

SLC25A38 is Necessary for Mitochondrial Pyridoxal 5'-Phosphate (PLP) Accumulation

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I was very pleased to see that the authors have submitted a revised manuscript given that this paper addresses a crucial aspect of cellular vitamin B6 homeostasis in humans, specifically how PLP is transported to the mitochondria for the PLP-dependent enzymes that require it. This is especially important given that PNPO, the enzyme that is involved in PLP synthesis, is located within the nucleoplasm and cytosol and has not been shown to localise to the mitochondria. Although the authors have still not been able to directly show that SLC25A38 transports PLP they have carried out some very elegant experiments implicating this transporter in PLP intracellular homeostasis.

Whilst the authors have satisfactorily addressed my previous comments and have undertaken additional experiments to support their findings, I notice that the updated version of the paper contains little reference to epilepsy. I feel that this should be discussed in more detail. It is well established that deficiencies of PLP, whether due to PNPO-, ALDH7A1- or PLPBP-deficiency, or dietary insufficiency of vitamin B6, results in an epilepsy and yet, as I mentioned in my comments previously, the clinical phenotype of congenital sideroblastic anemia (CSA) associated with a deficiency of SLC25A38 does not include epilepsy. Additionally, mutations in the glycine cleavage system cause non-ketotic hyperglycaemia, those in NFS1 can cause lactic acidemia and multiorgan system failure and abnormal mitochondria, whilst variants in SHMT2 cause a neurodevelopmental disorder with cardiomyopathy, spasticity and brain abnormalities. All of these mitochondrial enzymes require PLP. If mutations in SLC25A38 prevent import of PLP into the mitochondria the authors should discuss in the paper as to why they think the phenotype of congenital sideroblastic anaemia (CSA) is not like the above if PLP is not readily available for the PLP-dependent enzymes that require it within the mitochondria. The authors mention in their earlier reply that SLC25A38 appears to be an erythrocyte specific protein however as far as I can see this is not mentioned in the manuscript. Could it be that different cells may possibly have different mitochondrial PLP transporters?

Additional comments:

- The authors mention that PLP is the predominant B6 vitamer in the mitochondria in legend for Figure 1. Did injection of the same concentration of PN, PL, PMP and PLP give the same peak area? If not have they taken this into consideration?
- Whilst it is clear from the data presented by the authors that SLC25A38 is somehow involved in mitochondrial PLP homeostasis whether directly or indirectly in the cells looked at in this study, the authors do not hypothesise how this may be occurring. PLP has been reported to be an inhibitor of various of the SLC25 transporters (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3806213/> and Tonazzi et al., BBA 1718 (2005) 53-60), and whilst the genes encoding for these SLC25 transporters are not within the highest delta gene-scored genes in the genome-wide CRISPRi screen, could the mechanisms involved in the inhibition of these SLC25 members, i.e., through its reaction with lysine residues, have parallels with how SLC25A38 may be involved in the homeostasis of mitochondrial PLP?
- Also interestingly, the gene with the most significance in supplementary table 5 is SLC19A1. This is a gene that has been reported to transport pyridoxine however the authors do not comment on the significance of this finding in the manuscript.

Version 1:

Reviewer comments:

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(Remarks to the Author)

This paper is well written and addresses a crucial aspect of cellular vitamin B6 homeostasis in humans, specifically how PLP is transported to the mitochondria in erythroid cells for the PLP-dependent enzymes that require it. The authors have addressed my previous comments satisfactorily.

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We thank Reviewer 1 for their time in carefully considering our manuscript and below we address their points. In green we quote the comments we received, which are followed by our responses in black.

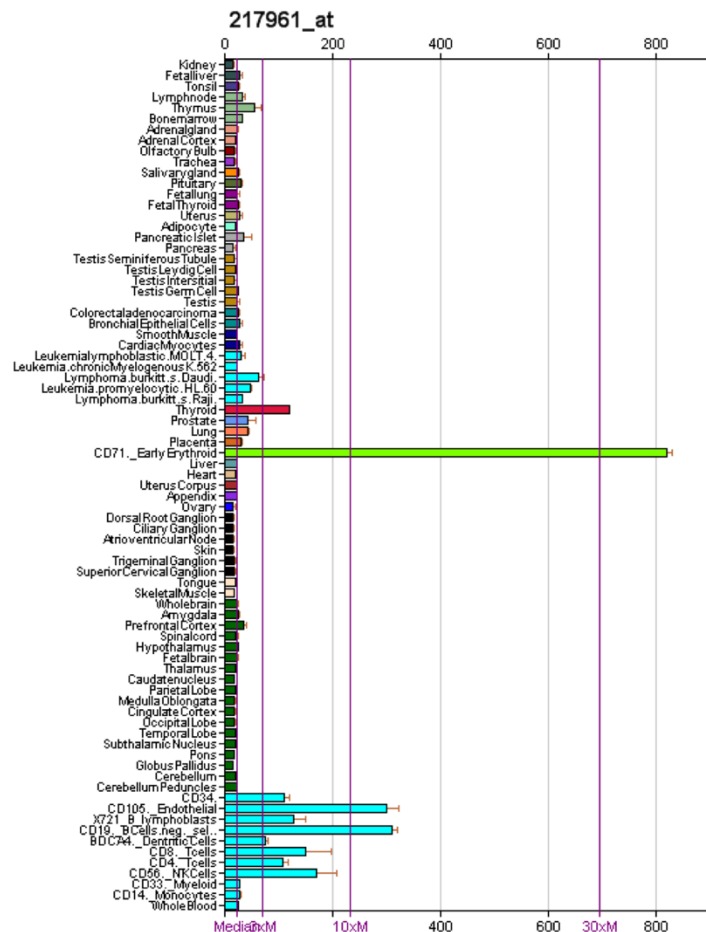
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We appreciate the reviewer’s time in carefully considering our manuscript and for the enthusiasm noted for the new experiments. We are very thankful for the comments received and we have improved the manuscript Discussion section following the reviewer’s recommendations.

“Whilst the authors have satisfactorily addressed my previous comments and have undertaken additional experiments to support their findings, I notice that the updated version of the paper contains little reference to epilepsy. I feel that this should be discussed in more detail. It is well established that deficiencies of PLP, whether due to PNPO- , ALDH7A1- or PLPBP-deficiency, or dietary insufficiency of vitamin B6, results in an epilepsy and yet, as I mentioned in my comments previously, the clinical phenotype of congenital sideroblastic anemia (CSA) associated with a deficiency of SLC25A38 does not include epilepsy. Additionally, mutations in the glycine cleavage system cause non-ketotic hyperglycaemia, those in NFS1 can cause lactic acidemia and multiorgan system failure and abnormal mitochondria, whilst variants in SHMT2 cause a neurodevelopmental disorder with cardiomyopathy, spasticity and brain abnormalities. All of these mitochondrial enzymes require PLP. If mutations in SLC25A38 prevent import of PLP into the mitochondria the authors should discuss in the paper as to why they think the phenotype of congenital sideroblastic anaemia (CSA) is not like the above if PLP is not readily available for the PLP-dependent enzymes that require it within the mitochondria. The authors mention in their earlier reply that SLC25A38 appears to be an erythrocyte specific protein however as far as I can see this is not mentioned in the manuscript. Could it be that different cells may possibly have different mitochondrial PLP transporters?

We appreciate this point of discussion that we believe to be of the utmost importance. We have updated our discussion to express the cell/tissue-specific expression and requirement of SLC25A38 in erythroid cells, and our hypothesis that a protein playing a redundant role on mitochondrial PLP homeostasis must exist in other cell types relevant to the abovementioned diseases, including epilepsy and other neurological conditions. As can be seen in this figure, SLC25A38 is very specifically enriched in erythroid cells but not in the brain (graph below copied from Guernsey et al 2009, supplemental figure 2, PMID: 19412178 showing tissue-specific expression of SLC25A38).



Supplementary Figure 2 from Guernsey et al 2009 (PMID: 19412178) Tissue specific expression profile of SLC25A38.

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- The authors mention that PLP is the predominant B6 vitamer in the mitochondria in legend for Figure 1. Did injection of the same concentration of PN, PL, PMP and PLP give the same peak area? If not have they taken this into consideration?

During our method development we noticed that, indeed, the signal/peak area obtained when injecting the different B6 vitamers is not the same (see example below). When injecting the same concentrations of analytical standards, the signal is usually an order of magnitude lower for PLP (and PLP-D3) compared to the unphosphorylated vitamer PL. PMP also had lower signal compared to PM. This is also seen in other methods in the literature, such as van der Ham et al. 2012 (PMID 22177072). In the van der Ham et al. 2012 method, peak intensities of 1.86×10^5 and 1.7×10^6 are obtained when injecting PLP-D3 at 132nM and PL-D3 at 47nM, for example. It is unclear why the signal is generally lower for PLP, but we can speculate it to be due to lower ionization in positive mode (ESI+) or instability (e.g., more prone to in-source fragmentation). Despite this, PLP is still the vitamer showing highest signal in the mitochondrial IPs. Given that our method allows for qualitative comparisons, not quantitative, we have decided to remove the statement in the Figure 1 legend noting that “PLP is the predominant B6 vitamer in the mitochondria in our preparation” and now state that “PLP and PMP are reliably detected in mitochondria IPs”. The observation of PLP and PMP in the mitochondria in our study is also in line with early studies in rat liver mitochondria (PMID: 6263901).

- Whilst it is clear from the data presented by the authors that SLC25A38 is somehow involved in mitochondrial PLP homeostasis whether directly or indirectly in the cells looked at in this study, the authors do not hypothesise how this may be occurring. PLP has been reported to be an inhibitor of various of the SLC25 transporters (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3806213/> and Tonazzi et al., BBA 1718 (2005) 53-60), and whilst the genes encoding for these SLC25 transporters are not within the highest delta gene-scored genes in the genome-wide CRISPRi screen, could the mechanisms involved in the inhibition of these SLC25 members, i.e., through its reaction with lysine residues, have parallels with how SLC25A38 may be involved in the homeostasis of mitochondrial PLP?

This is indeed a very good point. Most mitochondrial carriers can be inhibited by PLP; however, such studies use a very high dose (the “inhibitor-stop” method), likely unachievable physiologically. The studies mentioned in PMID: 23988125 and Tonazzi et al., BBA 1718 (2005) 53-60) used PLP concentrations of 10mM-20mM. Considering that cell/plasma levels of PLP are on the nanomolar scale, we believe that this is likely an artificial/non-specific effect. Most members of the SLC25 family of proteins have conserved lysine residues that could potentially form Schiff base/imine linkages with PLP in such high concentrations, especially at the second cytoplasmic salt bridge network. Some transporters also have lysine residues as part of the substrate contact sites, which could also potentially and artificially bind PLP at such high concentrations. Both (and other sites) could result in inhibition of the transport activity in excess PLP, such as the concentrations used of 10-20mM. It would be important to test whether this

inhibition can be achieved in physiologically relevant concentrations of PLP, which is unclear thus far.

With regards to how SLC25A38 may be affecting mitochondrial PLP accumulation, there are several possible mechanisms: (1) it may act as a direct transporter of PLP; (2) PLP import by another mitochondrial carrier may be dependent on interaction with SLC25A38; (3) SLC25A38 transporter activity may indirectly regulate a PLP mitochondrial carrier either dependent on glycine exchange from the mitochondria (antiport), another product of glycine-related mitochondrial metabolism, or symport of a compound transported out of the mitochondria by SLC25A38.

• Also interestingly, the gene with the most significance in supplementary table 5 is SLC19A1. This is a gene that has been reported to transport pyridoxine however the authors do not comment on the significance of this finding in the manuscript.

We thank the reviewer for pointing this out. To our knowledge, SLC19A1 is the reduced folate carrier in the plasma membrane, and we could not find any published manuscripts implicating this protein into pyridoxine transport. However, a recent paper showed that both thiamine transporters SLC19A2 and SLC19A3 can indeed carry out a pH-dependent pyridoxine plasma membrane transport (only in acidic environment, pH 5.5) (PMID: 33008889). The authors have also tested SLC19A1 which did not show any pyridoxine uptake. SLC19A2 and SLC19A3 had small delta gene scores (close to zero) in our genome-wide CRISPRi screen, which may suggest that these transporters are not relevant to essentiality in low vitamin B6 in neutral environments (RPMI media is pH 7.4). However, it is remarkable indeed that the folate transporter SLC19A1 displayed a high positive gene score (Ext Data Table 5: -B6 gene score: 0.022, +B6 gene score: 1.43), meaning its loss conferred a positive fitness to K562s growing in low vitamin B6. This pattern was observed in both the genome-wide and the targeted library screens indicating it has a high chance of being a true effect. We hope to generate knockout cell lines in the near future to test this effect and to investigate its molecular basis, but we believe these experiments to be beyond the scope of this manuscript.