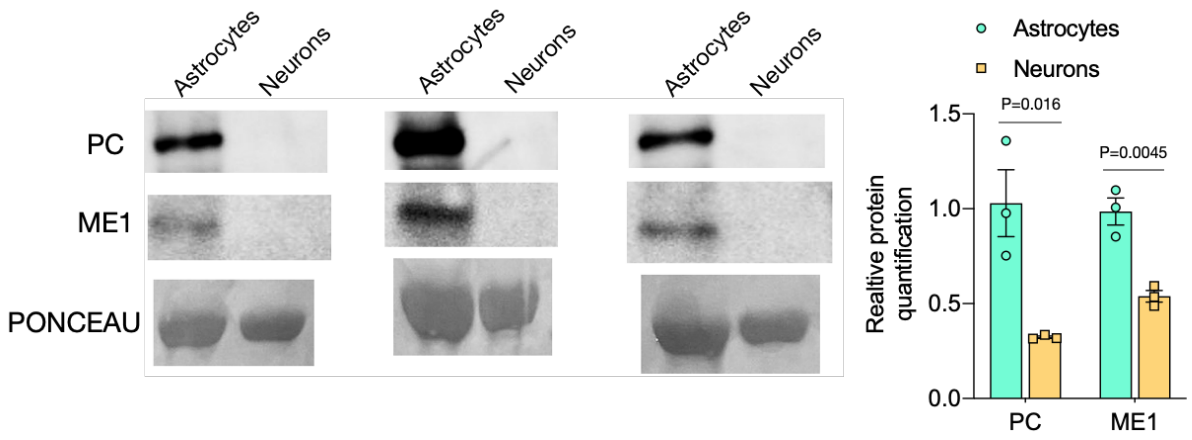

Figure 2. Immunoblot and quantification confirming increased PC and ME1 protein expression in astrocytes compared to neurons.

- Alleviation of neuroinflammation in Fig. 6D is quantified by the area positive for Iba1, a well established microglia marker. While expression of Iba1 is undoubtedly increased in reactive microglia, Iba1 should also label resting microglia, whereas AAV9_ME1 samples seem devoid of any signal. Can the authors explain this? I would also suggest to provide a zoom in for the Iba1/GFP channels shown in Fig. 6D, to disclose cellular morphological details and immunoreactivity of resting microglia.

In response to this concern, we have incorporated a new figure, Figure 6SE, which includes detailed zoom-in pictures showing Iba1 staining corresponding to microglia in the lesions of the ventral nucleus (VN) of both AAV9_Veh and AAV9_ME1 injected brain samples. These images aim to provide a clearer depiction of cellular/tissular morphological details and immunoreactivity of microglia, thus addressing the referee's suggestion and ensuring transparency in our analysis.

- While ME1 overexpression has beneficial effects on motor coordination Ndufs4KO mice, there is no indication that the mechanism suggested in cultured neurons is truly applicable in neurons in vivo. The authors acknowledge that AAV9 delivery is not specific for neurons, however immunohistochemistry and /or TEM analysis of AAV9_veh and AAV9_ME1 slices should be performed to analyze morphological and ultrastructural features of infected neurons to provide some insight if boosting ME1 function is indeed beneficial for neurons. Likewise, the same metabolomic analysis shown for Ndufs4 brain samples in Fig S1E should be repeated in these settings to show if boosting ME1 function is restoring the levels of PPP-associated metabolites.

To address whether ME1 overexpression in the brain leads to improved neuronal fitness, we performed immunofluorescence (IF) using the NeuN antibody, which is widely used to assess the functional state of neurons, in vehicle- or ME1-injected mice (Figure 3). We observed significant variability in the samples, likely due to the intrinsic damage present in the tissue. The vestibular nuclei are among the regions most affected in this KO model, and the tissue at this stage of the disease is quite damaged. Thus, we repeated the immunostaining using the Neurofilament Marker SMI-312. Here, we did observe significant results after quantification of the images. We want to

emphasize that these data do not fully rule out the possibility that the beneficial effects exerted by the increased expression of ME1 are due to enhanced functionality of infected neurons rather than a correction in the overall morphology of the cells or the ultrastructure of the tissue.

We have also conducted metabolomic analyses as suggested by the reviewer, and the new data are included in Figures 6B and 6SB. We observed that some of the PPP intermediates are corrected by the overexpression of ME1.

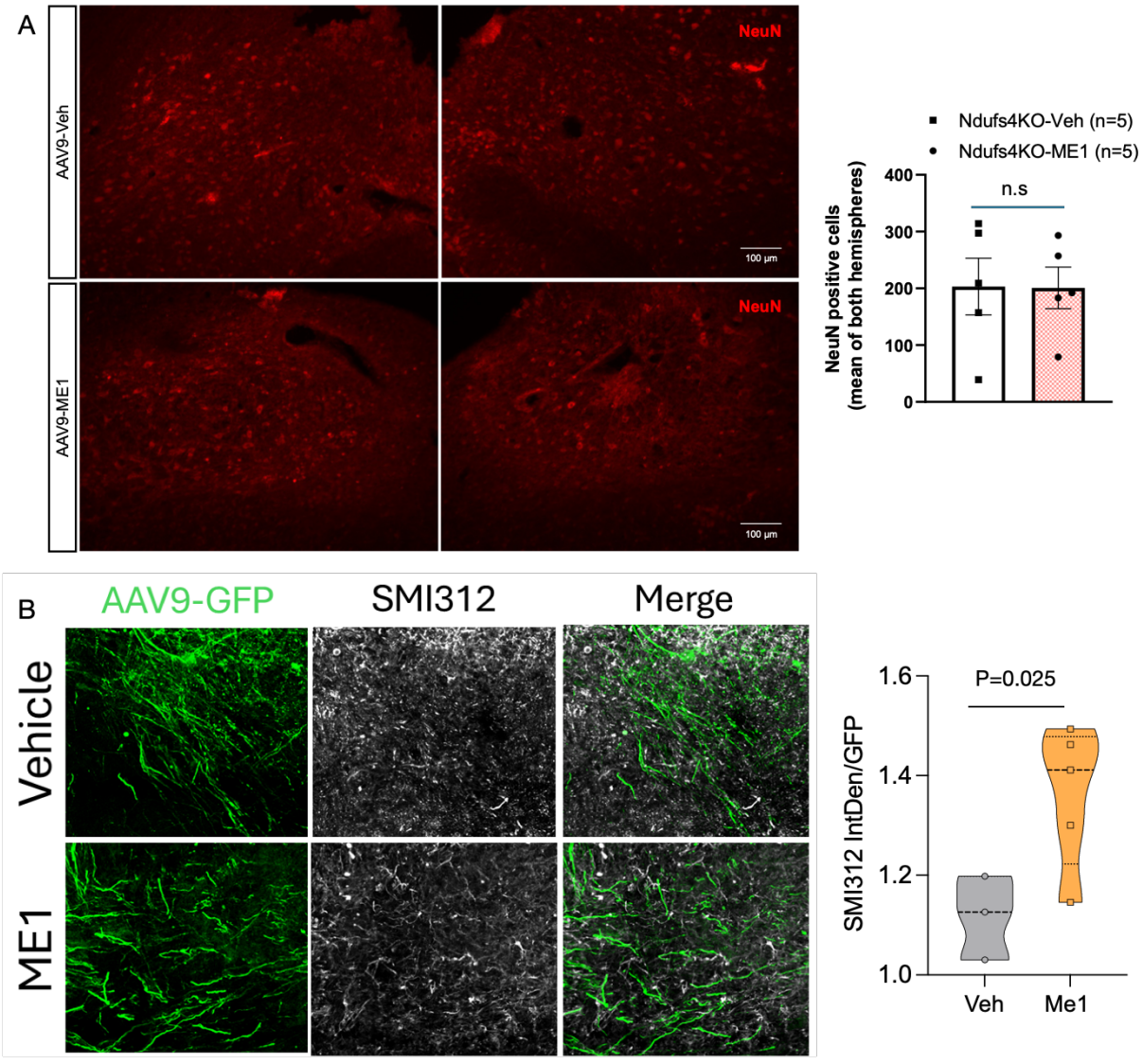

Figure 3. (A). Immunofluorescence images and quantification were performed using a NeuN 2 antibody on the ventral nucleus (VN) in AAV9-vehicle or ME1-injected Ndufs4 KO animals. (B) Representative images and quantification of SMI312 (axonal marker) staining normalised vs AAV9-GFP expression in the vestibular nucleus (VN) region of the brain.

Minor points:

- The panel order is difficult to follow in Fig. 2, S2 and S5. There is no panel E in Fig. S3

- The text contains some typos: line 176 “lactated”, line 216 “focued”, line 434 “Figure C”

We appreciate the referee's attention to detail regarding the panel order and typos present in the manuscript. In response to these observations, we have made the necessary corrections in the new version of the manuscript.

Reviewer #2 (Remarks to the Author):

In this manuscript, Prado, et al identify that inhibition of ETC complexes induces a dependency on the pentose phosphate pathway (PPP) for NADPH production. Upon dual inhibition of the PPP and ETC, malic enzyme 1 functions as an alternative source of NADPH. The expression of malic enzyme 1 can predict sensitivity of cells to ETC inhibition, including astrocytes and neurons in the brain. In a preclinical mouse model of complex I deficiency, overexpression of malic enzyme 1 mitigates neuroinflammation and motor function, establishing the physiological relevance of this metabolic adaptation to ETC inhibition.

This manuscript is both exciting and novel and warrants publication in Nature Communications after revision. Among the most exciting aspects of the paper include the fundamental connection between complex I activity and NADPH pools in mammalian metabolism. Moreover, complex I inhibition causing increased pyruvate carboxylase activity is quite interesting. Finally, the authors propose an interesting diversion of glutamine-derived carbon through the reductive carboxylation pathway and subsequently malic enzyme 1 as a means to generate NADPH upon complex I inhibition.

Below are suggested experiments to strengthen the claims made by the authors:

• In figure 1 the authors demonstrate a synthetic lethality of inhibition of the pentose phosphate pathway (PPP) with complex I inhibition. They show elevated PPP intermediates upon ETC inhibition, which could mean increased PPP activity but could also mean inhibition of downstream reactions such as nucleotide synthesis. To strengthen the claim that ETC inhibition increases the PPP the authors should perform 13C6-glucose tracing into the PPP intermediates in vehicle, complex I, and complex III inhibited cells +/- G6PD depletion. Additionally, 13C6-glucose tracing should be done in GFPT1, PHGDH, and MPC KO cells +/- complex I and III inhibition to demonstrate no difference in PPP incorporation, as these pathways were not synthetic lethal with ETC inhibition.

Our evidence of increased PPP activity is not inferred from measuring PPP intermediates but rather from direct measurements of PPP activity using a sensitive and validated approach involving radiolabeled glucose. Analysis of brain PPP intermediates was conducted from prior published results and is merely correlative. However, we acknowledge the importance of further substantiating our findings.

According to our model, the synthetic lethality between OXPHOS and PPP arises from the dual contribution of both pathways to NADPH generation: directly through PPP and indirectly via OXPHOS, which generates essential metabolites such as malate. In this context, it is not expected that the other glucose-dependent pathways (hexosamine pathway, serine biosynthesis pathway, or

the prevention of pyruvate entry into the mitochondria) have any impact on NADPH generation. Nevertheless, as suggested by the referee, we have conducted PPP activity measurements in GFPT1, PHGDH, and MPC knockout cells using the gold standard radioactive methodology. We consider that including the condition with Piericidin is not relevant in these settings, as we have already demonstrated that inhibiting CI leads to an increase in PPP. However, we have included a positive control using the well-validated inhibitor of glucose-6-phosphate dehydrogenase, G6PDi (Figure 4).

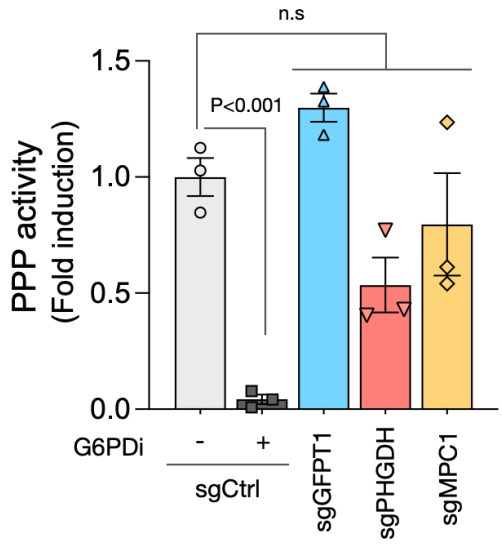

Figure 4. Measurements of PPP activity in 143B WT and KO cells for GFPT1, PHGDH, and MPC1. The selective inhibitor of glucose-6-phosphate dehydrogenase (G6PDi) was used as a positive control to ablate PPP activity.

As expected, none of the GFPT1, PHGDH, and MPC knockout cells significantly impacted PPP activity, compared to G6PDi treatment, which completely abrogated PPP activity.

We believe that these new experiments significantly strengthen our claim regarding the relationship between ETC inhibition and increased PPP activity.

• 13C6-glucose tracing into the PPP intermediates should be done in astrocytes versus neurons to demonstrate this difference in the two cell types.

In response to the referee's suggestion, we have conducted 13C6-glucose tracing experiments in both astrocytes and neurons, specifically focusing on PPP intermediates. The results of these experiments are presented in the graphs below. Overall and depending on the isotopologue, we do see some increases in certain PPP intermediates such as erythrose 4-P or ribose-5-P in neurons

compared to astrocytes, suggesting that neurons rely more on the PPP under basal conditions (Figure 5).

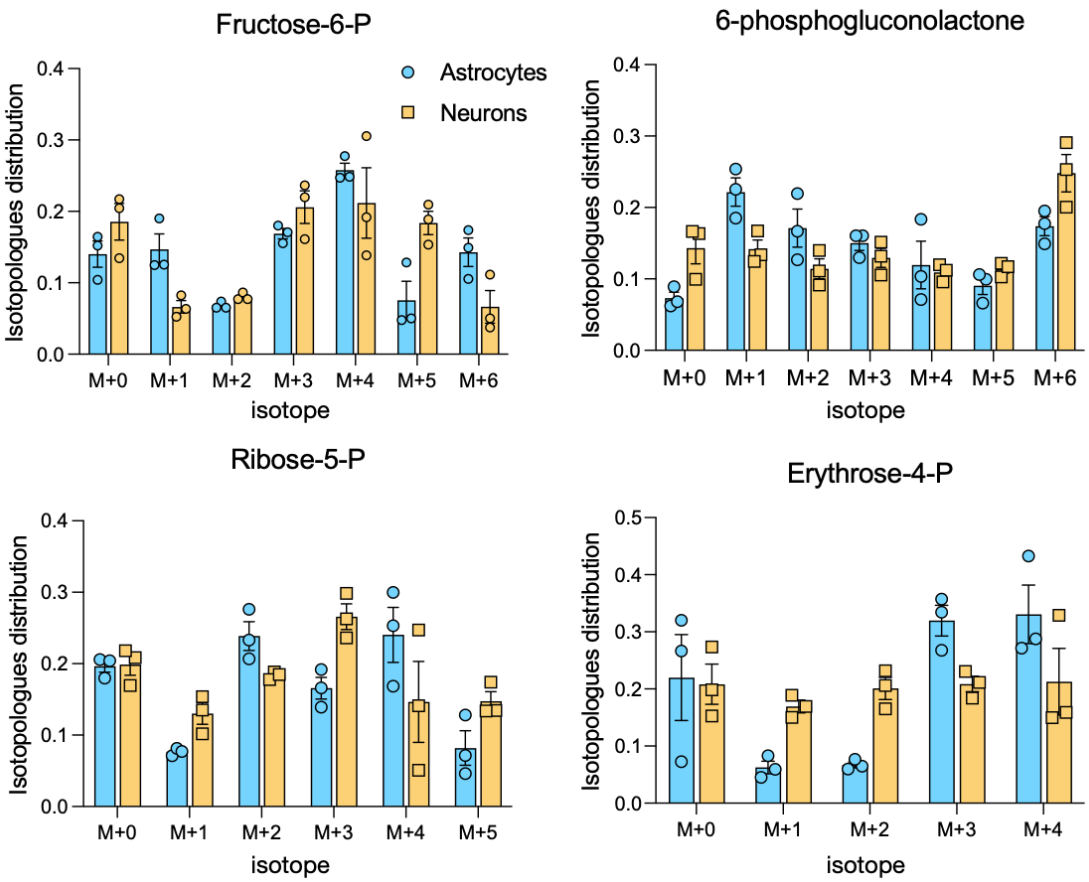

Figure 5. 13C labeling patterns of PPP intermediate metabolites from mouse primary astrocytes and neurons fed with [U-13C]-glucose.

• The authors suggest that ME1 activity increases in cells with dual PPP and complex 1 inhibition. It would strengthen the manuscript to directly test ME1 activity in their cells treated with vehicle, PPP inhibition, complex I inhibition, and the combination. One assay the authors could use to test ME1 activity is 13C4-aspartate tracing, which will label the 13C4-oxaloacetate, 13C4-malate, and 13C3-pyruvate. An alternative ME1 activity assay would also be acceptable.

It's important to clarify that our assertion is not that ME1 activity is increased by dual PPP and CI inhibition, but rather that it is indispensable for the observed phenotype. In support of this, the Rabinowitz lab has demonstrated that while the flux through ME1 increases in genetic models of PPP deficiency, its intrinsic activity remains unchanged[1].

We have measured ME1 activity in PPP and CI inhibited cells and in line with our previous explanation and cited literature we did not observe significant differences (Figure 6).

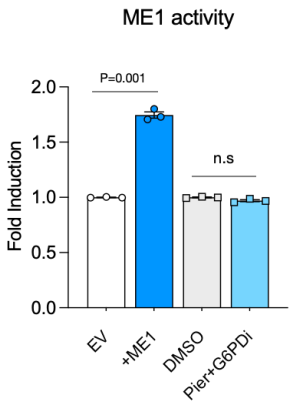

Figure 6. ME1 enzymatic activity do not change with dual inhibition of Piericidin and G6PDi. Cell overexpressing ME1 were used as positive control.

• The authors should perform metabolomics on the AAV-induced ME1 overexpression mouse model to measure NADPH and malate levels in the brain samples.

We have conducted metabolomic analyses as suggested by the reviewer, and the new data are included in Figures 6B and 6SB. Overall, we detected elevated levels of NADPH and associated metabolites, including GSH, in the VN of AAV9-ME1-injected mice. Notably, there was a concurrent decrease in certain PPP intermediates, likely due to the restoration of NADPH levels. Malate levels were unchanged, as shown in Figure 7.

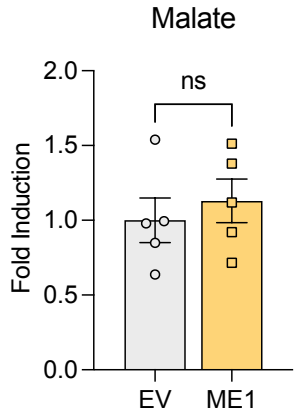

Figure 7. Malate levels assessed by mass spectrometry on the vestibular nuclei (VN) of mice injected with AAV9-Veh or AAV9-ME1.

• The authors provide a lot of experimental evidence that the pentose phosphate pathway and malic enzyme are regulators of NADPH upon complex I inhibition. Another possible way to link complex I inhibition to NADPH pools is the NAD kinase reaction, whereby complex I inhibition decreases NAD+ levels, decreasing the amount of NADP generated via NAD kinase. It would be great if the authors could either experimentally test this potential connection between complex I and NADPH pools or add some text about it to the discussion.

We appreciate the referee's innovative interpretation of the data and the suggestion regarding the potential connection between complex I inhibition and NADPH pools through the NAD kinase reaction. However, upon further examination of our experimental results, we have not observed a decrease in NADP+ levels following complex I inhibition, PPP inhibition, or dual inhibition. In fact, our data consistently show that NADP+ levels remain constant under these conditions. To illustrate, we provide several examples demonstrating the stability of NADP+ levels after treatment with complex I inhibitors, PPP inhibitors, or both (see below in Figure 8). These findings suggest that while the NAD kinase reaction presents an intriguing hypothesis, our experimental evidence does not support a direct link between complex I inhibition and alterations in NADPH pools via this mechanism.

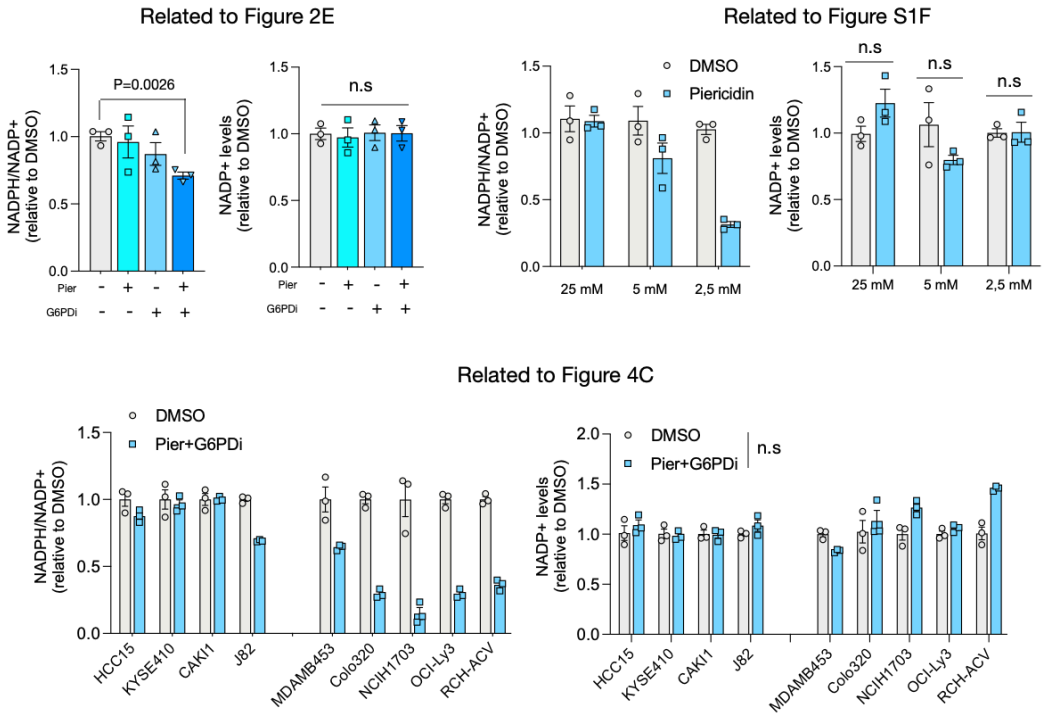

Figure 8. Re-analysis of NADPH/NADP+ ratios and NADP+ levels across multiple cell lines and conditions of CI inhibition and or PPP inhibition.

• Throughout the manuscript, the authors sometimes report NADPH levels and other times report the ratio of NADPH:NADP. It would be ideal if both the relative levels of NADPH and NADP, and their ratio, is reported for each experiment

We appreciate the referee's suggestion regarding the reporting of NADPH levels and the NADPH/NADP+ ratio in the manuscript. To our knowledge, Figure 2E was the only one where the values of NADPH were presented instead of the ratio. In response to this feedback, we have now included the ratio of NADPH/NADP+ for all figures throughout the manuscript, ensuring consistency in our reporting. At this stage, we find it unnecessary to include separate values, given that, as previously mentioned, our findings indicate no changes in NADP+ levels, only in NADPH.

• Throughout the manuscript the authors refer to their perturbations as “ETC inhibition.” Since most of the assays only test complex I inhibition, it would be better for the authors to specify which complexes are being inhibited for each experiment, instead of broadly calling it ETC inhibition.

In response to this feedback, we have made the necessary corrections throughout the manuscript to specify the complexes being inhibited for each experiment.

• The authors show the expression of ME1 in a panel of cell lines can predict their ETC dependence. Where do 143B cells, which were used in the initial experiments, fall on this ME1 expression scale? What about neurons and astrocytes?

We appreciate the referee's inquiry regarding the ME1 expression levels in 143B cells used in the initial experiments, as well as in neurons and astrocytes. Unfortunately, 143B cells were not included in the proteomic analysis[2]. which could explain why they are sensitive to dual inhibition in the long term. Nevertheless, we conducted western blot analyses to compare the levels of ME1 in 143B cells to those in ME1 high (CAKI1) and ME1 low (Colo320) cell lines. 143B cells exhibited intermediate levels of ME1, which could explain their sensitivity to dual inhibition over the long term (Figure 9). We also provide a graph below displaying the correlation between the protein expression levels of ME1 and G6PD in astrocytes and neurons. While minimal differences are observed in the levels of G6PD, astrocytes exhibit higher levels of ME1 (Figure 9).

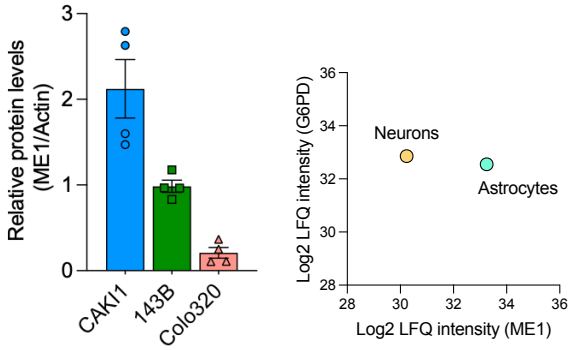

Figure 9. Quantification of the levels of ME1 in ME1-high (CAKI1) and ME1-low (Colo320) cell lines compared to 143B cells (left). Protein expression levels of ME1 and G6PD in astrocytes and neurons (right). Data obtained from[3]

• In Figure 3A there is a small error in the tracing schematic, where malate and aspartate should have 3 of their dots filled in to represent heavy carbons.

We appreciate the referee's attention to detail regarding the tracing schematic in Figure 3A. The error has been identified and corrected accordingly.

• In Figure 2J it would be great if the authors could present the impact of the combination treatment in the EV control as well

We apologize for the omission of this control in the initial version of Figure 2J. This oversight has been corrected, and the control has been added in the new version of the manuscript.

• The title of this paper "Compensatory activity of the PC-ME1 metabolic axis underlies differential sensitivity to mitochondrial dysfunction between brain cell types" does not do justice to many of the novel aspects of the paper, especially because much of the work in astrocytes and neurons only begins in figure 4. It would be better if the authors altered the title to highlight their discovery that a novel function of the ETC is to maintain cellular NADPH homeostasis.

We appreciate the referee's suggestion regarding the title of the paper. In response to this feedback, we have modified both the title and the abstract to better highlight our discovery regarding the novel function of the ETC in maintaining cellular NADPH homeostasis.

Reviewer #3 (Remarks to the Author):

The study from Prado and colleagues provide evidence for cytosolic NADP-reducing activity as required to sustain viability in respiration-deficient cells. Their study focuses initially on the NADP-reducing activity of the oxidative arm of the pentose phosphate pathway (oxPPP), but also investigate the cytosolic forms of malic enzyme (ME1) and isocitrate dehydrogenase (IDH1). The authors show that in cells with deficient mitochondrial respiration, rather than ATP deficiency, it is maintenance of the cellular antioxidant pool that determines whether the cell can survive or not. They show first that this is through activity of the oxidative PPP.

There is a substantial literature regarding the increase in mitochondrial ROS production in respiration deficient cells, which in turn put cells under significant oxidative stress burden, with increased reliance on the oxidative PPP – shown in multiple organisms. Indeed, it was already shown that ox PPP deficiency led to collapse of NADPH pool in the presence of oxidants (Castagna et al., 2011, Pandolfi et al., 1995 and Filosa et al., 2003.

We appreciate the referee's acknowledgment of the substantial literature on mitochondrial dysfunction leading to increased ROS production and oxidative stress burden, often associated with reliance on the oxidative PPP, as shown in multiple organisms. However, our data offers complementary insights to these observations. We suggest that while excessive ROS production is a well-established consequence of mitochondrial dysfunction, our findings indicate that oxidative stress may also arise from a decrease in antioxidant capacity due to reduced NADPH levels. This reduction in NADPH could have broader implications beyond ROS regulation, potentially impacting other metabolic pathways such as lipogenesis or toxin elimination via cytochromes P450, both of which require NADPH.

Critically for this manuscript, there is a previous publication in 2020 in Nature Communications from the last author of this manuscript that identified malic enzyme as the critical means by which cells avoid death from oxidative stress in respiration deficient cells (Balsa et al., 2020). They also showed that oxPPP isn't sufficient to respond, and that the phenotype of respiration deficiency is not through ATP generation problems. This suggests that the novelty of the current manuscript lies not in the novelty of the biology, but of the difference between cell types.

However, this is a somewhat minor aspect of the manuscript, and overshadowed by the initial oxPPP findings, which could be argued as more important.

Unlike the previous work cited by the referee, here we delve into the mechanistic details using isotopic tracing metabolomics, various KO cell lines and explore the biological relevance of these findings. Specifically, we demonstrate the impact on tumor biology and brain metabolism, as well as identify ME1 activation as a potential therapeutic target in mitochondrial diseases such as Leigh syndrome. These novel insights and therapeutic implications distinguish our manuscript and highlight its significance.

After showing initially that respiration deficient cells are highly dependent on oxPPP activity for NADPH regeneration, the authors then look for the next pathways whose activity might be required when both oxPPP and respiration are compromised – the relevance of this question being unclear. They show that under these specific conditions, ME1 becomes a critical pathway for cell survival. They show that in complex I deficient circumstances, reductive carboxylation of glutamine becomes the major pathway to generate citrate (published over 10 years ago), and broaden this to suggest that all respiration deficiencies might work in this way – something that requires more nuance given previous studies (Chen et al. Cell Metabolism, 2018; Mullen et al. Nature 2011).

We acknowledge the well-documented adaptation of reductive carboxylation in response to ETC failure or hypoxia, primarily studied in cancer cell lines. It is understood to be a mechanism for generating citrate and aspartate, crucial for supporting cellular proliferation. However, there has been limited attention paid to malate in these conditions, and our work emphasizes its importance in the context of mitochondrial dysfunction. Importantly, we delineate the pathways by which cells generate malate, not only through reductive carboxylation but also via pyruvate carboxylase from glucose-derived pyruvate. This broader perspective adds nuance to the understanding of metabolic adaptations in respiration-deficient cells and highlights the significance of our findings.

Around Figure 2 and 3, the authors transition from an interest in oxPPP to ME1, the rationale for which is not clear. If oxPPP is the major means of compensation, the later focus on ME1 is a little unexpected given that it has little physiological relevant (cancer cell sensitivity was under conditions where both respiration and oxPPP were inhibited).

The later parts of the manuscript, comparing the expression of ME1 and PC between astrocytes and neurons is interesting, but ignores the first part that shows the oxPPP is the most important in the systems that were studied. While of interest, these results do not follow from the rest of the manuscript.
Overall, the manuscript narrative is not currently presented in a logical format, and the first part at least lacks significant novelty (see references cited above).

We acknowledge the challenges of presenting scientific narratives within the constraints of a manuscript format. However, we respectfully disagree with the referee's assessment and believe that the flow of our narrative is appropriate and engaging. Our initial hypothesis led to the discovery of synthetic lethality between oxPPP and ETC dysfunction, a finding validated in an in

vivo model (Drosophila). Subsequently, we discovered that ME1, rather than IDH1, efficiently rescued this phenotype. The remainder of the manuscript delves into the mechanistic understanding and biological relevance of this phenomenon in cancer biology and brain metabolic adaptations to ETC dysfunction. We have used multiple approaches, techniques, cellular models, as well as in vivo models to test and strengthen our findings.

Minor comments:

1. The presence of pyruvate (1mM) in their media might mask changes in lactate that are only due to changes in glucose metabolism.

We appreciate the referee's insight regarding the potential influence of pyruvate supplementation on lactate levels. It's important to clarify that the specific experiments related to measuring lactate release were conducted without the supplementation of pyruvate in the media and this is now detailed in the methods section.

2. G6PDi being selective – a lot of the data is requiring this to be the fact, yet they don't show that their results can be phenocopied through genetic knockdown – this would be important. However, in addition to this, they only see a 50% reduction in apparent pathway activity upon inhibition, suggesting that they are not achieving high levels of inhibition (Extended Data Figure 2B) – is this enough? Indeed, the combination of this and the piericidin in a double pharmacological hit being used in many of the experiments needs additional genetic proofs where results are key.

The selectivity of the G6PD inhibitor has been established in the literature[45], providing confidence in its specificity. Additionally, we have observed that long-term genetic depletion of G6PD resulted in severe proliferation defects, which is consistent with previous literature[6]. Although we encountered challenges in maintaining a stable G6PD knockout cell line (even clones) for extended periods, experiments with G6PD knockout cells were conducted within the first 2-3 weeks after generating the knockout.

Our results suggest that achieving 100% inhibition with the G6PD inhibitor may not be necessary, as evidenced by the consistent phenotype observed in our experiments. This provides confidence in the relevance of our findings.

3. Metabolomic analysis – is sedoheptulose wrongly assigned or a spelling error – sedoheptulose should be 7-phosphate, not bis-phosphate as currently. (Extended Data Figure 2C).

This typo has been corrected.

Reviewer #4 (Remarks to the Author):

In this manuscript Prado et al follow up on their initial observation that respiration deficient cells are highly sensitive to glucose withdrawal. They show that loss of the pentose phosphate pathway (PPP) inhibits proliferation in respiration deficient cell lines and primary neurons. They go on to show that combined complex I (CI) and PPP inhibition results in decreased NADPH levels in 143B cells, while malic enzyme (ME1) overexpression, which regenerates cytosolic NADPH, rescues

the cell proliferation phenotype caused by combined CI/PPP inhibition. Stable isotope labelling experiments indicate that during complex I inhibition the TCA cycle is reconfigured to ensure a continued supply of malate. They then use a panel of cancer cell lines, combined with loss and gain of ME1 expression to demonstrate that reduced cell proliferation caused by combined CI/PPP inhibition is dependent on ME1 in vitro and in vivo. Finally, the authors focus on neurons and astrocytes, which have dramatically different ME1 expression levels, and show that CI inhibition does not alter ATP levels but reduces NADPH levels in neurons. Overexpression of ME1 in neurons rescued the decrease in NADPH and improved capacitance of neurons in vitro. Overexpression of ME1 in Ndufs4 KO mice did not improve the lifespan, bodyweight or locomotion phenotypes but gave some improvement in coordination/balance and reduced neuroinflammation.

This is a very interesting study with an impressive range of in vitro and in vivo techniques that are used to dissect the metabolic rewiring of cells during CI inhibition. The ability of ME1 overexpression to restore proliferation of cells with combined CI/PPP inhibition is convincing and the cancer cell panel experiments are a very nice demonstration of the wide applicability of these findings. The translational potential of ME1 overexpression to rescue CI inhibition phenotypes in the nervous system is less convincing. I have the following comments:

1. The authors frequently describe ‘cell viability’ phenotypes (line 132, 292, 306 etc.) when they are measuring cell number not specifically viability. Also, on line 219 the authors state ‘cell survival’ in the text describing Fig 2H, but the graph reads cell number. These should be corrected.

We acknowledge the referee's observation regarding the misleading descriptions of 'cell viability' and 'cell survival' when referring to measurements of cell number in various instances throughout the manuscript. We have made the necessary corrections to ensure accurate terminology is used consistently throughout the manuscript.

2. The methods state that all western blots were repeated at least 3 times. These repeats should be used for quantification and statistical analyses of the western blot data and quantifications included. Also, the molecular weight ladder or another indication of the MW of the bands for the western blots should be included.

We have now included quantification for all the western blots in accordance with the referee's suggestion (Figure 10). We do not consider quantification of the KO to be crucial for the manuscript; however, we have included them below for reference. If the referee deems their inclusion important for publication, we are willing to include them in the manuscript.

Additionally, we have ensured that molecular weight ladders or another indication of the MW of the bands are included in the western blots.

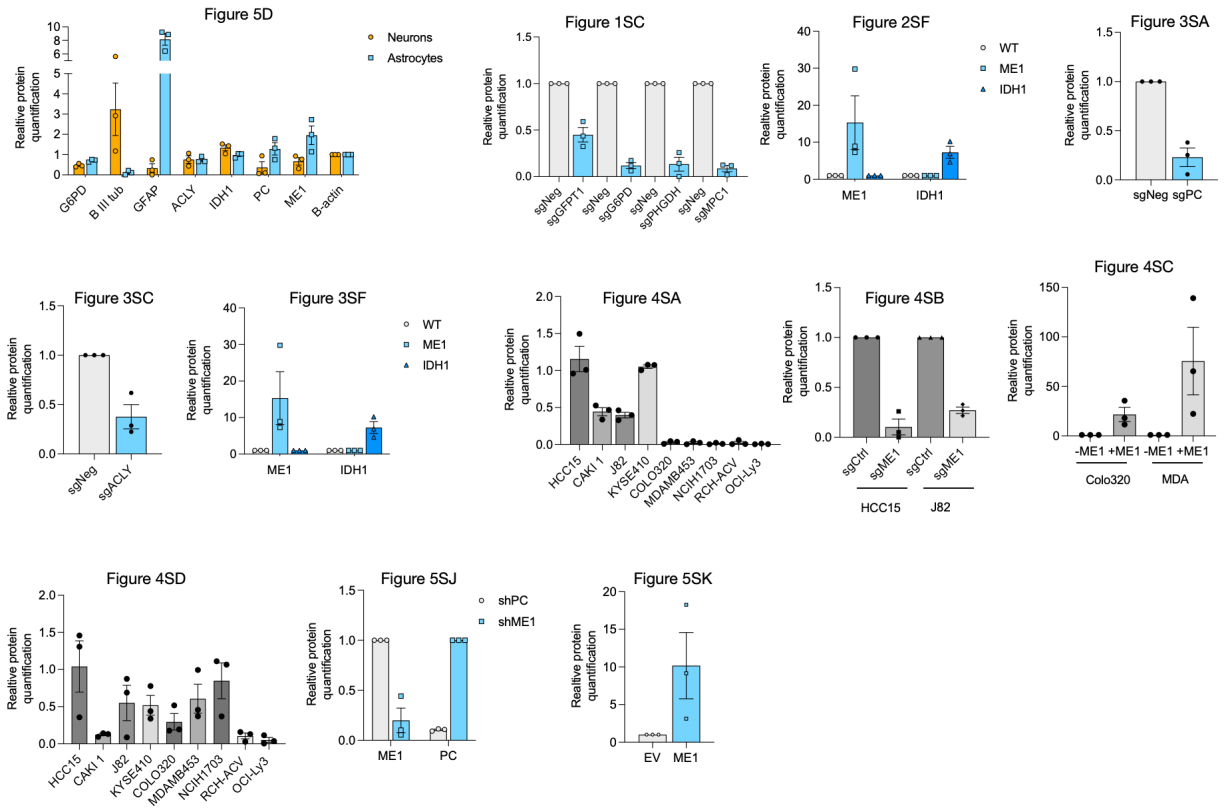

Figure 10. Quantification of the immunoblots presented in the manuscript.

3. The Drosophila experiments in Figure 1E are very poorly explained and the model is not validated. It is not explained in which tissues ND49 and ND51 are knocked down. The rn-Gal4 driver expresses in certain regions in some imaginal discs but there is no justification for why this driver was used, nor is there any validation that ND49 and ND51 expression is reduced by RNAi expression. The methods state that the flies were raised at 17 degrees but Drosophila experiments are usually performed at the optimum temperature of 25 degrees. There is no explanation for why 17 degrees was used. Gal4 activity is temperature sensitive and so knockdown would be much weaker at 17 degrees than 25 degrees. It's also not explained whether the Pgd and Zw mutations were heterozygous or homozygous and there is no validation that the PPP is affected by these mutations. It's also hard to fully understand the viability data. 'Phenotype aggressiveness' is also the wrong term to use, phenotype severity would be more appropriate. It would be much better to use one of the several existing Drosophila CI deficiency models that are published and validated. I would recommend using one of these models or simply removing the Drosophila data completely from the manuscript.

We apologize for the lack of clarity in explaining the experiments presented in Figure 1E. These results are indeed crucial as they represent the first documentation of synthetic lethality between PPP and ETC dysfunction in an in vivo model. The selection of the rn-Gal4 driver was based on its known expression in certain regions of imaginal discs and in the nervous system (Flybase). However, we acknowledge the need for better justification and validation of ND49 and ND51 knockdown, which we have addressed by performing qPCR validation (see graph below in Figure

11). As a matter of fact, these models of CI deficiency have been extensively used and validated in previous studies[78] which is the main reason we had selected them. While 25 degrees is often the optimal temperature for Drosophila experiments, we selected 17 degrees in this case because the Gal4 protein is temperature dependent. At 25 degrees larvae do not progress into pupae (presumably due to the expression in the nervous system) while at 17 degrees we observe some escapers. The Pgd and Zw mutations are both on the X chromosome and this model has been used previously in several studies[910]. Furthermore, we have revised the terminology from 'phenotype aggressiveness' to 'phenotypic severity' for better clarity. All these details have now been included in the methods section, and we apologize for the oversight in the previous version.

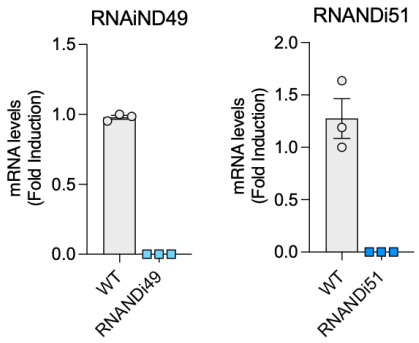

Figure 11. qPCR analysis to assess the levels of knockdown of ND49 and ND51. The housekeeping gene rpl32 was used for normalization.

4. The KO cell lines are validated by western blot but there is no validation that ME1 is overexpressed in the CNS in the Ndufs4 KO mice. Data showing ME1 overexpression in the Ndufs4 KO mice expressing AAV9-ME1 using e.g. western blot or immunostaining should be included.

We agree with the reviewer that the manuscript lacks this important control. In the new Figure 6SA, we now show an immunoblot and its quantification, confirming increased ME1 protein expression in the vestibular nuclei (VN) of mice injected with AAV9-ME1.

5. The implication of the study, as mentioned in the discussion and illustrated in the model in Figure 7, is that when CI/PPP are inhibited in cancer cells reduced NADPH levels cause increased oxidative stress which leads to reduced cell proliferation. In neurons, CI deficiency alone leads to decreased NADPH and causes reduced capacitance and excitability through the same mechanism. One prediction of the model is that reducing oxidative stress should have similar beneficial effects to overexpression of ME1. The authors should test their model by analysing whether treatment with antioxidants improves the phenotypes in cancer cells and neurons, similar to ME1 overexpression.

We thank the referee for pointing out the potential implications of our model. We concur with your suggestion and have indeed explored the effects of antioxidants on the observed phenotypes. As demonstrated in Figure 2SD, supplementing cells with GSH increased cell number in cells treated with Piericidin and G6PDi, supporting the notion that reducing oxidative stress can ameliorate

some of the observed phenotypes in cancer cells. Regarding neurons, GSH did not rescue the phenotype (Figure 12). This outcome could be due to multiple factors. It is possible that the cell-permeable derivative of glutathione, Glutathione Monoethyl Ester (GSH-MEE), is not the most effective strategy for use in neurons. Alternatively, the phenotype might be linked to other functions of NADPH, such as lipid synthesis.

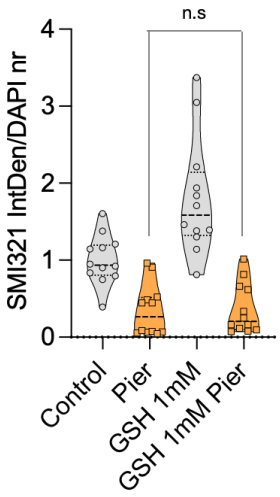

Figure 12. Quantification of axon degeneration using SMI-312 marker, measured in neurons treated with DMSO, piericidin and/or GSH for 24 hours.

6. The title of the manuscript and the abstract are misrepresentative. Nearly all the data in figures 1-4 are from non-neural proliferating cells and cancer cells, while the title and abstract give the reader the impression that the paper is focused on the brain. I recommend removing the word brain from the title and modifying the abstract so that it is more representative of the data and that most of the experiments are performed in non-neural cells. Moreover, the in vivo Ndufs4 KO ME1 overexpression data are the least convincing part of the story but are described as 'striking' in the abstract, while the data in the cancer cell lines is much more compelling but is not mentioned in the abstract. I would recommend re-writing the abstract to address these issues.

We recognize that although the figures related to brain metabolism make up roughly 30% of the total content, they play a crucial role in highlighting the importance of the PC-ME1 axis within the context of mitochondrial dysfunction across various brain cell types. Nevertheless, in response to the referee's suggestion, we have diligently revised the title and abstract to better capture the overall manuscript's scope.

Minor points

1. On line 180/181 the authors suggest that neurons are highly dependent on ATP levels. This is inconsistent with the latter findings and the points made in the discussion and so should be clarified. This statement aligns with the traditional belief that the adverse effects observed in neurons and other cell types following ETC inhibition are primarily attributed to ATP level disruption, a notion that our findings and others have begun to challenge in recent years.

2. There are lots of typos throughout the manuscript including typos between lines 208 and 230 in the supplemental figure names. Also on line 320. Inmunoblot spelling should be corrected. We appreciate the referee's observation regarding the typos and misspellings in the manuscript, including those between lines 208 and 230 in the supplemental figure names, as well as on line 320 ('Inmunoblot'). We have diligently corrected these errors.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts."

References:

1. Chen, L. *et al.* NADPH production by the oxidative pentose-phosphate pathway supports folate metabolism. *Nat Metab* **1**, 404–415 (2019).
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4. Ghergurovich, J. M. *et al.* A small molecule G6PD inhibitor reveals immune dependence on pentose phosphate pathway. *Nat Chem Biol* **16**, 731–739 (2020).
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6. Chen, L. *et al.* NADPH production by the oxidative pentose-phosphate pathway supports folate metabolism. *Nat Metab* **1**, 404–415 (2019).
7. Foriel, S. *et al.* A Drosophila Mitochondrial Complex I Deficiency Phenotype Array. *Front Genet* **10**, 245 (2019).

8. Perez-Gomez, R., Magnin, V., Mihajlovic, Z., Slaninova, V. & Krejci, A. Downregulation of respiratory complex I mediates major signalling changes triggered by TOR activation. *Sci Rep* **10**, 4401 (2020).
9. Nero, D., Bowditch, N., Pickert, S. & MacIntyre, R. J. A genetic and molecular analysis of P-induced mutations at the glucose-6-phosphate dehydrogenase locus in Drosophila melanogaster. *Mol Gen Genet* **219**, 429–438 (1989).
10. Sieber, M. H., Thomsen, M. B. & Spradling, A. C. Electron Transport Chain Remodeling by GSK3 during Oogenesis Connects Nutrient State to Reproduction. *Cell* **164**, 420–432 (2016).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all my concerns and improved the manuscript accordingly. I am happy to support the revised version for publication.

Reviewer #2 (Remarks to the Author):

I am satisfied with the revised manuscript other than three minor points.

1. For one experiment, the authors did not perform the experiment I requested.
I requested "To strengthen the claim that ETC inhibition increases the PPP the authors should perform 13C6-glucose tracing into the PPP intermediates in vehicle, complex I, and complex III inhibited cells +/- G6PD depletion. Additionally, 13C6-glucose tracing should be done in GFPT1, PHGDH, and MPC KO cells +/- complex I and III inhibition to demonstrate no difference in PPP incorporation, as these pathways were not synthetic lethal with ETC inhibition." The authors responded to this by doing no additional assays to corroborate the complex I and III contributions to PPP flux and especially did not perform the requested glucose tracing study. The authors stated "Nevertheless, as suggested by the referee, we have conducted PPP activity measurements in GFPT1, PHGDH, and MPC knockout cells using the gold standard radioactive methodology. We consider that including the condition with Piericidin is not relevant in these settings, as we have already demonstrated that inhibiting CI leads to an increase in PPP. However, we have included a positive control using the well-validated inhibitor of glucose-6-phosphate dehydrogenase, G6PDi (Figure 4). As expected, none of the GFPT1, PHGDH, and MPC knockout cells significantly impacted PPP activity, compared to G6PDi treatment, which completely abrogated PPP activity. We believe that these new experiments significantly strengthen our claim regarding the relationship between ETC inhibition and increased PPP activity."
The aforementioned experiment does not strengthen the claim of the relationship between ETC inhibition and increased PPP activity because it does not inhibit the ETC and measure PPP activity.
2. The second point is related to my comment "Throughout the manuscript, the authors sometimes report NADPH levels and other times report the ratio of NADPH:NADP. It would be ideal if both the relative levels of NADPH and NADP, and their ratio, is reported for each experiment."
The authors reply: "We appreciate the referee's suggestion regarding the reporting of NADPH levels and the NADPH/NADP+ ratio in the manuscript. To our knowledge, Figure 2E was the only one where the values of NADPH were presented instead of the ratio. In response to this feedback, we have now included the ratio of NADPH/NADP+ for all figures throughout the manuscript, ensuring consistency in our reporting. At this stage, we find it unnecessary to include separate values, given that, as previously mentioned, our findings indicate no changes in NADP+ levels, only in NADPH."
I think for completeness it would be best for the authors to include the individual levels of NADP+ and NADPH for each experiment.

3. The glucose tracing performed in neurons versus astrocytes is nice but should include some

statistical analysis of the comparison between neurons and astrocytes to demonstrate the difference. Also, specific discussion of the expected labeling for PPP flux using U-13C6-glucose into 13C6 - 6-phosphoglucolactone, 13C5 -ribose-5-P, and 13C4 -erythrose-4-P should be emphasized in the text, as these appear to be greater in the neurons (pending statistical analysis). Glucose-6-phosphate labeling should also be included in this panel to demonstrate equal (or near equal) enrichment of this labeling in the astrocytes and neurons.

Reviewer #3 (Remarks to the Author):

The authors have provided a rebuttal of all comments made in the previous review.

Reviewer #4 (Remarks to the Author):

The authors have mostly addressed my comments but several points need to still be addressed:

1. The western blot quantifications included in the rebuttal are important data and should be added to the supplemental figures in the revised manuscript.
2. Can the authors please cite papers supporting the statement in the methods that the "rn-Gal4 driver is expressed in certain regions of imaginal discs and in the nervous system".
3. In response to point 6 "As demonstrated in Figure 2SD, supplementing cells with GSH increased cell number in cells treated with Piericidin and G6PDi..." I cannot see these data in the revised manuscript so they should be added.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all my concerns and improved the manuscript accordingly. I am happy to support the revised version for publication.

Reviewer #2 (Remarks to the Author):

I am satisfied with the revised manuscript other than three minor points.

1. For one experiment, the authors did not perform the experiment I requested.
I requested "To strengthen the claim that ETC inhibition increases the PPP the authors should perform 13C6-glucose tracing into the PPP intermediates in vehicle, complex I, and complex III inhibited cells +/- G6PD depletion. Additionally, 13C6-glucose tracing should be done in GFPT1, PHGDH, and MPC KO cells +/- complex I and III inhibition to demonstrate no difference in PPP incorporation, as these pathways were not synthetic lethal with ETC inhibition." The authors responded to this by doing no additional assays to corroborate the complex I and III contributions to PPP flux and especially did not perform the requested glucose tracing study. The authors stated "Nevertheless, as suggested by the referee, we have conducted PPP activity measurements in GFPT1, PHGDH, and MPC knockout cells using the gold standard radioactive methodology. We consider that including the condition with Piericidin is not relevant in these settings, as we have already demonstrated that inhibiting CI leads to an increase in PPP. However, we have included a positive control using the well-validated inhibitor of glucose-6-phosphate dehydrogenase, G6PDi (Figure 4). As expected, none of the GFPT1, PHGDH, and MPC knockout cells significantly impacted PPP activity, compared to G6PDi treatment, which completely abrogated PPP activity. We believe that these new experiments significantly strengthen our claim regarding the relationship between ETC inhibition and increased PPP activity."
The aforementioned experiment does not strengthen the claim of the relationship between ETC inhibition and increased PPP activity because it does not inhibit the ETC and measure PPP activity.

To assess the activity of PPP, using 13C6 glucose tracing is not the best approach, as it could lead to ambiguous interpretations. Conversely, the radioactive-based experiments we have performed are considered in the field as the gold-standard approach to accurately measure PPP activity with enhanced sensitivity. We have clearly demonstrated (Fig. 1F, S1D, and S1F) that Complex I inhibition leads to increased PPP activity, while depletion of GFPT1, PHGDH, or MPC does not (Figure 4 rebuttal letter). Thus, our results provide strong arguments for an intimate relationship between ETC inhibition and increased PPP activity, which are further corroborated by our in vivo experiments using Drosophila models.

2. The second point is related to my comment "Throughout the manuscript, the authors sometimes report NADPH levels and other times report the ratio of NADPH:NADP. It would be

ideal if both the relative levels of NADPH and NADP, and their ratio, is reported for each experiment."
The authors reply: "We appreciate the referee's suggestion regarding the reporting of NADPH levels and the NADPH/NADP+ ratio in the manuscript. To our knowledge, Figure 2E was the only one where the values of NADPH were presented instead of the ratio. In response to this feedback, we have now included the ratio of NADPH/NADP+ for all figures throughout the manuscript, ensuring consistency in our reporting. At this stage, we find it unnecessary to include separate values, given that, as previously mentioned, our findings indicate no changes in NADP+ levels, only in NADPH."
I think for completeness it would be best for the authors to include the individual levels of NADP+ and NADPH for each experiment.

We have included the individual levels of NADPH and NADP+ in the revised version of the manuscript.

3. The glucose tracing performed in neurons versus astrocytes is nice but should include some statistical analysis of the comparison between neurons and astrocytes to demonstrate the difference. Also, specific discussion of the expected labeling for PPP flux using U-13C6-glucose into 13C6 - 6-phosphoglucolactone, 13C5 -ribose-5-P, and 13C4 -erythrose-4-P should be emphasized in the text, as these appear to be greater in the neurons (pending statistical analysis). Glucose-6-phosphate labeling should also be included in this panel to demonstrate equal (or near equal) enrichment of this labeling in the astrocytes and neurons.

We have included this data in the revised version of the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have provided a rebuttal of all comments made in the previous review.

Reviewer #4 (Remarks to the Author):

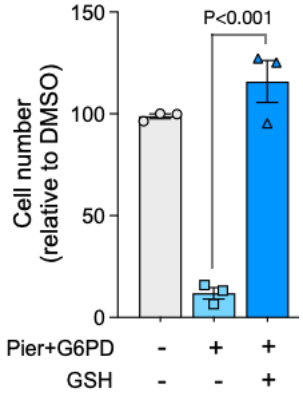
The authors have mostly addressed my comments but several points need to still be addressed:

1. The western blot quantifications included in the rebuttal are important data and should be added to the supplemental figures in the revised manuscript.
All the western blot quantifications have been included in the new version of the manuscript.

2. Can the authors please cite papers supporting the statement in the methods that the "rn-Gal4 driver is expressed in certain regions of imaginal discs and in the nervous system".
Reference regarding the expresion of rn-Gal4 has been included in the methods section (PMID: 24268416).

3. In response to point 6 "As demonstrated in Figure 2SD, supplementing cells with GSH increased cell number in cells treated with Piericidin and G6PDi..." I cannot see these data in the revised manuscript so they should be added.

In supplementary figure 2f of the manuscript is clearly demostrated that GSH supplementation rescued cell number of cells exposed to Piericidin and G6PDi. See below.



Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.