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Impaired Coenzyme A metabolism affects histone and tubulin acetylation in *Drosophila* and human cell models of Pantothenate Kinase Associated Neurodegeneration

Katarzyna Siudeja, Balaji Srinivasan, Lanjun Xu, Anil Rana, Jannie de Jong, Ellen A.A. Nollen, Suzanne Jackowski, Lynn Sanford, Susan Hayflick and Ody C.M. Sibon

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Transaction Report:

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

1st Editorial Decision

16 May 2011

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

You will see that they find the topic of your manuscript potentially interesting. However, they raise substantial concerns on your work, which should be convincingly addressed in a major revision of the manuscript.

Although the referees are rather supportive, referee #1 and #2 feel that the data need to be strengthened and they make constructive suggestions for that. The major concerns refer to the following points:

- definitive causative effect of CoA depletion
- clinical relevance of the study

Given the balance of these evaluations, we would consider a revision of your manuscript if you can address the concerns that have been raised by reviewers in a detailed point-by-point letter.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, unless arranged with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

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

Referee #1 (Other Remarks):

Siudeja and coworkers use mutants with dramatically reduced Acetyl Coenzyme A levels to assess the effect on protein acetylation and find a profound effect on the acetylation status of tubulin and histones. They link their findings to decreased DNA damage response (likely due to reduced histone acetylation) and decreased locomotor function (potentially due to decreased tubulin acetylation). Interestingly, the defects observed (including the defects in acetylation as well as the functional defects) can be rescued by pantethine or HDAC inhibitors, further signifying the importance of protein acetylation.

This is in my view a very timely paper, and given the involvement of protein acetylation (in particular tubulin acetylation) in several neurological diseases, the work holds important implications for disease (not only PKAN). I am in general positive about the work but have a few points that should be addressed before proceeding with publication.

1) Previous work from this lab has indicated that AcCoA levels fall below detection levels upon loss of fumble/pank. They now show that heavily acetylated proteins, tubulin and histones, are less acetylated, but labeling is not absent. Even more surprising, looking at the blots shown, many other proteins are still acetylated. Can the authors comment on this in the discussion? I suppose that either the conditions used do not remove all AcCoA from the system, and very little is enough for protein acetylation, or, alternatively there are other pathways of protein acetylation still active?

2) I believe an essential strength of the paper is that the findings of histone acetylation are coupled back to functionally relevant cellular events. However, the data for tubulin acetylation may be strengthened. Acetylation of tubulin only occurs on stable fibers and this recruits motor proteins. Consequently, microtubule dependent transport may be affected when Ac-CoA levels are down and tubulin acetylation is reduced. Can the authors test this hypothesis (in flies or in cells)? Could this feature contribute to pathogenesis?

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

As detailed below, I think this is a nice paper. It would be stronger if they can more directly connect coA impairment to the other changes in the absence of PANK deficiency. Some work in patient derived cell lines would also increase both the medical impact and novelty.

Referee #2 (Other Remarks):

In this paper the authors extend upon their previous work in a drosophila model of NBIA where they had found lower levels of acetyl CoA associated with Pank deficiency. Here they connect this with altered acetylation of numerous proteins, including tubulin and certain histones. These alterations and one viability assay are responsive to the HDAC inhibitor TSA. Lastly they show altered histone acetylation in hek293 cells where they acutely knock down pank2 and this is reversible by VPA treatment.

Overall, I like this paper and I think it extends their previous findings nicely. In addition, the paper is well written and laid out. However, I think they have not proven their central hypothesis that pank deficiency induces CoA deficiency and that this alone causes acetylation changes, DNA damage sensitivity, and the behavioral phenotypes seen. Below are a few suggestions that I think would strengthen their case. I feel most strongly about points #1 and #2. The other experiments suggested may extend beyond the scope of this particular manuscript, but if so, then some of the claims/language should be tempered a bit.

1) If lower levels of CoA are the critical downstream regulator of PKAN mediated neuro-degeneration, then they should be required to demonstrate this more directly by showing that knockdown of CoA in cells and in the fly produces the same phenotypes. There are likely to be other

pathways affected by Pank deficiency, so a more direct demonstration that ac CoA deficiency is sufficient to induce these effects would significantly strengthen their claims.

If they are unable to do this, then statements in the title and abstract such as "We show that decreased Coenzyme A levels induce a reduction in histone and tubulin acetylation and this coincides with an impaired DNA damage response, decreased locomotor function and decreased survival" are inaccurate. They show these changes are induced by PANK loss or and that these changes are also associated with CoA deficiency, but they do not demonstrate that CoA deficiency is causative of the stated changes. To make a causative statement, they need causative data, not associative data.

2) Previous work by this group has used other readouts besides viability/eclosion that would be interesting for their TSA trial in these flies. Also, use of a second (preferably mechanistically distinct) HDACi in these fly trials would strengthen their conclusions. After all, their claims suggest HDACi's as a therapeutic strategy in the human disease. Although I would not yet emphasize this conclusion based on the data presented, it could end up being the most important finding of this paper in the long run.

3) Do they have a PANK siRNA knockdown fly line? This would be useful to allow them to direct expression to specific (ie neuronal) tissues. This would also be helpful when studying a neurodegenerative model by letting them analyze adult onset rather than developmentally induced effects.

4) Do they have access to patient fibroblasts? If so, demonstrating altered acetylation and increased sensitivity to ionizing radiation in those cells would be more convincing than the HEK293 or S2 cell experiments.

5) Similarly to point #1, although they have shown that acetylation changes of certain proteins are associated with the phenotype, they have not shown these particular alterations are causative of the phenotypes, so statements that suggest this (which are present throughout the document) should be edited to reflect this point.

Referee #3 (Comments on Novelty/Model System):

I gave the highest possible score in each categories except for novelty. That is because the original discovery of decreased level of CoA in *Drosophila* PKAN model and its restoration by pantethine feeding was already published by the same group recently in PNAS (Rana et al, 2010)

Referee #3 (Other Remarks):

The paper, Siudea et al is an excellent work. The relevant questions were systematically asked and the right answers rigorously pursued. The work has high importance in the putative treatment of PKAN disease. I am also sure that other laboratories, working with disease models in which case the effect of under-Acetylation was also confirmed such as Huntington disease, will soon follow their path.

I seemed to find some funny English at certain places but, as I am not a native speaker myself, I am not the best person to correct them. However I think it certainly would be prudent to show the manuscript to someone fluent in English before the final submission. In summary, I recommend the manuscript for publication after fixing the language issues.

1st Revision - Authors' Response

29 July 2011

Dear Editor,

We would like to thank the editor and the reviewers for their in-depth reading of our manuscript and their overall positive comments. Some interesting questions and discussion points were raised which

we have carefully considered and which to our opinion now have strengthened our manuscript. Please find below our responses (*in italics*) to the comments (in regular) of the editor and the reviewers. We have also added a draft summary for “The paper explained” paragraphs of our article highlighting: the medical issue we are addressing, the results obtained and their clinical impact.

Although the referees are rather supportive, referee #1 and #2 feel that the data need to be strengthened and they make constructive suggestions for that. The major concerns refer to the following points:

- definitive causative effect of CoA depletion

(specifically addressed by our answer to point 1 of reviewer 2)

- clinical relevance of the study

(specifically addressed by our answer to point 2 of reviewer 1 and our answer to point 4 of reviewer 2 and in our draft summary for “The paper explained”).

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

Referee #1:

Siudeja and coworkers use mutants with dramatically reduced Acetyl Coenzyme A levels to assess the effect on protein acetylation and find a profound effect on the acetylation status of tubulin and histones. They link their findings to decreased DNA damage response (likely due to reduced histone acetylation) and decreased locomotor function (potentially due to decreased tubulin acetylation). Interestingly, the defects observed (including the defects in acetylation as well as the functional defects) can be rescued by pantethine or HDAC inhibitors, further signifying the importance of protein acetylation.

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1) Previous work from this lab has indicated that AcCoA levels fall below detection levels upon loss of fumble/pank. They now show that heavily acetylated proteins, tubulin and histones, are less acetylated, but labeling is not absent. Even more surprising, looking at the blots shown, many other proteins are still acetylated. Can the authors comment on this in the discussion? I suppose that either the conditions used do not remove all AcCoA from the system, and very little is enough for protein acetylation, or, alternatively there are other pathways of protein acetylation still active?

Response:

This reviewer raises interesting discussion points concerning the detected levels of Coenzyme A in our previous and current manuscript and how, under the tested conditions, protein acetylation can still be observed. Firstly, we would like to clarify that our previous and current work only addresses levels of Coenzyme A and not levels of acetyl-Coenzyme A. We have now added an additional sentence (“Coenzyme A is required for acetyl-Coenzyme A synthesis and acyl groups from the latter are transferred to lysine residues of proteins, in a reaction regulated by acetyltransferases“.) to the abstract to avoid further confusion. In our assays all acetyl-Coenzyme A and other acyl-CoAs are converted into Coenzyme A and subsequently levels of total Coenzyme A are measured. We have also now made this more clear in the text by adding the word “total”. In light of these comments we like to stress that so far it was completely unknown whether or not levels of the metabolite Coenzyme A influence protein acetylation levels, and if so, which proteins specifically are affected. Our data presented here addressed for the first time these questions.

In our previous manuscript (Rana et al., PNAS 2010) we used a previously published method (Demoz et al., J Chromatogr B Biomed Appl 1995) to estimate the levels of total Coenzyme A. With the method used in our previous manuscript we concluded that levels of Coenzyme A were below the detection limit in dPANK/fbl homozygous mutants and Coenzyme A levels were reduced to 20-30% in dPANK/Fbl RNAi-treated S2 cells. When levels of Coenzyme A are below the detection limit as in the dPANK/fbl mutant flies it can not be concluded that the levels are zero and remains likely there are still some residual levels of Coenzyme A and acetyl-Coenzyme A present. We were well aware about the rather insensitive method we used and therefore we were careful in our statements and we “only” concluded that levels of Coenzyme A in de dPANK/fbl mutants were below the detection limit. Using this Coenzyme A quantification method for the S2 cells we showed that in dPANK/Fbl RNAi treated cells Coenzyme A levels are decreased by about 70%. Based on this, we can conclude that there is a residual Coenzyme A pool left in dPANK/Fbl RNAi-treated S2 cells and this residual pool is obviously not sufficient to maintain the normal levels of tubulin and histone acetylation but is enough to maintain acetylation levels of other acetylated proteins visible at the Western Blots. This residual Coenzyme A pool is also sufficient to partly rescue levels of acetylated tubulin and histones after addition of HDAC inhibitors. These results do show that acetylation of histones and tubulin are sensitive and responsive to a decrease in Coenzyme A. The Drosophila dPANK/fbl mutant used in this study is a hypomorph (Afshar et al., Genetics 2001; Bosveld et al., Hum Mol Gen 2008) meaning that levels of the pantothenate kinase are strongly reduced but not absent. Complete loss of Drosophila pantothenate kinase activity is lethal (Afshar et al., Genetics 2001). Therefore, in dPANK/fbl mutants there is probably some residual Coenzyme A pool left, sufficient for acetylation of the unaffected proteins visible on the Western blots probed with the acetyl-lysine antibody. We therefore agree with the reviewer that most likely the conditions used do not remove all Coenzyme A from the system. This in turn suggests that the reduced amount of Coenzyme A is enough for the observed levels of protein acetylation. We agree with this reviewer that this is an interesting discussion and this was not sufficient explained and addressed in our original version. We now address these issues in the discussion of the revised version of the manuscript. We have added the following paragraph to the discussion:

“Our results reveal that levels of tubulin and histone acetylation are decreased but still detectable under conditions of CoA reduction. In addition, acetylation levels of other proteins recognized by the acetyl-lysine antibody seem to be unaffected. It will be of interest to investigate how much residual CoA is required to maintain acetylation levels of specific proteins. Until now no literature exists addressing these issues. Previously, we demonstrated that in adult dPANK/fbl (hypomorph) mutant flies levels of CoA are undetectable with the method used [13], however we can not conclude that CoA levels are actually zero. In dPANK/Fbl RNAi depleted Drosophila S2 cells, levels of CoA decrease to 30% [13] and after HoPan treatment CoA levels drop to 50% (this manuscript). Under all these circumstances, acetylation of tubulin and histones is still detectable but clearly decreased. Most likely under these conditions there is a residual CoA and acetyl-CoA pool left, sufficient for the detected protein acetylation or alternatively there exist an additional source other than acetyl-CoA for protein acetylation. It will be of interest to investigate in a more detailed way, how levels of CoA in specific subcellular compartments influence the acetylation of specific proteins over time and whether this is tissue specific and how this affects specific cellular processes”.

I believe an essential strength of the paper is that the findings of histone acetylation are coupled back to functionally relevant cellular events. However, the data for tubulin acetylation may be strengthened. Acetylation of tubulin only occurs on stable fibers and this recruits motor proteins. Consequently, microtubule dependent transport may be affected when Ac-CoA levels are down and tubulin acetylation is reduced. Can the authors test this hypothesis (in flies or in cells)? Could this feature contribute to pathogenesis?

This reviewer suggests strengthening our tubulin acetylation data by coupling it back to functionally relevant cellular events. This reviewer is correct that acetylated microtubules are enriched in stable microtubule fibers (Hammond et al., Curr Opin Cell Biol 2008) and it has also been demonstrated in mammalian neuronal cells that normal levels of tubulin acetylation are required for Kinesin-1 (motor protein) binding and transport along microtubules (Reed et al., Current Biology 2006). More recently Hammond and coworkers (Mol Biol Cell 2010) demonstrated that tubulin acetylation

enhances the motility of Kinesin 1 in mammalian cultured neuronal cells. However, a direct link between transport and tubulin acetylation is absent in Drosophila and it is currently unknown whether decreased tubulin acetylation levels affect transport along microtubules in Drosophila (cells and flies).

However recently, with the use of the model organism Caenorhabditis elegans in a series of elegant experiments it was demonstrated specifically that acetylation of tubulin at K40 is required for touch sensation (Shida et al., PNAS 2010). Shida and co-workers identified a highly effective α -tubulin k40 acetyltransferase (referred to as α TAT1) in C. elegans, that is not a histoneacetyltransferase. Elimination of α TAT1 reduced levels of tubulin acetylation to undetectable levels and diminished touch sensation. These data demonstrate that touch sensation in C.elegans is strongly dependent upon the presence of acetylated tubulin. We hypothesized therefore that impairment of Coenzyme A biosynthesis in C.elegans would lead to decreased levels of acetylated tubulin and will subsequently result in an abnormal touch response. Fortunately C. elegans mutants are available that carry a mutation in the C. elegans ortholog of pantothenate kinase 2. We now have added additional data that these mutants showed a decrease in the levels of acetylated tubulin and an abnormal touch response. The phenotype (reduced tubulin acetylation and a reduced touch response) was rescued by feeding pantethine.

To further test the evolutionary conservation of our findings, we used HoPan to inhibit pantothenate kinase in HEK293 cells. In order to test our findings in a human neuronal background we also used SHSY-5Y cells, a human derived neuroblastoma cell line (Biedler et al., Cancer Res. 1987) Impairment of pantothenate kinase induced a decrease in levels of acetylated tubulin in SHSY-5Y cells. These data show that impairment of Coenzyme A biosynthesis coincides with decreased levels of tubulin acetylation in a variety of organisms and points to an evolutionarily conserved link between Coenzyme A metabolism and protein acetylation. We believe that together these data strengthen our findings concerning tubulin acetylation and we have now added these data in the revised version of the manuscript. We also have added to the discussion the following paragraph concerning how decreased acetylation of tubulin may contribute to the pathogenesis of PKAN:

“Our data demonstrate that defects in pantothenate kinase functioning in various cells and organisms is associated with a decrease in acetylated tubulin. This is of interest because there is extensive literature in which abnormal tubulin acetylation is linked to impaired neuronal functioning. Amongst others, acetylated tubulin is associated with stable tubulin filaments and defects in tubulin acetylation are associated with abnormal transport in neuronal cells [24], abnormal branching of projection neurons [25], neuromuscular defects [15] and impaired function of touch receptor neurons [31], for a recent review on the topic tubulin acetylation and neurodegenerative disorders see Perdiz et al [32]. Although it remains to be proven it is attractive to speculate that the neurological defects observed in PKAN affected individuals may be partly explained by abnormal tubulin acetylation”.

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

As detailed below, I think this is a nice paper. It would be stronger if they can more directly connect coA impairment to the other changes in the absence of PANK deficiency. Some work in patient derived cell lines would also increase both the medical impact and novelty.

We now have added additional experiments and a more thorough explanation/discussion to connect our findings with impairment in Coenzyme A (see also our response to point 1 of this Reviewer 2) and we now have performed experiments using patient material and a human derived neuroblastoma cell line (see also our response to point 4 of this reviewer).

Referee #2 (Other Remarks):

In this paper the authors extend upon their previous work in a drosophila model of NBIA where they had found lower levels of acetyl CoA associated with Pank deficiency. Here they connect this with altered acetylation of numerous proteins, including tubulin and certain histones. These alterations and one viability assay are responsive to the HDAC inhibitor TSA. Lastly they show altered histone acetylation in hek293 cells where they acutely knock down pank2 and this is reversible by VPA treatment.

Overall, I like this paper and I think it extends their previous findings nicely. In addition, the paper is well written and laid out. However, I think they have not proven their central hypothesis that pank deficiency induces CoA deficiency and that this alone causes acetylation changes, DNA damage sensitivity, and the behavioural phenotypes seen. Below are a few suggestions that I think would strengthen their case. I feel most strongly about points #1 and #2. The other experiments suggested may extend beyond the scope of this particular manuscript, but if so, then some of the claims/language should be tempered a bit.

1) If lower levels of CoA are the critical downstream regulator of PKAN mediated neuro-degeneration, then they should be required to demonstrate this more directly by showing that knockdown of CoA in cells and in the fly produces the same phenotypes. There are likely to be other pathways affected by Pank deficiency, so a more direct demonstration that ac CoA deficiency is sufficient to induce these effects would significantly strengthen their claims.

If they are unable to do this, then statements in the title and abstract such as "We show that decreased Coenzyme A levels induce a reduction in histone and tubulin acetylation and this coincides with an impaired DNA damage response, decreased locomotor function and decreased survival" are inaccurate. They show these changes are induced by PANK loss or and that these changes are also associated with CoA deficiency, but they do not demonstrate that CoA deficiency is causative of the stated changes. To make a causative statement, they need causative data, not associative data.

This reviewer states that we should demonstrate that the knockdown of CoA in cells and in the flies produce the same phenotype as compared to the knock down of PANK. However, as far as we know it is not possible to knockdown Coenzyme A other than by downregulating/inhibiting one of its producing enzymes like PANK and CoA biosynthesis is the only known biochemical fate for the product of PANK activity. We achieve downregulation/inhibition of PANK in our manuscript by 1) RNAi, 2) analyzing Drosophila mutants carrying mutations in the dPANK/fbl gene, and 3) by HoPan treatment. Coenzyme A is a metabolite that is synthesized de novo and it is only possible to reduce the levels of Coenzyme A by depleting the enzymes (such as dPANK/Fbl) that are required to synthesize Coenzyme A. We agree with this reviewer that a phenotype induced by downregulation of dPANK/Fbl can be explained by decreased levels of Coenzyme A or can be explained by decreased levels of dPANK/Fbl. In our manuscripts we demonstrate that we are able to rescue the phenotypes by addition of pantethine. Pantethine is a compound capable of restoring CoA levels in a dPANK/Fbl depleted background (Rana et al., PNAS 2010). Therefore, phenotypes that are being rescued by pantethine are due to decreased levels of Coenzyme A and not due to decreased levels of dPANK/Fbl. HoPan is a specific chemical inhibitor of pantothenate kinase, as it binds competitively to pantothenate (vitamin B5) for binding to the kinase (Zhang et al., Chem Biol 2007). HoPan is a structural analog of pantothenate and inhibits CoA synthesis by substituting for pantothenate in the kinase reaction. HoPan is phosphorylated by PANK but is not further metabolized to CoA, thereby depleting the CoA Pool. HoPan does not modify the activity or function of PANK. The results obtained with HoPan therefore further confirm that CoA depletion is causing the observed phenotypes and not the inhibition of PANK. To further strengthen our claims and to address the comment of this reviewer we did additional experiments and added CoA to the medium of dPANK/Fbl depleted cells. This addition rescued the decreased acetylation of microtubules and histones and further demonstrates that levels of tubulin and histone acetylation fall and rise with

levels of Coenzyme A. Based on this we believe that it is fair to conclude that decreased levels of Coenzyme A induce decreased levels of acetylated tubulin and histones. We agree with this reviewer that a direct causative effect of decreased tubulin and histone acetylation on neurodegeneration is not demonstrated, decreased levels of CoA and decreased levels of histone and tubulin acetylation are “only” associated/coincide with specific neurodegenerative phenotypes. We have adjusted the text regarding this point throughout the manuscript. In addition, we now have removed the arrow in Figure 7 linking reduced protein acetylation to impaired survival, DNA damage response and locomotor function, because this is a correlative and not a causative effect. We have adjusted the text in the Figure legend accordingly.

2) Previous work by this group has used other readouts besides viability/eclosion that would be interesting for their TSA trial in these flies. Also, use of a second (preferably mechanistically distinct) HDACi in these fly trials would strengthen their conclusions. After all, their claims suggest HDACi's as a therapeutic strategy in the human disease. Although I would not yet emphasize this conclusion based on the data presented, it could end up being the most important finding of this paper in the long run.

We investigated whether TSA feeding during larval development rescued adult locomotor behavior, however, we could not demonstrate a significant improvement. As suggested by this reviewer we have now also tried to supplement the Drosophila food with different HDAC inhibitors. We tested valproic acid (VPA) and sodium phenylbutyrate (PBA) both in a concentration range of 0,1 to 10 mM. We did not observe a clear protective effect of these inhibitors on the survival (eclosion rates) of the dPANK/fbl mutant flies. It has to be noted that TSA is the most potent and broad spectrum inhibitor acting on all Drosophila HDACs (Cho et al., Genomics 2005; Foglietti et al., JBC 2006). Thus it is possible that TSA is more potent and therefore VPA and PBA fail to rescue.

3) Do they have a PANK siRNA knockdown fly line? This would be useful to allow them to direct expression to specific (ie neuronal) tissues. This would also be helpful when studying a neurodegenerative model by letting them analyze adult onset rather than developmentally induced effects.

A dPANK/fbl RNAi line (VDRC) does exist and it is of interest to investigate tissue specific consequences of decreased levels of dPANK/fbl, however we also agree with this reviewer that this falls outside the scope of our manuscript.

4) Do they have access to patient fibroblasts? If so, demonstrating altered acetylation and increased sensitivity to ionizing radiation in those cells would be more convincing than the HEK293 or S2 cell experiments.

We do agree with this reviewer that it will be highly relevant to investigate whether decreased acetylation is also present in patient material. However, this is complicated to perform. The only tissue reported to be affected in PKAN patients is brain tissue in specific areas (globus pallidus) (Kruer et al., Brain 2011), it is currently unknown why this specific area is affected. Brain material from PKAN patients is extremely sparse and PKAN is a rare disease. It is possible to investigate protein acetylation levels in non-brain tissue of patients, and in order to address the comment of this reviewer we did the following:

In order to test whether a similar phenotype observed in Drosophila cells or tissue cultured mammalian cells could be observed in material derived from patients, we investigated histone acetylation levels in lymphoblasts derived from various patients. Lymphoblasts were obtained from 5 individuals carrying the following set of mutations in the PANK2 gene (patient 1: allele 1:206_228del (deletion frameshift) and allele 2:243del (deletion frameshift) ; patient 2: allele 1:502C>T (missense mutation) and allele 2: 1231G>A (missense mutation); patient 3: allele 1:519C>G (missense mutation) and allele 2: 1231G>A (missense mutation); patient 4: allele 1: 1021C>T (nonsense mutation) and allele 2: 1231G>A (missense mutation); patient 5: allele 1: IVS2+3A>G (5' splice site) and allele 2: IVS2+3A>G (5' splice site). Cells were obtained and

cultured as previously described (Wall et al., , F.E., In Vitro Cell. Dev. Biol. Anim. 1995). Cells were analyzed using Western Blot analysis for ratios of acetylated histone 3: total levels of histone 3. As an additional loading control tubulin was used. For these experiments over 18 Western blots were analyzed. The levels of histone acetylation differed considerably between samples and no significant difference in histone acetylation was observed between lymphoblasts derived from healthy controls and lymphoblasts derived from patients. Based on this we concluded that histone 3 acetylation is not detectably decreased in lymphoblasts derived from the tested PKAN patients. It should be noted that lymphoblast cells of patients are not affected and therefore it will be of interest to investigate protein acetylation levels in tissues (when available in the future) that are affected in PKAN patients such as the globus pallidus.

In order to test whether impaired Coenzyme A biosynthesis affects protein acetylation in a human neuronal background we tested now also SHSY-5Y cells and we demonstrated that in these cells also levels of histone and tubulin acetylation are decreased upon incubation with HoPan and this is rescued by addition of TSA or pantethine (see also our comments to the first reviewer).

5) Similarly to point #1, although they have shown that acetylation changes of certain proteins are associated with the phenotype, they have not shown these particular alterations are causative of the phenotypes, so statements that suggest this (which are present throughout the document) should be edited to reflect this point.

We have now changed this accordingly (see also our response to point 1 of this reviewer).

Referee #3 (Comments on Novelty/Model System):

I gave the highest possible score in each categories except for novelty. That is because the original discovery of decreased level of CoA in *Drosophila* PKAN model and its restoration by pantethine feeding was already published by the same group recently in PNAS (Rana et al, 2010)

We like to mention that indeed the restoration of Coenzyme A levels by feeding pantethine has been previously published by us (Rana et al., PNAS 2010), however decreased acetylation of specific proteins as a consequence of decreased levels of Coenzyme A is a novel finding and demonstrates a link between a metabolic cofactor, post translational modifications and neurodegeneration. This to our knowledge has never been addressed before.

Referee #3 (Other Remarks):

The paper, Siudea et al is an excellent work. The relevant questions were systematically asked and the right answers rigorously pursued. The work has high importance in the putative treatment of PKAN disease. I am also sure that other laboratories, working with disease models in which case the effect of under-Acetylation was also confirmed such as Huntington disease, will soon follow their path.

I seemed to find some funny English at certain places but, as I am not a native speaker myself, I am not the best person to correct them. However I think it certainly would be prudent to show the manuscript to someone fluent in English before the final submission. In summary, I recommend the manuscript for publication after fixing the language issues.

A native (English) speaker has corrected the revised version of our manuscript.

2nd Editorial Decision

04 August 2011

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

Referee #2 and I would appreciate if you could incorporate the negative data generated in response to referee #2 within the manuscript as supplementary data and discuss them in the main text.

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

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

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

Referee #1:

The authors have addressed my concerns and I have no further comments and thus recommend publishing the work

Referee #2:

I think the authors should be applauded for addressing the concerns of the reviewers. Unfortunately, some of the data they generated at least partially undermines the larger significance of the paper. I do feel that they have adequately addressed the questions they set out to test. I do not feel, however, that it is appropriate to exclude contradictory data that they have generated from the paper. The authors should be required to include the negative data they generated in the supplemental data and comment on it in the paper. Specifically, I would like them to include the VPA and Sodium Butyrate data showing no effects on fly eclosion and the lymphoblast patient derived cell line data demonstrating no changes in basal histone H3 acetylation. I understand why they excluded it- it is negative data and does not support their arguments very well, and they feel they have reasons to explain why it was negative. However, its inclusion will prevent others from doing these types of experiments as well as put the work into proper context.

2nd Revision - Authors' Response

31 August 2011

We now have incorporated in the supplementary file (S5 and S6) the requested information showing the experiments with VPA and PBA on the eclosion rate and the experiments with the lymphoblasts derived from the patients.

We also now have added the following parts to the main text

Page 9:

First we investigated whether addition of various HDAC inhibitors (valproic acid (VPA), sodium phenylbutyrate (PBA) or TSA) to the larval food increased the eclosion rate of homozygous *dPANK/fbl* flies. VPA and PBA did not result in a significant rescue (Figure S5) however, TSA addition increased the survival rate of the homozygous mutant progeny in a concentration dependent

manner (Figure 5D). VPA and PBA could only be used in relatively low concentrations, because the concentrations commonly used for an efficient HDAC inhibition (above 1 mM) induced lethality when fed during larval development. TSA, on the other hand, is less toxic. Moreover, TSA is a potent and broad spectrum inhibitor acting on all *Drosophila* HDACs [31, 32].

Page 9 and 10:

In light of the above data, it is highly relevant to investigate levels of histone and tubulin acetylation in material derived from PKAN patients. We investigated acetylation levels in available patient-derived lymphoblasts. In these cells no significant difference in histone acetylation could be observed (Figure S6). However, it should be noted that lymphoblast cells of patients do not show any phenotype and therefore it will be of higher relevance to investigate protein acetylation levels in tissues (when available in the future) that are affected in PKAN patients, such as the globus pallidus [33].

We also have changed the title because more human cell lines were used to underscore our findings and we have now written the name PKAN in full.

The initial title was: "Impaired Coenzyme A metabolism affects histone and tubulin acetylation in a *Drosophila* and human cell model of the neurodegenerative disorder PKAN"

The current title is: "Impaired Coenzyme A metabolism affects histone and tubulin acetylation in *Drosophila* and human cell models of Pantothenate Kinase Associated Neurodegeneration"

We are looking forward to your reply,