

4'-Phosphopantetheine corrects CoA, iron and dopamine metabolic defects in mammalian models of PKAN

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

10 April 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

You will see from the set of comments below that both referees find the study important, and while ref. 1 is supportive of publication, ref. 2 is more hesitant and raises some pertinent issues: relevance of the mouse model, phosphopantetheine treatment rescue-effect to dissect, as well as sub-cellular localisation of PPCS and PPCDC to assess. We would also like you to fix some technical limitations and provide more details and explanations. Both referees agree that the link between the two papers should be made stronger (same comment went to the other paper).

Upon our cross-commenting exercise, ref. 2 added "As it stands, the only link highlighted by both papers in that the impaired mtACP biogenesis result reported in the Sibon ms [A single experiment (Fig. 2) conducted in S1 insect cells, supported by one of the kd experiments shown in Fig. 4 (E' and F')]. For back-to-back papers, I would have liked to see greater interdependency. In some respects (as also noted by Ref. 1) the two papers currently actually mutually detract, and consequently reduce the overall impact of the other paper." Further this referee also added "The enzyme activity data is not convincing. And the authors do not correctly represent our current knowledge of the subcellular localization of the CoA biosynthetic enzymes."

We would therefore welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

The exact mechanism used to generate the mouse ko should be described or referenced. It appears to be a total ko- what was the approach used to make this model

Referee #1 (Remarks for Author):

This paper submission represents the culmination of years of preparatory work and it overcomes previous roadblocks. The dissection of the brain into sections, focusing on GP, proved to very useful. The molecular amelioration of feeding 4 phosphopantetheine to animals is impressively clear. In the next paper, the authors could consider using IH with some of their markers to see if a specific subset of GP cells are affected. It was probably very good for their study that the mice did not develop advanced disease.

Minor

They need to explain in greater detail exactly which mouse model is used here and how it was generated.

Referee #2 (Comments on Novelty/Model System for Author):

One of the central points of the manuscript is the study of the model system to determine its utility/adequacy; in this sense this question is therefore not entirely relevant.

Referee #2 (Remarks for Author):

In the manuscript by Jeong et al. the authors set out to establish an improved mammalian model of PKAN, the neurodegenerative disease associated with a genetic defect in the first enzyme of coenzyme A (CoA) biosynthesis, pantothenate kinase (PanK). This was achieved by isolating brain tissues known to be affected by the disease from healthy tissue and by identifying biomarkers in the isolated tissues that show changes that correlate with disease status, and that can be corrected by intervention with a CoA biosynthetic pathway intermediate that bypasses the PanK defect.

The described research is an excellent observational study rich in data obtained from a variety of samples and different treatment conditions. However, the data-rich results, and specifically the manner in which it is presented unfortunately makes it difficult to determine a) what the main goal of the study was and b) whether the data interpretation is unambiguous, i.e. whether other conclusions would not be equally valid. Consequently, it is not obvious how the outcome should be evaluated. This reduces the overall impact of the manuscript in its current form.

To highlight a few specific concerns:

1. Model organism and biomarker identification: The manuscript's biggest strength is in the studies done to establish an animal model for PKAN, and to identify biomarkers of the disease. The finding that a Pank2 KO mouse that does not show a strong and immediate disease phenotype indeed shows differences in the expression of key genes (determined through qRT-PCR) in the disease-vulnerable globus pallidus (GP) is an important finding. However, while implied by the nature of the analysis, the authors should concede that the utility of the model and the biomarkers may be limited due to the invasive nature of the analysis, which requires sacrifice of the animals. Or they need to be

clearer in how they see the model being used in the study of the disease and treatments - this relates to their use of fibroblasts as surrogate for analysis (See also point 4 below).

2. Biomarker analysis: The bulk of the biomarker analysis is done by qRT-PCR. In this context it is important to provide evidence of the robustness of the method and the associated statistical analysis. Specifically, the authors used the Comparative CT method (ddCT) to perform quantification. This methodology assumes 100% PCR efficiency in all samples; if there are differences in the PCR efficiency between the reference gene and the gene of interest, this method will yield greatly skewed results. The biomarker analysis will be significantly improved if a method is used that takes this variation in efficiency into account, such as the one proposed by Pfaffl (PMID: 11328886). Moreover, the authors chose to report all the data in a set as % values relative to the mRNA level related to the gene of interest in the GP. Are they certain that the reference gene's expression levels are the same across all brain regions? If not, then the comparison cannot be made in this manner. Finally, I'm concerned about the statistical analysis when there is such a large variation in the number of independent samples used in a specific experiment. E.g. in the case of the data presented in Figs. 2a and 3c the value of n varies between 3 and 11. Why such a big difference? Although ANOVA analysis usually takes account of such differences, it would be preferable if the sample sizes were more equally matched. Moreover, for the data in Figure 1, two sets of n values are given but it is not clear what each refers to. Overall, considering that the conclusions of the study stands and falls with the biomarker analyses, I would like to see a more thorough presentation of the method and the statistical treatment of the results.

3. Enzyme activity analysis: The authors report activity assays for aconitase (fig. 2c), PDH in the GP (fig. 2d) and complex I in the GP (Fig. 2e). However, I have several concerns about this data. First, the authors are not clear how the results are normalized to total protein in the sample, or how the sample sizes (i.e. amount of cells used for preparations of the lysates) are kept similar. The relevant statements in the methods section is: "Total protein...[]...was extracted". Was the amount of tissue used the same? How was the protein extracted? For the aconitase assay, how much protein was loaded? Was this standardised? For the PDH assay, can the authors be sure that the Dipstick was not saturated with PDH? The assays as presented here are all in reporting specific activities, which is an indication of the amount of active enzyme relative to the total amount of protein. It is therefore important to be clear on how the total amount of protein was determined and how it was kept comparable between the samples.

4. Correlation of data between systems: The authors indicate that their findings in mouse was corroborated by using primary cells from PKAN patients (fibroblasts and lymphoblasts). Yet, in the previous section the focus was to show a distinction in the expression levels of the biomarkers in the different brain tissues in the mouse. Why do they expect the expression in primary blood cells to correspond with those in the GP? How can they be sure that this correlation relates to the disease phenotype, particularly if they spend so much effort in showing that there is NO correlation between the expression in different brain tissues, and between brain and liver?

5. Effects of phosphopantetheine treatment: The authors demonstrate effects on the biomarker expression levels following phosphopantetheine treatment, but not following treatment with CoA, pantetheine, or Vit B5. However, the authors acknowledge in the discussion that this is not unexpected as the other test compounds are all expected to be broken down. Consequently, the value of such a negative result is rather questionable. In regards to the effect on Coasy expression following phosphopantetheine treatment the authors conclude that high levels repress its expression in both WT and KO animals; yet the levels are similar in the treated and not treated KO. It is therefore unclear if any repression is actually happening, or if the lower levels of phosphopantetheine just alleviates the basal repression seen in the KO animal. Again, as alluded to in the previous point, it is unclear how the results obtained from the human fibroblasts can be related to those from the mouse GP.

6. Hypothesis of PKAN pathogenesis: The authors use the hypothesis proposed by Lambrechts et al (the paper submitted together with theirs) to explain their results. Indeed, in my opinion this is the only reason why the current paper deserves to be combined by the one by Lambrechts; however, it could equally be evaluated and understood separately from it. Be that as it may, a key point not explained by this model, and which the authors fail to address, is why the phosphopantetheine produced by the cytosolic biosynthesis of CoA fails to provide similar rescue as seen when

phosphopantetheine is exogenously applied. Indeed, a recent report indicated that activation of the cytosolic PanK enzymes could alleviate PKAN-related phenotypes (PMID: 30352999). Moreover, the model doesn't explain why the effects are uniquely detrimental to the GP, while the impact on CoA, mtACP and all corresponding factors should be the same in all cells. The key question here is subcellular localization of the PPCS and PPCDC enzymes, and the permeability of the mitochondrial membrane to CoA intermediates - and whether this differs by cell type/tissue. Although the authors state in the introduction that CoA biosynthesis is cytosolic, to my knowledge no evidence has been published on the subcellular localization of the PPCS and PPCDC enzymes. Since these enzymes are able to form phosphopantetheine from the product of the PanK reaction, having this knowledge is key to the complete understanding of PKAN pathogenesis, and treatment options.

Apart from addressing the questions above, the authors should also pay attention to the overall writing and structure of the manuscript. For the reader not familiar with the disease or the genes in question, it would be helpful to have a bit more context provided when these are first mentioned (eg. the genes referred to in the first paragraph on p. 8). There also seem to be an inconsistent handling of the gene and protein nomenclature in regards to capitals/italics in the text and figures/figure legends (note specifically the various forms of Coasy). Finally, some of the figure legends are not completely clear without reference to the text.

Taken together, I believe that it would be premature to publish the manuscript in its current form.

1st Revision - authors' response

10 July 2019

Referee #1 (Comments on Novelty/Model System for Author):

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See below.

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Minor

They need to explain in greater detail exactly which mouse model is used here and how it was generated.

We appreciate Referee #1's suggestion and will explore cell specific changes in a future manuscript.

We clarified that the mouse model is a whole-body, germline Pank2 null mutant mouse in the 'Main' and 'Methods' sections and referenced the original report.

Referee #2 (Comments on Novelty/Model System for Author):

One of the central points of the manuscript is the study of the model system to determine its utility/adequacy; in this sense this question is therefore not entirely relevant.

Referee #2 (Remarks for Author):

In the manuscript by Jeong et al. the authors set out to establish an improved mammalian model of PKAN, the neurodegenerative disease associated with a genetic defect in the first enzyme of coenzyme A (CoA) biosynthesis, pantothenate kinase (PanK). This was achieved by isolating brain tissues known to be affected by the disease from healthy tissue and by identifying biomarkers in the isolated tissues that show changes that correlate with disease status, and that can be corrected by intervention with a CoA biosynthetic pathway intermediate that bypasses the PanK defect.

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samples and different treatment conditions. However, the data-rich results, and specifically the manner in which it is presented unfortunately makes it difficult to determine a) what the main goal of the study was and b) whether the data interpretation is unambiguous, i.e. whether other conclusions would not be equally valid. Consequently, it is not obvious how the outcome should be evaluated. This reduces the overall impact of the manuscript in its current form.

See above General responses.

To highlight a few specific concerns:

1. Model organism and biomarker identification: The manuscript's biggest strength is in the studies done to establish an animal model for PKAN, and to identify biomarkers of the disease. The finding that a Pank2 KO mouse that does not show a strong and immediate disease phenotype indeed shows differences in the expression of key genes (determined through qRT-PCR) in the disease-vulnerable globus pallidus (GP) is an important finding. However, while implied by the nature of the analysis, the authors should concede that the utility of the model and the biomarkers may be limited due to the invasive nature of the analysis, which requires sacrifice of the animals. Or they need to be clearer in how they see the model being used in the study of the disease and treatments - this relates to their use of fibroblasts as surrogate for analysis (See also point 4 below).

We have added more specific information about the utility and limitations of this animal model for studies of disease pathogenesis and potential therapeutics. While the need to sacrifice animals to study specific brain abnormalities does pose limitations, this is a common challenge with mammalian models of neurological disorders.

2. Biomarker analysis: The bulk of the biomarker analysis is done by qRT-PCR. In this context it is important to provide evidence of the robustness of the method and the associated statistical analysis. Specifically, the authors used the Comparative CT method (ddCT) to perform quantification. This methodology assumes 100% PCR efficiency in all samples; if there are differences in the PCR efficiency between the reference gene and the gene of interest, this method will yield greatly skewed results. The biomarker analysis will be significantly improved if a method is used that takes this variation in efficiency into account, such as the one proposed by Pfaffl (PMID: 11328886). Moreover, the authors chose to report all the data in a set as % values relative to the mRNA level related to the gene of interest in the GP. Are they certain that the reference gene's expression levels are the same across all brain regions?

If not, then the comparison cannot be made in this manner. Finally, I'm concerned about the statistical analysis when there is such a large variation in the number of independent samples used in a specific experiment. E.g. in the case of the data presented in Figs. 2a and 3c the value of n varies between 3 and 11. Why such a big difference? Although ANOVA analysis usually takes account of such differences, it would be preferable if the sample sizes were more equally matched. Moreover, for the data in Figure 1, two sets of n values are given but it is not clear what each refers to. Overall, considering that the conclusions of the study stands and falls with the biomarker analyses, I would like to see a more thorough presentation of the method and the statistical treatment of the results.

We appreciate the Referee's concern regarding the primer efficiency and the equal expression of Gapdh as a housekeeping gene, which are important factors that can change the overall results. Therefore, we re-tested all the primer efficiencies used in study using serially diluted cDNA samples. All primer efficiencies were between 90-110%, which is expected for a good SYBR green QPCR reaction.

Target	R ² value	PCR Efficiency (%)
<i>Coasy</i>	0.9995	101.6
<i>Drd1</i>	0.9951	110.7
<i>Gapdh</i>	0.9986	103.1
<i>Ireb2</i>	0.9991	101.0
<i>Pank1α</i>	0.9961	100.3
<i>Ppcs</i>	0.9989	104.1
<i>Tfrc</i>	0.9976	102.8

***We also confirmed that expression of the housekeeping gene (Gapdh) is not skewed between tri-
sected areas. [Unpublished data removed at the authors' request.] Moreover, mRNA expression
data from the Allen Brain Atlas also shows relatively equal expression of Gapdh throughout
mouse brain [unpublished data removed at the authors' request]. Finally, we have clarified
number of samples used for each experiment.***

3. Enzyme activity analysis: The authors report activity assays for aconitase (fig. 2c), PDH in the GP (fig. 2d) and complex I in the GP (Fig. 2e). However, I have several concerns about this data. First, the authors are not clear how the results are normalized to total protein in the sample, or how the sample sizes (i.e. amount of cells used for preparations of the lysates) are kept similar. The relevant statements in the methods section is: "Total protein...[]...was extracted". Was the amount of tissue used the same? How was the protein extracted? For the aconitase assay, how much protein was loaded? Was this standardised? For the PDH assay, can the authors be sure that the Dipstick was not saturated with PDH? The assays as presented here are all in reporting specific activities, which is an indication of the amount of active enzyme relative to the total amount of protein. It is therefore important to be clear on how the total amount of protein was determined and how it was kept comparable between the samples.

We agree with the Referee that the PDH and complex I activity assays have potential limitations of saturation of the 'capturing antibody'. Therefore, we ran control experiments first in order to examine this issue. Below is the PDH activity dipstick assay using only WT GP samples with increasing amount of protein. Based on these data, we used 6ug of total protein for each group. The manufacturer recommends using ~ 3/4 of total protein within the linear range. The same control experiments were performed for the complex I activity dipstick assay, and 2ug of total protein was used for this assay. [Unpublished data removed at the authors' request.

As for the normalization, we used the same amount of total protein extracted from mouse brain and human cells. This could have posed a problem if there were atrophy in mouse brain or significant differences in cell size between genotypes. However, we have observed no protein synthesis problem or differences in cell size of any samples. As a matter of fact, total protein yield per wet tissue weight (see table below) did not differ between WT and KO mouse GP. We affirm that this information was not reported in detail in the Methods section and have now modified the manuscript accordingly.]

Genotype	GP total protein / wet tissue weight (mg/mg)	Student's t test between genotypes
WT	9.06 $\pm$ 0.84	
KO	8.46 $\pm$ 0.51	0.07

4. Correlation of data between systems: The authors indicate that their findings in mouse was corroborated by using primary cells from PKAN patients (fibroblasts and lymphoblasts). Yet, in the previous section the focus was to show a distinction in the expression levels of the biomarkers in the different brain tissues in the mouse. Why do they expect the expression in primary blood cells to correspond with those in the GP? How can they be sure that this correlation relates to the disease phenotype, particularly if they spend so much effort in showing that there is NO correlation between

the expression in different brain tissues, and between brain and liver?

We were surprised to find the same patterns of biomarker changes in fresh blood cells and cultured fibroblasts, and we lack a strong hypothesis to explain this. Nevertheless, we did observe similar differences in biomarkers in blood and brain at baseline and in response to phosphopantetheine treatment in mice. We do not, however, expect the blood biomarkers to be directly influenced by levels of the brain biomarkers. Instead, we view the blood biomarkers as quantifiable abnormalities that can be used to determine if oral treatment reaches the circulation, is taken up by cells, and alters gene expression (COASY) or protein function (complex I) in an accessible cell type. We hypothesize that a candidate therapeutic that is delivered systemically to humans and crosses the blood-brain barrier would change expression in blood cells in parallel to changing expression in neurons. In this way, the blood biomarkers may be able to serve as a surrogate for brain changes in human studies. We have reviewed the text to ensure that this idea is clearly presented and appreciate further comments if not.

5. Effects of phosphopantetheine treatment: The authors demonstrate effects on the biomarker expression levels following phosphopantetheine treatment, but not following treatment with CoA, pantetheine, or Vit B5. However, the authors acknowledge in the discussion that this is not unexpected as the other test compounds are all expected to be broken down. Consequently, the value of such a negative result is rather questionable.

Readers who are not immersed in the CoA field may wonder about the effects of administering these related compounds. Moreover, in published cell culture systems, CoA has had some therapeutic effect. Therefore, we propose to retain Fig 6a within the larger figure. At the Editor's request, we could move Fig 6a to supplemental data.

In regards to the effect on Coasy expression following phosphopantetheine treatment the authors conclude that high levels repress its expression in both WT and KO animals; yet the levels are similar in the treated and not treated KO. It is therefore unclear if any repression is actually happening, or if the lower levels of phosphopantetheine just alleviates the basal repression seen in the KO animal.

We acknowledge that this is an alternate explanation of the KO data; however, the observation of a similar decrease in Coasy expression with higher doses of 4'-phosphopantetheine in the WT animals would then require a second explanation. Therefore, we propose the most parsimonious interpretation of our data relying on a single mechanism for decreased expression at higher doses in both genotypes. That said, we have introduced the Referee's concept regarding these results, and we appreciate the alternate idea.

Again, as alluded to in the previous point, it is unclear how the results obtained from the human fibroblasts can be related to those from the mouse GP.

See 4 above.

6. Hypothesis of PKAN pathogenesis: The authors use the hypothesis proposed by Lambrechts et al (the paper submitted together with theirs) to explain their results. Indeed, in my opinion this is the only reason why the current paper deserves to be combined by the one by Lambrechts; however, it could equally be evaluated and understood separately from it.

We agree that they papers could stand alone; however, our view is that Jeong amplifies the medical relevance of Lambrechts, and Lambrechts provides strong experimental data implicating mtACP in support of hypothesis presented in Jeong. Therefore, we prefer to keep the papers together.

Be that as it may, a key point not explained by this model, and which the authors fail to address, is why the phosphopantetheine produced by the cytosolic biosynthesis of CoA fails to provide similar rescue as seen when phosphopantetheine is exogenously applied.

We acknowledge this as a limitation of our current knowledge of the pathway and have now stated that explicitly.

Indeed, a recent report indicated that activation of the cytosolic PanK enzymes could alleviate PKAN-related phenotypes (PMID: 30352999).

This comment raises an important point that we have addressed directly in our paper. The model reported in the referenced paper is not a PKAN-related phenotype; it requires a double-hit to cause global impairment, and the phenotype is no more PKAN-like than is that of any other gait-

impaired mouse. As an example, cerebellar loss of Pank1+Pank2 may cause ataxia and gait abnormalities, one of many feasible explanations that were not explored. While those authors represent that mouse as PKAN-like, their conclusion is not supported; they created a neuronal CoA-depleted mouse and treated it with a CoA-replenishing compound. Such a model may or may not have relevance to PKAN, as we state in our paper.

Moreover, the model doesn't explain why the effects are uniquely detrimental to the GP, while the impact on CoA, mtACP and all corresponding factors should be the same in all cells.

We acknowledge this as a limitation of our current knowledge of the pathway and have now stated that explicitly. We elected not to present the myriad possibilities that distinguish GP from other regions, including differences in energy demands, differences in cell membrane composition, differences in vascular anatomy, and vulnerability to hypoxia. We have added this list to the discussion around 'why GP?'.

The key question [ONE key question] here is subcellular localization of the PPCS and PPCDC enzymes, and the permeability of the mitochondrial membrane to CoA intermediates - and whether this differs by cell type/tissue.

This is an important point, and we appreciate its being raised. We performed new experiments and evaluated public databases to support our conclusion that PPCS is non-exclusively found in mitochondria and PPCDC is in cytoplasm. Moreover, we analyzed their subcellular localization in each brain region and found no differences nor any significant regional variation in their quantities. The Referee raises one possible explanation, which our mouse data now refute, but there are numerous other possible explanations, including differences in demand for mFAS, Fe-S cluster biogenesis or CoA demand, etc. We have corrected our statements about subcellular localization of the PPCS and PPCDC enzymes and have added to the discussion about possible bases for the observed regional differences as noted above.

Although the authors state in the introduction that CoA biosynthesis is cytosolic, to my knowledge no evidence has been published on the subcellular localization of the PPCS and PPCDC enzymes. Since these enzymes are able to form phosphopantetheine from the product of the PanK reaction, having this knowledge is key to the complete understanding of PKAN pathogenesis, and treatment options.

Based on this insightful comment about alternate substrates for pantothenate kinase (specifically pantetheine), we added this idea to our discussion and referenced the published evidence. We thank the Referee.

Apart from addressing the questions above, the authors should also pay attention to the overall writing and structure of the manuscript. For the reader not familiar with the disease or the genes in question, it would be helpful to have a bit more context provided when these are first mentioned (eg. the genes referred to in the first paragraph on p. 8). There also seem to be an inconsistent handling of the gene and protein nomenclature in regards to capitals/italics in the text and figures/figure legends (note specifically the various forms of Coasy). Finally, some of the figure legends are not completely clear without reference to the text.

We have edited the manuscript to introduce genes and proteins/enzymes early in the paper. In reference to mouse/human and gene/protein nomenclature, we have strived to accurately format these to reflect which we are talking about at every point in the manuscript. If questions remain, we appreciate our being pointed to specific questions of nomenclature. The legends have been modified to enable them to be understood without reference to the text.

2nd Editorial Decision

29 July 2019

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee who was asked to re-assess it. As you will see the reviewer is now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending minor editorial amendments and a response addressing the minor changes commented by referee 2.

Please submit your revised manuscript within two to three weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #2 (Remarks for Author):

The authors clearly attempted to address all the concerns raised in an appropriate manner. This has raised the quality and presentation of the manuscript to a level where publication would be warranted following appropriate revision of the following points:

- 1) The authors carefully responded to the questions raised regarding the qRT-PCR, but I do not see this information included in the manuscript, only in the rebuttal. I would suggest that it is included in the supplementary materials to support the quality of the analysis.
- 2) As requested, the authors have now clarified the number of samples used for the various experiments. However, the authors did not indicate whether they are concerned about the influence of the large variance in sample sizes (in some cases) on the statistical analysis. I'm not a statistician, and I will not push this point; yet in the context of validating biomarkers I would want to be certain that the data analysis is not skewed because of the use of unequal samples sizes.
- 3) I do not see the information that total protein yield per wet tissue weight was the same for WT and KO mouse GP included in the manuscript/SM. This is useful and should be added (perhaps as a statement in the methods?)
- 4) In regards to the localization of PPCS/PPCDC, the authors state in the rebuttal: "We performed new experiments and evaluated public databases to support our conclusion that PPCS is non-exclusively found in mitochondria and PPCDC is in cytoplasm. Moreover, we analyzed their subcellular localization in each brain region and found no differences nor any significant regional variation in their quantities."

However, apart from the following statement found in the discussion:

"The non-exclusive mitochondrial localization of several CoA synthesis enzymes, including PANK2, PPCS, and COASY, raises the intriguing possibility that CoA pathway intermediates such as 4'-phosphopantetheine might traverse organellar membranes and serve to replenish compartmental pools of CoA"

I do not see anything in the revised manuscript that reflects the outcome of these "new experiments" and analysis. The authors do provide references associated with this statement, but since they have gone through the trouble of doing the analysis I want to suggest that they are explicit with the reasons/basis for the statement.

In addition, I find the use of "non-exclusive mitochondrial localization" in the quoted statement somewhat problematic. First, this phrase also refers to PANK2, which is stated to be **UNIQUELY** localized in the mitochondria in the introduction. Using "uniquely" in the first instance and then "non-exclusively" here seems contradictory; if it isn't, the authors should be clear in what they mean so that there is no confusion.

Second, either PPCS or PPCDC *must* be exclusively localized in one of the subcellular compartments in order to give credence to the idea that there are compartmental pools of CoA. Do the authors propose that PPCDC is not found in the mitochondria? If so, they again must be explicit in this regard, and explain their reasoning for this proposal.

5) Gene/Protein nomenclature: The possible confusion here is in the reference of mouse vs human genes/proteins. On p.18, in the line "With higher doses of 4'-phosphopantetheine, we observed progressively lower levels of Coasy expression in GP" I believe Coasy should be in italics.

6) "phosphopantothenoylcysteine" is spelled without a hyphen.

2nd Revision - authors' response

7 August 2019

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1) The authors carefully responded to the questions raised regarding the qRT-PCR, but I do not see this information included in the manuscript, only in the rebuttal. I would suggest that it is included in the supplementary materials to support the quality of the analysis.

We appreciate the comment and have included these data in Appendix Figure S1A

2) As requested, the authors have now clarified the number of samples used for the various experiments. However, the authors did not indicate whether they are concerned about the influence of the large variance in sample sizes (in some cases) on the statistical analysis. I'm not a statistician, and I will not push this point; yet in the context of validating biomarkers I would want to be certain that the data analysis is not skewed because of the use of unequal samples sizes.

As Reviewer #2 noted in the previous review, ANOVA analysis factors the number of samples as a part of the analysis. Moreover, our statistical analyses for Figure 1C were within the brain region between genotypes and were never an intra-regional analysis. Therefore, these analyses were performed with relatively matching number of samples (11 vs. 8 in GP, 4 vs. 4 in SN and cerebellum). We also included all the exact n numbers in the figure legends to clarify this issue.

3) I do not see the information that total protein yield per wet tissue weight was the same for WT and KO mouse GP included in the manuscript/SM. This is useful and should be added (perhaps as a statement in the methods?)

We have added this information in the Materials and Methods section.

4) In regards to the localization of PPCS/PPCDC, the authors state in the rebuttal: "We performed new experiments and evaluated public databases to support our conclusion that PPCS is non-exclusively found in mitochondria and PPCDC is in cytoplasm. Moreover, we analyzed their subcellular localization in each brain region and found no differences nor any significant regional variation in their quantities."

However, apart from the following statement found in the discussion:

"The non-exclusive mitochondrial localization of several CoA synthesis enzymes, including PANK2, PPCS, and COASY, raises the intriguing possibility that CoA pathway intermediates such as 4'-phosphopantetheine might traverse organellar membranes and serve to replenish compartmental pools of CoA"

I do not see anything in the revised manuscript that reflects the outcome of these "new experiments" and analysis. The authors do provide references associated with this statement, but since they have gone through the trouble of doing the analysis I want to suggest that they are explicit with the reasons/basis for the statement.

We now also reference our own studies.

In addition, I find the use of "non-exclusive mitochondrial localization" in the quoted statement somewhat problematic. First, this phrase also refers to PANK2, which is stated to be UNIQUELY localized in the mitochondria in the introduction. Using "uniquely" in the first instance and then "nonexclusively" here seems contradictory; if it isn't, the authors should be clear in what they mean so that there is no confusion.

Second, either PPCS or PPCDC **must** be exclusively localized in one of the subcellular compartments in order to give credence to the idea that there are compartmental pools of CoA. Do the authors propose that PPCDC is not found in the mitochondria? If so, they again must be explicit in this regard, and explain their reasoning for this proposal.

We have clarified the language in both places in the manuscript.

5) Gene/Protein nomenclature: The possible confusion here is in the reference of mouse vs human genes/proteins. On p.18, in the line "With higher doses of 4'-phosphopantetheine, we observed progressively lower levels of Coasy expression in GP" I believe Coasy should be in italics.

We thank the Reviewer for directing us to this error in formatting, which now has been corrected.

6) "phosphopantothienoylcysteine" is spelled without a hyphen.
We have corrected this in the text.

We include updated data for Figure 4B. We removed the restriction in E-14 on Author's checklist because we expanded our fresh blood COASY QRT-PCR analysis from 'classical PKAN only' to 'all PKAN samples'. This has changed our Figure 4B and strengthened our statistical analysis. We have updated Author's checklist, main text, figure, and legend accordingly.

	<i>n</i>	<i>n</i>
	<i>Previously</i>	<i>Updated</i>
<i>Control</i>	<i>11</i>	<i>51</i>
<i>PKAN</i>	<i>9</i>	<i>35</i>

	<i>Previously</i>	<i>Updated</i>
<i>P</i>	<i>0.00595077</i>	<i>0.0000000282</i>

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Hayflick
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2019-10489

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For human primary cells, sample size was determined based on the number of available patient and their matching controls.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, minimum of three animals per genotype and treatment group to maximum of 11 was used to ensure statistical analysis (one-way or two-way ANOVA)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from this study
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Both mice and human samples were assigned with non-descriptive, anonymizing ID number and equally divided into treatment and vehicle groups.
For animal studies, include a statement about randomization even if no randomization was used.	See above
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Both mice and human samples were assigned with non-descriptive, anonymizing ID number to hide genotype and the treatment status. They were revealed and matched after data collection and analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	See above
5. For every figure, are statistical tests justified as appropriate?	We used either one-way or two-way ANOVA analysis depending on the number of variables (e.g. genotype, treatment group, day of sample collection, etc)
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used ANOVA for the statistical analyses (SigmaStat) and all the dataset passed the Shapiro-Wilk normality test
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	All the dataset passed the Brown-Forsythe equal variance test

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>
<http://1degreebio.org>
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>

<http://grants.nih.gov/grants/olaw/olaw.htm>
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>
<http://ClinicalTrials.gov>
<http://www.consort-statement.org>
<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun>

<http://datadryad.org>

<http://figshare.com>

<http://www.ncbi.nlm.nih.gov/gap>

<http://www.ebi.ac.uk/ega>

<http://biomodels.net/>

<http://biomodels.net/miriam/>
<http://jij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Mouse anti-COASY (Santa Cruz, sc-393812, PMID: 21471969), rat anti-DRD1 (Sigma, D2944, PMID: 28316027), mouse anti-TFR1 (ThermoFisher, 13-6800, knock-down verification, https://www.thermofisher.com/antibody/product/Transferrin-Receptor-Antibody-clone-H68-4-Monoclonal/13-6800), rabbit anti-PDH (Cell Signaling, 3205, PMID: 29572438), rabbit anti-VDAC (Cell Signaling, 4661), mouse MSH6 (BD, 610918), and mouse b actin (ThermoFisher, AM4302) antibodies have been used as loading controls in the lab.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Primary fibroblast lines were generated from 2mm skin biopsies. Primary lymphoblasts were generated using fresh blood. Sanger sequencing was used to confirm patient mutations. All cell lines are tested negative for mycoplasma contamination via DAPI staining.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Pank2 null mice and their littermate control on C57BL/6 background were used. Mice were between 3-6 months of age and both genders were used. This line was generated in the Hayflick laboratory (PMID: 15525657) and housed at Oregon Health & Science University, Department of Comparative Medicine's vivarium. Animals were housed under 12 hours light cycle and cared with daily monitoring while having free access to chow & water.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental protocols were pre-approved by OHSU IACUC (protocol no; IP00000450) and following NIH and NC3Rs guidelines.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLOS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We had followed NIH OLAW (Office of Laboratory Animal Welfare) guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	OHSU Institutional Review Board
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All the human sample collection were performed following informed consent. All subjects and the sample collection for the repository are part of OHSU's IRB-approved protocol e7232 with additional work covered by protocol e14. The transformation of human blood cells is approved by OHSU Institutional Biosafety Committee (protocol no; IBC-11-28).
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n/a
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n/a

F- Data Accessibility

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

G- Dual use research of concern

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