Mutations in *PROSC* Disrupt Cellular Pyridoxal Phosphate Homeostasis and Cause Vitamin B_6-Dependent Epilepsy

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Abstract

Pyridoxal 5'-phosphate (PLP), the active form of vitamin B_6, functions as a cofactor in humans for more than 140 enzymes, many of which are involved in neurotransmitter synthesis and degradation. A deficiency of PLP can present, therefore, as seizures and other symptoms which are treatable with PLP and/or pyridoxine. Deficiency of PLP in the brain can be caused by inborn errors affecting B_6 vitamer metabolism or by inactivation of PLP; by compounds accumulating as a result of inborn errors of other pathways or by ingested small molecules. Whole exome sequencing of 2 children from a consanguineous family with pyridoxine-dependent epilepsy revealed a homozygous nonsense mutation in proline synthetase co-transcribed homolog (bacterial) (*PROSC*), a PLP-binding protein of hitherto unknown function. Subsequent sequencing of 29 unrelated indivduals with pyridoxine-responsive epilepsy identified 4 additional children with biallelic *PROSC* mutations. Pre-treatment cerebrospinal fluid samples showed low PLP concentrations and evidence of reduced activity of PLP-dependent enzymes. However, cultured fibroblasts showed excessive PLP accumulation. An *E.coli* mutant, lacking the *PROSC* homologue (Δ*YggS*) is pyridoxine-sensitive; complementation with human *PROSC* restored growth whilst *hPROSC* bearing p.Leu175Pro, p.Arg241Gln and p.Ser78Ter did not. PLP, a highly reactive aldehyde, poses a problem for cells - how to supply enough PLP for apoenzymes while maintaining free PLP concentrations low enough to avoid unwanted reactions with other important cellular nucleophiles. Whilst the mechanism involved is not fully understood our studies suggest that PROSC is involved in intracellular homeostatic regulation of PLP, supplying this cofactor to apoenzymes while minimizing any toxic side reactions.

Introduction

Pyridoxal 5'-phosphate (PLP) is a highly reactive aldehyde. This allows it to act as the cofactor for over 140 enzyme-catalysed reactions, however, it also poses a problem for the cell - how to supply enough PLP for all the newly synthesised apo-B_6 enzymes while keeping the cellular level of free PLP so low that it does not react with other nucleophiles (e.g. sulphydryl and amino compounds and proteins that are not B_6 enzymes). Excess intracellular PLP is hydrolysed by phosphatases and, in the liver, pyridoxal is oxidized to pyridoxic acid. However, the mechanisms of regulation of PLP homeostasis within the cell remain an important, as yet unresolved, issue.[1]

The pathways from dietary B_6 vitamers to brain PLP, the PLP recycling pathways and the pathway for catabolism of excess PLP are shown in Figure 1. There are several disorders that are known to affect these pathways. In 1995, Waymire et al. showed that mice lacking tissue non-specific alkaline phosphatase (TNSALP) have a reduced concentration of PLP in the brain which disrupts neurotransmitter metabolism and causes seizures.[2] In man, the clinical picture of hypophosphatasia (*ALPL* [MIM: 171760]) caused by a lack of TNSALP is usually dominated by the bone disease but pyridoxine-responsive seizures can occur.[3] In 2004, Gachon et al showed that, in the mouse, knockout of the 3 transcription factors that activate pyridoxal kinase leads to low brain levels of PLP, dopamine and serotonin, and to severe epilepsy.[4] In 2005, we described a cohort of infants with neonatal epileptic encephalopathy and changes in CSF concentrations of neurotransmitter amine precursors and metabolites indicating deficient activity of aromatic L-amino acid decarboxylase, the PLP-dependent enzyme required for synthesis of dopamine and serotonin[5]. Raised levels of threonine and glycine in the CSF suggested there may be a general defect of B_6-dependent enzymes and, while the infants' seizures did not show much response to treatment with pyridoxine (PN), they responded dramatically to PLP. We were able to show that this cohort of individuals had homozygous mutations in *PNPO* encoding pyridox(am)ine 5'-phosphate oxidase, that substantially reduced the catalytic efficiency of the enzyme. Deficiency of PNPO (MIM: 610090) impairs PLP synthesis and recycling as is clear from Figure 1. More recently we have been able to show that individuals with PNPO deficiency can be pyridoxine-dependent rather than only PLP-dependent.[6] In 2006 we

showed that pyridoxine-dependent epilepsy is usually caused by accumulation of a metabolite that reacts with PLP, Δ^1-piperideine-6-carboxylate. This metabolite accumulates because of a block in the pipecolic acid pathway of lysine catabolism (ALDH7A1 deficiency; MIM: 266100);[7] a similar mechanism occurs with the accumulation of Δ^1-pyrroline-5-carboxylate in hyperprolinaemia type II (*HYRPRO2* [MIM: 239510])[8]. Finally, there is a group of disorders in which alkaline phosphatase cannot be anchored because of a defect in the glycosylphosphatidylinositol anchor pathway (GPI-AP deficiencies). Circulating alkaline phosphatase levels are high (hyperphosphatasia) and, in some individuals seizures respond to treatment with pyridoxine.[9]

These days, faced with a child with seizures that show a response to either pyridoxine or to PLP, it is possible to exclude hypophosphatasia and hyperphosphatasia, PNPO deficiency and ALDH7A1 deficiency by DNA analysis, enzymology or metabolite analyses (or a combination). When we do this, we are left with a group of children for whom the underlying cause remains obscure. This paper describes the results of investigation of a consanguineous Syrian family in which there have been 3 children affected by pyridoxine-dependent epilepsy. This led to the implication of a gene of hitherto unknown function (*PROSC* [GenBank: NM_007198.3]). Subsequent investigation of a cohort of 29 children / young adults with B_6-dependent epilepsy revealed biallelic mutations in *PROSC* in 4 additional individuals. Analysis of body fluids and fibroblasts of PROSC-deficient individuals suggest that it is an intracellular binding protein that is involved in the homeostatic regulation of free PLP levels.

PROSC is ubiquitously expressed in human tissues and is highly conserved throughout evolution suggesting an important cellular function.[10] The gene product is a cytoplasmic protein that has a PLP-binding barrel domain similar to the N-terminus of bacterial alanine racemase and eukaryotic ornithine decarboxylase[11] to which PLP binds without affecting the quaternary structure[12] and, whilst PROSC deficiency in bacteria affects amino acid metabolism, the protein has no definitive enzyme activity.[11] Recently YggS, the *E.coli* homolog, was implicated in PLP homeostasis.[12] In the absence of the PROSC homolog (Δ*YggS* strain), a pyridoxine containing disc produces a ring of bacterial growth inhibition. We have used this to test the function of human PROSC; we

demonstrated that transfection with wild type *hPROSC* abolishes the ring of growth inhibition whilst *hPROSC* bearing mutations found in the infants with B_6-responsive epilepsy (p.Leu175Pro [c.524T>C], p.Arg241Gln [c.722G>A] and p.Ser78Ter [c.233C>G]) did not.

Subjects and Methods

Subjects and Samples

All samples from affected individuals and their families were obtained following the approval of the study by the Ethics committee at the University of Gothenburg and/or the National Research Ethics Service (NRES) Committee, (London, Bloomsbury: REC Ref. 3/LO/0168). Written informed consent was obtained for all subjects/families. Total genomic DNA was extracted from EDTA blood according to the manufacturer's instructions (Qiagen) or using an AutoGenFlex STAR automated system according to the manufacturer's protocol. Stored fibroblasts from Subjects 2, 4 and 5 were cultured using standard methods. All fibroblasts were tested for mycoplasma contamination prior to any experiments being performed.

Homozygosity Mapping and Whole Exome Sequencing

Copy-number neutral loss-of heterozygosity and whole exome sequencing analysis were undertaken on the index family. The pedigree of this family is shown in Figure 2A. DNA from two siblings, one affected (IV.6) and one unaffected (IV.5), and one affected cousin (IV.2) were analysed by GeneChip® Human Mapping 250K Nsp I Array from Affymetrix and analysed using the software CNAGv3.0. Several regions of copy-number neutral loss-of heterozygosity (cnn-LOH) were identified and of these, only one overlapped in both affected individuals but were absent in the healthy sister. This region was subsequently analysed by whole exome sequence analysis. DNA was enriched using the Agilent SureSelect Human All Exon (Agilent Technologies) and sequenced using Illumina HiScanSQ (Illumina). Quality assessment of the NGS sequence reads was performed by generating

QC statistics with FastQC (see Web Resources). Read alignment to the reference human genome (hg19, UCSC assembly, February 2009) was done using BWA[13] with default parameters. Quality score recalibration, indel realignment and variant calling were performed with the GATK package.[14] Variants were annotated with Annovar.[15] Filtering was done with regard to exonic changes in protein-coding genes located in the LOH-region on chromosome 8p11.23-p21.2, present in homozygous state in both affected individuals, and finally on predicted function (synonymous changes excluded).

Sanger Sequencing of *PROSC*

For sequence validation, subsequent mutation screening of a cohort of 29 children who had vitamin B_6-responsive seizures, and segregation analysis, the coding exons and intron/exon boundaries of *PROSC* (ENST00000328195) were PCR amplified from genomic DNA (primers listed in Table S1). Amplicons were purified using Shrimp Alkaline phosphatase and Exonuclease I prior to sequencing bi-directionally using the Big Dye Terminator Cycle Sequencing System version 1.1 (Applied Biosystems) on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems). Population frequencies of identified sequence changes were obtained from the Ensembl and ExAC databases (accessed 02/08/2016). ClustalW2 software (see Web Resources) was used to align sequences.

Cloning of human *PROSC* cDNA from fibroblasts of affected individuals and sequence analysis of cDNA.

Total RNA was extracted from fibroblasts using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was carried out using the SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Scientific) and oligo(dT)20 primers. Amplification of *PROSC* cDNA was carried out using the reaction conditions and primers detailed in Table S2. Individual cDNA products were gel purified using the QIAquik Gel Extraction Kit (Qiagen) and cloned into TOPO 2.1 (Thermo Scientific) and TOP10 competent cells (Invitrogen) transformed. *PROSC* cDNA inserts were sequenced using the conditions and primers detailed in Table S3.

qRT-PCR.

Expression analysis of *PROSC* was performed using fibroblast cDNA generated as described above. Real-time PCR was carried out on a StepOne Real-Time PCR System (Thermo Scientific) using the following TaqMan Gene Expression Assays: PROSC (Hs00200497_m1), β-actin (4333762T) and GAPDH (hs02758991_g1). Relative quantification of gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, with β-actin and GAPDH as reference genes.

Immunoblot analysis.

Proteins in fibroblast lysates were separated electrophoretically on a NuPAGE Novex 4 -12% Bis-Tris Protein Gel (Thermo Fisher) and transferred to a polyvinylidene difluoride (PVDF) membrane using an iBlot Dry Blotting System according to the manufacturer's instructions. Blots were probed with PROSC primary antibody raised in rabbit (1:100 in 5% TBST milk; HPA023646, Sigma) and after incubated with donkey anti-rabbit IgG-HRP (1:5000; sc-2317, Santa Cruz Biotechnology). The membrane was developed using the Novex ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher) and imaged using a ChemiDoc MP System (Bio-Rad) coupled to ImageLab 4.1 software. Equivalent loading was determined using mouse β-actin primary antibody (1:40,000; A1978, Sigma) and goat anti-mouse IgG antibody conjugated to HRP (1:3,000; P0447, DAKO).

B_6 vitamers and 4-pyridoxic acid analysis

The method used was essentially as described by Footitt et al.[16] for the measurement of plasma B_6 vitamers with the following minor modifications. Fibroblast pellets were lysed and 10 µl of supernatant added to 110 µl of master mix (containing 40 µl of dH_2O, 60 µl of 0.3N trichloroacetic acid (TCA) and deuterated internal standards; 100 nM D2-PLP, D2-PA and D3-PM, 10 nM D2-PN, 50 nM D3-PL). In the case of CSF 50 µl of sample was added to 70 µl of master mix (containing 60 µl of 0.3N TCA and deuterated internal standards; 100 nM D2-PLP, D2-PA and D3-PM, 10 nM D2-

PN, 50 nM D3-PL). Supernatants were analysed by LC-MS/MS (Waters Acquity Ultra Performance LC system linked to a triple Quadrupole Xevo TQ-S instrument) on an Acquity UPLC HSS T3 column (1.8 µm x 2.1 mm x 50 mm) fitted with a HSS T3 VanGuard guard column (Waters) using a mixture of mobile phase A (3.7% acetic acid with 0.01% HFBA) and B (100% methanol) at a flow rate of 0.4 ml/minute. Details of the mobile phase gradients are shown in Table S4. The quantification of the different analytes was performed using the transitions detailed in Table S5.

Fractionation of fibroblast cell lysates

Fibroblast cell lysate supernatants were fractionated using Amicon Ultra-0.5 ml 3 kDa Centrifugal Filters (Millipore). The B_6 vitamers in the resulting fractions (>3 kDa, < 3kDa) and in the unfiltered supernatant were analysed using the UPLC-MS/MS method described above and were corrected for the unfiltered protein concentration.

Cloning and Generation of *PROSC* Mutations by Site Directed Mutagenesis.

Wild-type human *PROSC* cDNA in pOTB7 (Thermo Scientific; BC012334.1; clone ID 3546307) was used as a template. Site-directed mutagenesis was carried out using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) in order to generate Ser78Ter. The primers used for site directed mutagenesis are as detailed in Supplementary Table 5. Wild-type *PROSC* and Ser78Ter *PROSC* were subsequently amplified using the following primers: SDHuyggS-fwd (5'-GGAATTCAGGAGGTATACACCATGTGGAGAGCTGGCAGCAT-3') and HuyggS-rev (5'-CGCGGATCCGGTACCTTAGTGCTCCTGTGCCACC-3'), SDHuyygS-fwd and SHuyggS-rev (5'-ATTATTGGATCCTTATTAGTCGACTTATGCTTTTTCTAGCAGTTCCTGAACGTAG-3') and the resulting PCR products subcloned into the EcoRV and BamHI sites of the multiple cloning site (MCS) of pBEY279.1[12] to generate pBEY332.1 and pBEY330.4. Wild-type and Ser78Ter *PROSC* were subsequently amplified from pBEY332.1 and pBEY330.4 using the following primers FW_PBAD33_SacI_RBS_ProsC 5'-TTAGCGAGCTCAGGAGGAATTCACCATGTGGAGAGCTGG

CA-3' and RV_PBAD33_SalI_ProsC 5'-TTATTAGTCGACTTAGTGCTCCTGTGCCACCTCCAGCG GG-3', FW_PBAD33_SacI_RBS_ProsC and RV_PBAD33_salI_PROSC_stop 5'-TTATTAGTC GACTTATGCTTTTTCTAGCAGTTCCTGAACGTAG-3' to enable sub-cloning of the gene into the SacI and SalI sites of the multiple cloning site of pBAD33 to generate the plasmid pLSP01 and pLSP02. The Q5 site-directed mutagenesis kit, (New England Biolabs) was used essentially as described by the manufacturer, to generate the missense mutations Arg241Gln, Leu175Pro and Pro87Leu using the plasmid pLSP01 as template and to give pLSP07, pLSP03 and pLSP05, respectively. The primers used for site directed mutagenesis are as detailed in Table S6.

***E.coli* complementation studies**.

The *E.coli* ΔyggS (VDC6594) were freshly transformed with the empty vector (pBAD33) as negative control and pLSP01, pLSP02, pLSP03, pLSP05, pLSP07 as described previously.[12] 2 sterile discs were set on the top of the agar and 20 µl of PN (0.1 mg/ml or 1 mg/ml) was added to the disc and plates were incubated overnight at 37 °C.[12] The sensitivity to pyridoxine is defined as the distance (in cm) from the disc where the ΔyggS strain does not grow.

Results

Homozygosity Mapping and Exome Sequencing of Index Family

In order to identify shared regions of homozygosity and potential candidate genes DNA of the index family from affected individuals IV.2 and IV.6 and the unaffected sister of IV.6 i.e. IV.5 were analysed on a Human mapping array. Several overlapping copy-number neutral loss-of-heterozygosity regions were identified in the affected individuals, however only one was absent in the healthy sister. However, none of the 77 protein-coding genes in this region (chromosome 8p11.23-p21.2; Table S7) obviously explained the seizures. Characterisation of this region using WES for IV.2 and IV.6 identified 18 exonic single nucleotide variants (SNVs) (Table S7) present in both affected individuals

after filtering. One had a stop-gain predicted function; c.233C>G (p.Ser78Ter) in *PROSC* (proline synthetase co-transcribed homolog (bacterial) GenBank: NM_007198.3), a gene of unknown function. This variant had not been reported in the dbSNP, 1000 Genomes or ExAC databases.

Sanger Sequencing of *PROSC*

Sequencing of *PROSC* in the index family (Subjects 1 - 3; Table 1) showed that this variant segregated within the family (Figure 2A). Genotyping of 237 Swedish and 89 Syrian controls (living in Sweden) for c.233C>G identified one heterozygote and no homozygotes. Subsequent sequencing of *PROSC* in a cohort of 29 children with B_6-repsonsive epilepsy (for whom ALDH7A1 and PNPO deficiency had been excluded using genetic and/or biochemical evidence) identified potential pathogenic variants in four additional subjects (Subjects 4 – 7) (Table 1, Figure 2B and Figures S1 and S2) and analysis of parental DNA for these families confirmed that the parents were heterozygous carriers. Whilst the nonsense mutation p.Gln71Ter (c.211C>T), and the splice site mutation c.320-2A>G have not been reported in the ExAc, dbSNP or Ensembl databases the missense mutations p.Pro87Leu (c.260C>T), p.Leu175Pro (c.524T>C) and p.Arg241Gln (c.722G>A), and the splice site mutation c.260C>T have but only in heterozygous form with a prevalence of <0.01%.

Clinical Histories

The index family is of Syrian descent and includes three affected members with vitamin B_6-dependent seizures in whom PNPO and ALDH7A1 deficiencies were excluded; 2 siblings (IV.1 and IV.2) and a cousin (IV.6). The pedigree in Figure 2A demonstrates an autosomal-recessive pattern of inheritance. Clinical characteristics of the affected children are summarized in Table 2. Detailed clinical histories of all 7 affected individuals are in the Supplemental Information and are tabulated in Table 2.

Comparison of the Clinical Phenotypes

Of the 7 individuals with *PROSC* mutations, 3 had abnormal intrauterine movements (including the one subject whose mother was being treated with pyridoxine throughout pregnancy), 4 showed signs of fetal distress and 4 had a birth head circumference on or below the 9th centile. All affected infants presented with seizures on day 1 of life, apart from Subject 7 who presented at 1 month. The commonest EEG finding was burst suppression (4/7). All responded to pyridoxine treatment with an immediate reduction in seizure frequency and severity; 2 showed respiratory depression. In the case of subject 1, concerns about respiratory depression led to the pyridoxine being discontinued for several weeks and this infant died. Some affected infants showed signs of systemic disease in addition to the seizures in the first few days of life: 4/7 infants had metabolic acidosis with raised blood lactate, 2/7 anemia and 3/7 gastrointestinal dysfunction - abdominal distension, vomiting, necrotizing enterocolitis. On follow up, four patients had their B_6 treatment changed from pyridoxine to PLP and all showed an improvement in seizure control. The majority (5/6) of the surviving subjects require treatment with anticonvulsants (clobazam, carbamazepine, levetiracetam and phenobarbitone) in addition to vitamin B_6 with 3/5 receiving levetiracetam for optimal seizure control. Neonatal MRI scans where available (4/7) and with the exception of the one individual treated *in utero*, showed global underdevelopment of the brain and periventricular germinolytic cysts (Figure 3, Table 3). Follow-up scans showed persistent white matter abnormalities and head circumference measurements revealed acquired microcephaly. Whilst all subjects showed some degree of developmental delay (Table 2) one attended normal school and leads a normal life. Minor dysmorphic features were evident for four of the affected individuals.

Analysis of Impact of *PROSC* Sequence Variants found in Subjects with B_6-Dependent Epilepsy

Sequence alignment (Figure S2) of PROSC and COG0325 family members to which PROSC belongs, shows two of the missense changes identified, p.Leu175Pro and p.Arg241Gln, affect

residues conserved across mammalian species and in bacteria and yeast. Pro87, however, is only conserved across higher organisms. Analysis of mRNA in fibroblasts indicated that p.Leu175Pro [c.524T>C; Subject 4] decreases *PROSC* expression ($p<0.0001$) (Figure 4A, Figure S3) resulting in no detectable PROSC protein in fibroblasts (Figure 4B). PROSC protein is also not detectable in fibroblasts of Subject 2 (Figure 4B), with p.Ser78Ter presumably resulting in nonsense-mediated mRNA decay (Figure 4A). Fibroblasts were not available to study p.Gln71Ter and p.Pro87Leu/p.Arg241Gln further.

Analysis of cDNA from Subject 5 revealed that c.207+1G>A and c.320-2A>G affect DNA splicing (Figure 4C) and result in decreased mRNA expression (Figure 4A). The largest cDNA product originates from c.207+1G>A and is due to inclusion of intron 2 (Figure S4) and results in nonsense-mediated decay due to the introduction of a premature stop codon (p.Val70IlefsTer6; Figure S4) whilst the smallest cDNA product arises due to skipping of exon 2 and an in-frame deletion of 36 amino acids (p.Asp34_Tyr69del) and corresponds to a protein of ≈26 kDa (Figure 4B). Sequencing of the intermediate product revealed c.320-2A>G does not cause skipping of exon 5, but results in deletion of the first ten amino acids of this exon and use of a cryptic splice site (p.Ala107_Thr116del).

Biochemical Profiles of Individuals with *PROSC* Mutations

The response of individuals with *PROSC* mutations to B_6 treatment indicated CNS PLP deficiency and biochemical analyses (where available) supported this. Subjects 4 and 5 had low CSF PLP concentrations prior to / just after starting B_6 supplementation (Table 4) and Subjects 2, 4 and 5 displayed features of deficiency of aromatic L-amino acid decarboxylase (AADC),[19] a B_6-dependent enzyme, including elevated CSF concentrations of 3-ortho-methyldopa, L-dopa and/or 5-hydroxytryptophan (Table 5) and urinary organic acid analysis showed raised vanillactate. Glycine, the substrate for the B_6-dependent glycine cleavage enzyme, was on occasions slightly elevated when Subjects 4 and 5 were off B_6 supplementation (Table S8) as was alanine; a substrate of B_6-

dependent alanine transaminase (Table S8). Whilst on B_6 supplementation CSF amino acid levels normalized with the exception of tyrosine (substrate of the PLP-dependent enzyme tyrosine aminotransferase) which was slightly raised on several occasions in Subject 2 (Table S9).

Pre-treatment CSF profiling of Subject 5 revealed that homocarnosine, a histidine dipeptide carbonyl scavenger,[20] was undetectable (Table S10).

Vitamin B_6 profiles of plasma and fibroblast samples from PROSC-deficient individuals

Analysis of B_6 vitamer profiles of plasma samples from subjects on B_6-supplementation revealed that PROSC-deficient individuals had high plasma PLP levels but, unlike individuals with PNPO deficiency,[16] do not accumulate pyridoxine, pyridoxamine, pyridoxine phosphate and pyridoxamine phosphate (Table S11), suggesting PNPO activity is not impaired. Plasma PLP levels, although not as high as those seen in hypophosphatasia caused by mutations in *ALPL*,[21] were 4–7 times higher than reported for individuals with PNPO and PDE deficiencies receiving comparable supraphysiological doses of B_6 (Table S11, including unpublished diagnostic data, National Hospital). TNSALP activity was normal (Table S12) in all PROSC-deficient individuals for whom it was measured and no pathogenic sequence variants in *ALPL* were detected in WES data available for Subjects 1 and 2.

Similarly to plasma, PLP levels were also found to be elevated in fibroblasts from PROSC-deficient individuals [when grown in standard media (containing pyridoxine)], being 2–3 times those of controls (Figure 4D). The concentration of pyridoxal was significantly elevated in cells from Subject 2 (Figure S5) and this may have arisen from phosphatase activity on elevated PLP levels. Comparison of other B_6 vitamers in affected and control fibroblasts was unremarkable.

PLP is a highly reactive aldehyde[22] and can bind not only via Schiff base linkages to the epsilon amino groups on lysine residues at the active site of B_6-dependent enzymes, but can also react with other residues of proteins[23], with metabolites such as Δ^1-piperideine 6-carboxylate,[7,8] with

many amino acids, particularly cysteine (forming thiazolidine adducts)[24,25] and with histidine dipeptides[20]. Therefore, the PLP distribution between low (<3K) and high (>3K) molecular weight fractions from control and PROSC-deficient fibroblasts was investigated. This was similar between fractions (Figure S6) suggesting that supra-physiological cellular concentrations of PLP react non-specifically with both proteins and small molecules.

Complementation of the Pyridoxine-Sensitive Phenotype of ΔyggS *E.coli*

To further explore the role of PROSC in PLP homeostasis we looked for complementation of the pyridoxine sensitive phenotype of the Δ*yggS E.coli*[12] by wild-type and mutant human *PROSC* [Ser78Ter, Pro78Leu, Leu175Pro and Arg241Gln] (Figure 5, Table S13). Wild-type human PROSC and Pro87Leu, which is not conserved between human PROSC and *E.coli* YggS, restored growth. The missense mutations Leu175Pro and Arg241Gln, however, which affect highly conserved amino acids across the YggS/PROSC/YBL036C family (COG0325), did not complement the Δ*yggS* pyridoxine toxicity phenotype nor did the nonsense variant Ser78Ter. Interestingly Arg241Gln appeared to exacerbate the toxicity effect of pyridoxine on the ΔyggS *E.coli* mutant.

Discussion

Homozygosity mapping and exome sequencing of a consanguineous family led to the discovery of a homozygous nonsense mutation, p.Ser78Ter in *PROSC*, a gene of hitherto unknown function, which segregates with a phenotype of prenatal / neonatal onset seizures that respond to treatment with pyridoxine and PLP. The phenotype is expressed in all 3 affected children and not in 9 heterozygotes and 2 homozygous wildtype individuals in 3 generations. In a cohort of individuals with B_6-responsive epilepsy, in which known causes of B_6-dependent epilepsy had been excluded, homozygous or compound heterozygous mutations were found in *PROSC* in 4 of the 29 children / young adults (14%). Little is known about the COG0325 family of proteins to which PROSC belongs.

Whilst structural studies of the yeast and *E.coli* family proteins[26] have shown that these proteins are similar to the N-terminal of alanine racemase and ornithine decarboxylase and bind PLP in a similar manner they are monomeric and have no racemase activity towards any of the 20 protein amino acids or their D-enantiomers.[11,12] Indeed PLP binding does not affect the quaternary structure of the protein.[12]

As with pyridoxine-dependent epilepsy due to ALDH7A1 (antiquitin) deficiency[27,28] and pyridoxine/PLP-dependent epilepsy due to PNPO deficiency,[6] the clinical phenotype is dominated by the B_6-dependent seizure disorder although systemic signs such as anemia, enterocolitis, electrolyte abnormalities and lactic acidosis may be present in the neonate. The birth head circumference was on or below the 9th centile for 4 out of the 6 subjects with PROSC deficiency for whom this measurement was available; birth HC is usually normal in PNPO deficiency and ALDH7A1 deficiency. Also, in PROSC deficiency we noted MRI features showing global underdevelopment of the brain (broad gyri and narrow sulci) and later underdevelopment of white matter. Periventricular cysts could be present in the neonatal scans. In the child treated during pregnancy, these changes were not seen but acquired microcephaly was evident on follow-up for him and all but one of the other PROSC-deficient individuals. All affected children had some degree of developmental delay whereas some individuals with PNPO deficiency and 25% of individuals with ALDH7A1 deficiency have a normal neurodevelopmental outcome.[28,29]

Analysis of CSF concentrations of neurotransmitter amine precursors and metabolites on PROSC-deficient subjects off treatment showed elevation of 3-O-methyldopa (3/3) and 5-hydroxytryptophan (2/2) – also consistent abnormalities in PNPO deficiency - but minimal or no reduction in homovanillic acid (HVA) or 5-hydroxyindoleacetic acid (5HIAA). HVA and 5HIAA were low in the first-described individuals with PNPO deficiency[5] but not consistently in subsequently described individuals with PNPO deficiency.[30] There may be a route of production of dopamine and hence its metabolite, HVA, that does not require AADC[31] and this pathway may be active to a variable degree in individuals with PROSC deficiency and PNPO deficiency. In the one PROSC-deficient subject in whom CSF homocarnosine was measured, it was undetectable. This may indicate that

this carbonyl scavenger is reacting with free PLP; whether this will prove to be a specific marker for PROSC-deficiency remains to be seen.

The plasma PLP concentration in PROSC-deficient individuals receiving pharmacological doses of pyridoxine/PLP was higher than we see in ALDH7A1 deficiency or PNPO deficiency but not as high as seen in hypophosphatasia[21]. Unlike PNPO deficiency, there was no accumulation of pyridoxine, pyridoxamine, pyridoxine phosphate or pyridoxamine phosphate in the plasma of the PROSC-deficient individuals. So the plasma B_6 vitamer profile on treatment could be a pointer to PROSC deficiency.

The mutations in *PROSC* in the children with B_6-dependent epilepsy include nonsense, missense and splice site mutations. The missense mutations, p.Leu175Pro and p.Arg241Gln affect residues that are highly conserved from bacteria through yeast to man. However Subject 7, who had a milder disease with onset of seizures at 1 month rather than on day 1, had a mutation on one allele that affected a residue (Pro87) that is only conserved across higher species.

We used fibroblasts to study the effects of the *PROSC* mutations found in the affected individuals on *PROSC* RNA and protein and B_6 vitamer levels. The nonsense mutation, p.Ser78Ter, led to a reduced *PROSC* mRNA level as would be expected from nonsense-mediated decay but reduced *PROSC* mRNA and PROSC protein were also seen in the fibroblasts of the child homozygous for p.Leu175Pro. The splice site mutations were confirmed to give rise to aberrant splicing. In fibroblasts cultured in normal medium, the amounts of PLP present in the cells of the PROSC-deficient individuals, was 2–3 times those of controls; an excess was present in both high molecular weight and low molecular weight cell lysate fractions suggesting that the excess PLP present was binding to proteins and small molecules indiscriminately.

An *E.coli* strain lacking the PROSC homologue (Δ*YggS*) shows growth inhibition when exposed to a high concentration of pyridoxine and this phenotype was rescued by transfection with wild type human *PROSC* but not by *hPROSC* bearing the p.Leu175Pro, p.Arg241Gln, and p.Ser78Ter mutations found in affected individuals. One mutation, however, did allow hPROSC to

rescue the pyridoxine sensitivity of Δ*YggS* – Pro78Leu. This is unsurprising as Pro78 is not conserved between human PROSC and *E.coli* YggS. Previous studies on *E.coli* Δ*YggS* showed an abnormality of the metabolism of amino acids and 2-oxoacids that were interpreted as indicating deficiency of coenzyme A (CoASH) and were reversed by supplementing with pantothenate.[11] It is possible that in the absence of PROSC, free PLP rises to a level at which it reacts with and inactivates CoASH.

Several mechanisms have been proposed to explain how the cell ensures that PLP is guided to apo-B_6 enzymes and does not react inappropriately with small molecules and proteins.[32-37] It has been suggested that PNPO and pyridoxal kinase, which are regulated by PLP feedback inhibition,[32] channel PLP to certain apo-B_6-enzymes.[36,37] Our data suggests PROSC is involved in cellular PLP homeostasis in man. Whilst exact mechanisms remain uncertain, we postulate that PROSC is a PLP-carrier which, prevents PLP from reacting with other reactive molecules, supplies it to apo-enzymes and protects it from intracellular phosphatases (Figure S7). In human PROSC deficiency there is clear evidence of low levels of CSF PLP which are accompanied by deficient activity of PLP-holoenzymes (e.g. AADC) and of B_6-dependent epilepsy. There is also evidence of carbonyl scavenger depletion *in vivo* and evidence, in cultured cells, of uncontrolled build-up of PLP with excessive binding to proteins and small molecules.

Treatment of PROSC-deficient individuals with PLP produced better seizure control than treatment with pyridoxine. The plasma B_6 vitamer profile of a subject on pyridoxine showed very high levels of pyridoxal and pyridoxic acid (Table S11). This suggests that the PLP produced in the liver from the pyridoxine supplement by the sequential actions of pyridoxal kinase and PNPO, is, in the absence of PROSC, vulnerable to degradation by intracellular phosphatases and aldehyde oxidase. All but one of the individuals with PROSC deficiency required treatment with antiepileptic drug(s) as well as B_6. This suggests that the pathophysiology of the seizure disorder involves more than CNS PLP deficiency; it is possible that the inappropriate reaction of PLP with proteins and small molecules contribute to the pathogenesis of the seizures. It is also possible that variants in other genes will be found to affect the phenotype of PROSC deficiency.

Supplemental Data

Supplemental Data includes 7 Figures and 13 Tables

Acknowledgements

PBM and PTC received Great Ormond Street Hospital (GOSH) Children's Charity Leadership awards (V2516 and V1254) and PBM, PTC and SH are supported by the National Institute for Health Research Biomedical Research Centre at GOSH for Children NHS Foundation Trust and University College London. VdC-L is funded by a US National Science Foundation (NSF) grant MCB1611711. We thank Georg F. Hoffmann (Division of Neuropediatrics and Pediatric Metabolic Medicine, University Children's Hospital Heidelberg, Germany) and Gajja S. Salomons (Metabolic Laboratory, VU University Medical Centre, Amsterdam, The Netherlands) for laboratory studies (regarding Subject 5), Andrew Hanson with whom early discussions helped formulate a possible link between PROSC and PLP homeostasis and Dr. Stephan Coburn (Department of Chemistry, Indiana University, Forte Wayne, USA) for the kind gift of the D2-PLP. The authors declare that they have no conflict of interest with regard to this publication.

Web Resources

The URLs for data presented herein are as follows:

OMIM, http://www.omim.org/

dbSNP, http://www.ncbi.nlm.nih.gov/SNP/

FastQC, http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc

Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org/

Ensembl, http://www.ensembl.org/index.html

ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/

REFERENCES

1. Whittaker, J.W. (2016). Intracellular trafficking of the pyridoxal cofactor. Implications for health and metabolic disease. Arch Biochem Biophys. *592*, 20-26.

2. Waymire, K.G., Mahuren, J.D., Jaje, J.M., Guilarte, T.R., Coburn, S.P., MacGregor,G.R. (1995). Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. Nat Genet. *11*, 45-512.

3. Belachew, D., Kazmerski, T., Libman, I., Goldstein, A.C., Stevens, S.T., Deward, S., Vockley, J., Sperling, M.A., Balest, A.L. (2013). Infantile hypophosphatasia secondary to a novel compound heterozygous mutation presenting with pyridoxine-responsive seizures. JIMD Rep. *11*, 17-24.

4. Gachon, F., Fonjallaz, P., Damiola, F., Gos, P., Kodama, T., Zakany, J., Duboule, D., Petit, B., Tafti, M. and Schibler, U. (2004). The loss of circadian PAR bZip transcription factors results in epilepsy. Genes Dev. *18*, 1397–1412.

5. Mills, P.B., Surtees, R.A., Champion, M.P., Beesley, C.E., Dalton, N., Scambler, P.J., Heales, S.J., Briddon, A., Scheimberg, I., Hoffmann, G.F., et al. (2005). Neonatal epileptic encephalopathy caused by mutations in the PNPO gene encoding pyridox(am)ine 5'-phosphate oxidase. Hum Mol Genet. *14*, 1077-1086.

6. Mills, P.B., Camuzeaux, S.S., Footitt EJ, Mills KA, Gissen P, Fisher L, Das KB, Varadkar SM, Zuberi S, McWilliam R, et al. (2014). Epilepsy due to PNPO mutations: genotype, environment and treatment affect presentation and outcome. Brain *137*, 1350-1360.

7. Mills, P.B., Struys, E., Jakobs, C., Plecko, B., Baxter, P., Baumgartner, M., Willemsen, M.A., Omran, H., Tacke, U., Uhlenberg, B., et al. (2006). Mutations in antiquitin in individuals with pyridoxine-dependent seizures. Nat Med. *12*, 307-309.

8. Farrant, R.D., Walker, V., Mills, G.A., Mellor, J.M., Langley, G.J. (2001). Pyridoxal phosphate de-activation by pyrroline-5-carboxylic acid. Increased risk of vitamin B6 deficiency and seizures in hyperprolinemia type II. J Biol Chem. *276*:15107-15116.

9. Ng, B.G., Freeze, H.H. (2015). Human genetic disorders involving glycosylphosphatidylinositol (GPI) anchors and glycosphingolipids (GSL). J Inherit Metab Dis. *38*, 171-178.

10. Ikegawa, S., Isomura, M., Koshizuka, Y., Nakamura, Y. (1999). Cloning and characterization of human and mouse PROSC (proline synthetase co-transcribed) genes. J Hum Genet. *44*, 337-342.

11. Ito, T., Iimori, J., Takayama, S., Moriyama, A., Yamauchi, A., Hemmi, H., Yoshimura, T. (2013). Conserved pyridoxal protein that regulates Ile and Val metabolism. J Bacteriol. *195*, 5439-5449.

12. Prunetti, L., El Yacoubi, B., Schiavon, C., Kirpatrick, E., Huang, L., ElBadawi-Sidhu, M., Harrison, K., Gregory, J.F. 3rd, Fiehn, O., Hanson, A.D. et al (2016). Evidence That COG0325 Proteins are involved in PLP Homeostasis. Microbiology. Feb 12. doi: 10.1099/mic.0.000255. [Epub ahead of print].

13. Li, H., Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics *25*, 1754-1760.

14. McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., DePristo, M.A. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res *20*, 1297 -1303

15. Wang, K., Li, M., Hakonarson, H. (2010). ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. *38*, e164.

16. Footitt, E.J., Clayton, P.T., Mills, K., Heales, S.J., Neergheen, V., Oppenheim, M., Mills, P.B. (2013). Measurement of plasma B6 vitamer profiles in children with inborn errors of vitamin B6 metabolism using an LC-MS/MS method. J Inherit Metab Dis. *36*, 139-145.

17. Albersen, M., Bosma, M., Jans, J.J., Hofstede, F.C., van Hasselt, P.M., de Sain-van der Velden, M.G., Visser, G., Verhoeven-Duif, N.M. (2015). Vitamin B6 in plasma and cerebrospinal fluid of children. PLoS One. *10*, e0120972.

18. Albersen, M., Groenendaal, F., van der Ham, M., de Koning, T.J., Bosma, M., Visser, W.F., Visser, G., de Sain-van der Velden, M.G., Verhoeven-Duif, N.M. (2012). Vitamin B6 vitamer concentrations in cerebrospinal fluid differ between preterm and term newborn infants. Pediatrics. *130*, e191-198.

19. Pons, R., Ford, B., Chiriboga, C.A., Clayton, P.T, Hinton, V., Hyland, K., Sharma, R., De Vivo, DC. (2004). Aromatic L-amino acid decarboxylase deficiency: clinical features, treatment, and prognosis. Neurology *67*, 1058-1065.

20. Vistoli, G., De Maddis, D., Straniero, V., Pedretti, A., Pallavicini, M., Valoti, E., Carini, M., Testa, B., Aldini, G. (2013). Exploring the space of histidine containing dipeptides in search of novel efficient RCS sequestering agents. Eur J Med Chem. *66*, 153-160.

21. Whyte, M.P., Mahuren, J.D., Fedde, K.N., Cole, F.S., McCabe, E.R., Coburn, S.P. (1988). Perinatal hypophosphatasia: tissue levels of vitamin B6 are unremarkable despite markedly increased circulating concentrations of pyridoxal-5'-phosphate. Evidence for an ectoenzyme role for tissue-nonspecific alkaline phosphatase. J Clin Invest. *81*, 1234-1239.

22. Phillips, R.S. (2015). Chemistry and diversity of pyridoxal-5'-phosphate dependent enzymes. Biochim Biophys Acta. *1854*, 1167-1174.

23. Kajita, R., Goto, T., Lee, S.H., Oe, T. (2013). Aldehyde stress-mediated novel modification of proteins: epimerization of the N-terminal amino acid. Chem Res Toxicol. *26*, 1926-1936.

24. Terzuoli, L., Leoncini, R., Pagani, R., Guerranti, R., Vannoni, D., Ponticelli, F., Marinello, E. (1998). Some chemical properties and biological role of thiazolidine compounds. Life Sci. *63*, 1251-1267.

25. Schonbeck, N.D., Skalski, M., Shafer, J.A. (1975) Reactions of pyridoxal 5'-phosphate, 6-aminocaproic acid, cysteine, and penicilamine. Models for reactions of Schiff base linkages in pyridoxal 5'-phosphate-requiring enzymes. J Biol Chem. *250*, 5343-5351.

26. Eswaramoorthy, S., Gerchman, S., Graziano, V., Kycia, H., Studier, F.W., Swaminathan, S. (2002). Structure of a yeast hypothetical protein selected by a structural genomics approach. Acta Crystallogr D Biol Crystallogr. *59*, 127-135.

27. Mills, P.B., Footitt, E.J., Mills, K.A., Tuschl, K., Aylett, S., Varadkar, S., Hemingway, C., Marlow, N., Rennie, J., Baxter, P., et al. (2010). Genotypic and phenotypic spectrum of pyridoxine-dependent epilepsy (ALDH7A1 deficiency). Brain *133*, 2148-59.

28. van Karnebeek, C.D., Tiebout, S.A., Niermeijer, J., Poll-The, B.T., Ghani, A., Coughlin, C.R. 2nd, Van Hove, J.L., Richter, J.W., Christen, H.J., Gallagher, R., Hartmann, H., Stockler-Ipsiroglu, S. (2016). Pyridoxine-Dependent Epilepsy: An Expanding Clinical Spectrum. Pediatr Neurol. *59*, 6-12.

29. Hatch, J., Coman, D., Clayton, P., Mills, P., Calvert, S., Webster, R.I., Riney, K. (2016). Normal Neurodevelopmental Outcomes in PNPO Deficiency: A Case Series and Literature Review. JIMD Rep. *26*, 91-97.

30. Hoffmann, G.F., Schmitt, B., Windfuhr, M., Wagner, N., Strehl, H., Bagci, S., Franz, A.R., Mills, P.B., Clayton, P.T., Baumgartner, M.R. et al. (2007). Pyridoxal 5'-phosphate may be curative in early-onset epileptic encephalopathy. J Inherit Metab Dis. *30*, 96-99.

31. Wassenberg, T., Willemsen, M.A., Geurtz, P.B., Lammens, M., Verrijp, K., Wilmer, M., Lee, W.T., Wevers, R.A., Verbeek, M.M. (2010). Urinary dopamine in aromatic L-amino acid decarboxylase deficiency: the unsolved paradox. Mol Genet Metab. *101*, 349-356.

32. Di Salvo, M.L., Contestabile, R., Safo, M.K. (2011). Vitamin B(6) salvage enzymes: mechanism, structure and regulation. Biochim Biophys Acta *1814*, 1597-1608.

33. Stepuro, I.I., Solodunov, A.A., Piletskaia, T.P., Iaroshevich, N.A., Ostrovskii, I. (1988). Distribution of pyridoxal-5-phosphate between proteins and low molecular weight components of plasma: effect of acetaldehyde. Ukr Biokhim Zh. *60*, 34-41.

34. Bohney, J.P., Fonda, M.L., Feldhoff, R.C. (1992). Identification of Lys190 as the primary binding site for pyridoxal 5'-phosphate in human serum albumin. FEBS Lett. *298*, 266-268.

35. Ink, S.L., Henderson, L.M. (1984). Effect of binding to hemoglobin and albumin on pyridoxal transport and metabolism. J Biol Chem. *259*, 5833-5837.

36. Safo, M.K., Musayev, F.N., Schirch, V. (2005). Structure of Escherichia coli pyridoxine 5'-phosphate oxidase in a tetragonal crystal form: insights into the mechanistic pathway of the enzyme. Acta Crystallogr D Biol Crystallogr. *61*, 599-604.

37. Di Salvo, M.L., Nogués, I., Parroni, A., Tramonti, A., Milano, T., Pascarella, S., Contestabile, R. (2015). On the mechanism of Escherichia coli pyridoxal kinase inhibition by pyridoxal and pyridoxal 5'-phosphate. Biochim Biophys Acta *1854*, 1160-1166.

Figure Titles and Legends

Figure 1. Enzymes and Transporters Involved in Mammalian CNS PLP Synthesis and Homeostasis and Known Human Genetic Vitamin B_6-Dependent Epilepsies

Pyridoxal 5'-phosphate (PLP); pyridoxamine 5'-phosphate (PMP); pyridoxal (PL); pyridoxine (PN); pyridoxine 5'-phosphate (PNP); pyridoxine-5'-β-D-glucoside (PNG); intestinal phosphatases (IP); transporter (identity unknown;T1); pyridoxal kinase (PK); pyridox(am)ine 5'-phosphate oxidase (PNPO); tissue non-specific alkaline phosphatase (TNSALP); pyridoxal-phosphatase (PLPase). AOX/DH (Aldehyde oxidase [Mo cofactor] / β-NAD dehydrogenase) (1) PNPO is controlled by feedback inhibition from PLP. (2) PLP functions as a co-factor, forming Schiff bases with the ε-amino group of lysine residues of proteins. (3) PLP can be formed by recycling the cofactor from degraded enzymes ('salvage pathway'). (4) PLP levels, in part, are maintained by circadian clock controlled transcription factors with PAR bZip transcription factors (DBP, HLF, TEF) targeting PK. (5) *PNPO* mutations cause a B_6-dependent epilepsy disorder. (6) Disorders resulting in accumulation of L-Δ^1-pyrroline-5-carboxylic acid (P5C) and Δ^1-piperideine-6-carboxylic acid (P6C), (hyperprolinaemia type II and pyridoxine-dependent epilepsy due to mutations in *ALDH4A1* and *ALDH7A1*, respectively) cause decreases in bioavailable PLP as do reactions with exogenous small molecules.

Figure 2. Pedigree of Index Family and Position of *PROSC* Variants

(A) Pedigree of index family (Subjects 1 – 3) and segregation analysis of c.233C>G; p.Ser78Ter for this family. Affected individuals are homozygous for GG. Analysis of extended family DNA demonstrated that both parents are heterozygous carriers of the identified variant and all other individuals were found to either be wildtype (CC) or heterozygous (GC) for this variant. Squares represent males, circles females, and a double line a consanguineous union. Black shapes represent affected individuals, shown subsequently to be homozygous for c.233C>G. The diagonal

line through the square indicates that this individual has deceased. Genotypes: II.2, II.4 = GC; III.1, III.2, III.3, III.4, III.5, = GC; III.6 = CC; IV.1, IV.2, IV.6 = GG; IV.3, IV.4 = GC; IV.5 = CC.

(B) Predicted Features of *PROSC* and Position of Mutations. This gene spans 17.17 kb and consists of 8 exons. The translated protein is 275 amino acids in length. Blue arrows indicate positions of mutations detected. The green line represents the PLP-binding barrel domain [amino acids 21–250] and the blue line the alanine racemase N-terminal domain [amino acids 17-251]. K47 is proposed to have an N6-pyridoxal-phosphate modification (by similarity). A cAMP & cGMP-dependent protein kinase phosphorylation site; RKGS [amino acids 132-135] and a putative N-linked glycosylation site; NTGS [amino acids 146-149] are also predicted. aa = amino acids.

Figure 3. CT and MRI Brain Features of Individuals with Mutations in *PROSC*

(A) CT head (axial) of Subject 1 at 2 months of age showing underdevelopment of brain with broad gyri and shallow sulci as well as a cyst adjacent to the left frontal horn (white arrow). (B) & (C) MRI scan (axial T2 weighted) of Subject 4 at 2 months of age showing underdevelopment of brain with broad gyri and shallow sulci, as well as subcortical and deep white matter oedema and white matter petechial haemorrhages (black arrow). (D) MRI scan (axial, T2 flair) from Subject 5 at 16 days of age showing global underdevelopment of brain with coarse gyral pattern and bilateral large cysts adjacent to the frontal horns. (E) MRI scan (axial, T2 flair) from Subject 5 at 1y 6mo showing global underdevelopment of the brain with a coarse gyral pattern, more severe at the frontal poles, and underdevelopment of white matter.

Figure 4. Loss of *PROSC* Expression Affects Intracellular PLP Homeostasis

(A) qRT-PCR showing *PROSC* expression in affected individuals and wild-type. Reference genes; *β-Actin* and *GAPDH*. Data presented as means ± s d. Statistical analysis performed using Student's

two tailed t test; *** p<0.001. Controls; n=8 and for samples from affected individuals and heterozygotes n=3.

(B) Western blot analysis of control fibroblasts, fibroblasts from PROSC-deficient individuals and a Ser78Ter PROSC heterozygote. β-Actin used as control. Full-length wild-type PROSC is 30 kDa, faint band (Subject 5) is 26 kDa.

(C) *PROSC* cDNA products from wild-type and Subject 5 fibroblasts.

(D) Total fibroblast PLP of controls, PROSC-deficient individuals and Ser78Ter heterozygote. PLP quantified relative to D2-PLP internal standard and normalized (mg cell protein). Data presented as means ± sd. Statistical analysis performed using Student's two tailed t test; comparison of controls with PROSC-deficient samples p<0.0001***. QC; quality control sample. QC n=5; Controls n = 18; PROSC-deficient subjects & Heterozygote n>3.

Figure 5. Complementation of *E. coli* Δ*yggS* with Human Wild-Type and Mutant *PROSC*

E.coli Δ*yggS* were transformed with empty vector (pBAD33) as negative control, human wild-type *PROSC*, Ser78Ter, Leu175Pro, Pro87Leu or Arg241Gln and grown in the presence of discs impregnated with a) 20 µl 0.1 mg/ml or b) 20 µl 1 mg/ml pyridoxine. Pyridoxine produces a ring or rings of growth inhibition around the disc. This is prevented by transfection with wild-type *PROSC* or *PROSC* bearing the Pro87Leu mutation. It is not prevented by transfection with *PROSC* bearing Ser78Ter, Leu175Pro or Arg241Gln. The latter exacerbates growth inhibition producing a filled circle rather than a ring.

Table 1. Genotypes of Subjects with *PROSC* Mutations

Subject	Position	Nucleotide	Predicted Effect	Allele Frequency[b]
1[a]	Exon 3	c.233C>G (M) + c.233C>G (P)	p.Ser78Ter	Novel
2[a]	Exon 3	c.233C>G (M) + c.233C>G P)	p.Ser78Ter	Novel
3[a]	Exon 3	c.233C>G (M) + c.233C>G (P)	p.Ser78Ter	Novel
4	Exon 6	c.524T>C (M) + c.524T>C (P)	p.Leu175Pro	1 in 121412
5	Intron 2	c.207+1G>A (M)	Splicing effect	4 in 121408
	Intron 4	c.320-2A>G (P)	Splicing effect	Novel
6	Exon 3	c.211C>T + c.211C>T	p.Gln71Ter	Novel
7	Exon 4	c.260C>T (M)	p.Pro87Leu	2 in 121410
	Exon 8	c.722G>A (P)	p.Arg241Gln	5 in 121407

(M) = maternal allele; (P) = paternal allele; [a]Related; [b]Data from ExAC; whilst individuals heterozygous for p.Pro87Leu, p.Leu175Pro, p.Arg241Gln and c.207+1G>A have been reported on the ExAC database the frequency of these variants is <0.01% and there are no reports of any homozygous individuals.

Table 2. Clinical Characteristics of Individuals with Mutations in *PROSC*

	Subject 1	Subject2[a]	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Gender	M	M	F	F	F	M	M
Current Age	Died 4.5 m	9 y	6 y	3 y 6 m	5 y 6 m	3 y 2 m	16 y
Ethnicity (Domicile)	Syrian (Sweden)	Syrian (Sweden)	Syrian (Sweden)	Indian (UK)	German (Germany)	Indian (UK)	Italian (Italy)
Consanguinity	+	+	+	+	-	+	-
Birth Gestational Age (wks)	32	40	36^{+6}	36^{+3}	35^{+1}	39	39
Abnormal Intrauterine Movements	-	+	+	+	-	-	-
Fetal Distress	+	-	-	+	+	+	-
Apgar Scores	5, 10, 10	8, 9, 10	8, 8, 9	7, 8, 8	8, 8, 9	9, 9, 9	9, 9, 10
Birth head circ. centile	25-50%	2-9%	9%	90%	9%	9%	25-50%
Anemia at birth	+	-	-	+	-	-	-
Acidosis	+	-	+	+	+	+	-
Raised Blood Lactate	+	-	-	+	+	+	Not known
Respiratory Distress	-	-	-	+	+	+	-
Hypertonia (Neonatal)	-	+	-	+	-	+	-
Hypotonia (Neonatal)	-	-	-	-	+	-	-
Abdominal distension/vomiting/feed intolerance	+ NEC	-	-	+	+	-	-
Irritability	Not known	Not known	Not known	+	+	+	-
Seizure onset within 24h	+	+	+	+	+	+	-[b]
Response to AEDs	-	Given with PN; some improvement	Given with PLP/PN; some improvement	Minimal	Partial	Given with PLP; better control	Partial[d]
EEG: Burst suppression	+	-	+	+	+	+	-
Reduced background Activity	+	+	+				-
Focal discharge(s)		+					-
Multifocal spikes					+		-
Seizure type[e]: Tonic	+	+			+	+	

Clonic	-	-	-	-	-	+	-
GTC	-	+	+	+	-	+	+
Myoclonic	+	+	-	-	+	-	-
Lip-smacking/ grimacing	+	-	-	+	+		
Response to pyridoxine	+	+	+	+	+	+	+
Respiratory depression upon treatment with PN in neonatal period	+	-	-	-	+	-	n/a
PN withdrawal undertaken (and led to recurrence of seizures)	+ (+)	-	-	-	+ (+)	-	-
Switch from PN to PLP (improved seizure control)	-	-	+ (+)	+ (+)	+ (+)	+ (+)	-
PLP withdrawal (recurrence of seizures)	n/a	n/a	-	-	+ (+)	+ (+)	n/a
Speech delay	n/a	+	+	+	+	+	-
Motor delay	n/a	+	+	+	+	-	-
Learning difficulties	n/a	+	+	+	+	+	+
Breakthrough seizures with fever	n/a	+	+	-	+	+	+
Acquired microcephaly	+	+	+	+	+	+	-
Minor dysmorphic features[c]	+	+	+	Not known	+	-	-

n/a = not applicable; m = months; y = years; AEDs antiepileptic drugs; GTC = generalised tonic-clonic; NEC = necrotising enterocolitis; circ. = circumference [a]subject treated prophylactically; mother given 100mg pyridoxine daily through pregnancy; [b]subject presented at 1 month of age; [c]2/4 thin upper lip, 1/4 long philtrum, 1/4 upslanting palpebral fissures, 1/4 convergent squint, 1/4 periorbital fullness; [d]Anticonvulsant treatment stopped at age of 8 years; [e]refers to time of presentation.

Table 3. MRI Brain Features of Individuals with Mutations in *PROSC*

	MRI Feature				
Subject (age)	Global underdevelopment of brain with broad gyri and shallow sulci	Microcephaly with global underdevelopment of white matter	Cyst(s)	White matter oedema Deep white matter petechial haemorrhages[a]	Normal
1 (2 m)	+	+[b]	1 [Close to L frontal horn]	-	-
2 (3½ m)	-	-	-	-	+[c]
2 (23 m)	-	-	-	-	+[c; c]
3 (10 d)	+		1 [Close to L frontal horn][d]	-	-
3 (2 y 2 m)		+		-	-
4 (10 d)	+		-	+	-
4 (2 m)	+		-	-	-
4 (2 y)		+	-	-	-
5 (16 d)	+[e]		2 [Bilateral, adjacent to frontal horns])[d]	-	-
5 (7 m)	+[f]	+[b]	Have coalesced with ventricular system	-	-
5 (1y 6 m & 3y 11 m)	+[g]	+	Coalesced	-	-
6	-	-	-	-	+[h]
7	-	-	-	-	+[h]

[a] Asymmetrical; [b]Too young to determine white matter development: [c]Treated in utero and from birth [d]With associated haemorrhage; [e]With failure of operculisation; [f] Magnetic resonance spectroscopy showed a low N-acetyl asparate (NAA) / creatine ratio, a high choline/creatinine ratio and high lactate peaks in parietal white matter; [g]More severe in frontal regions; [h]Not reviewed in London; m = months; d = days; y = years; L = left.

Table 4. CSF B_6 Vitamer Profiles of PROSC-Deficient Individuals

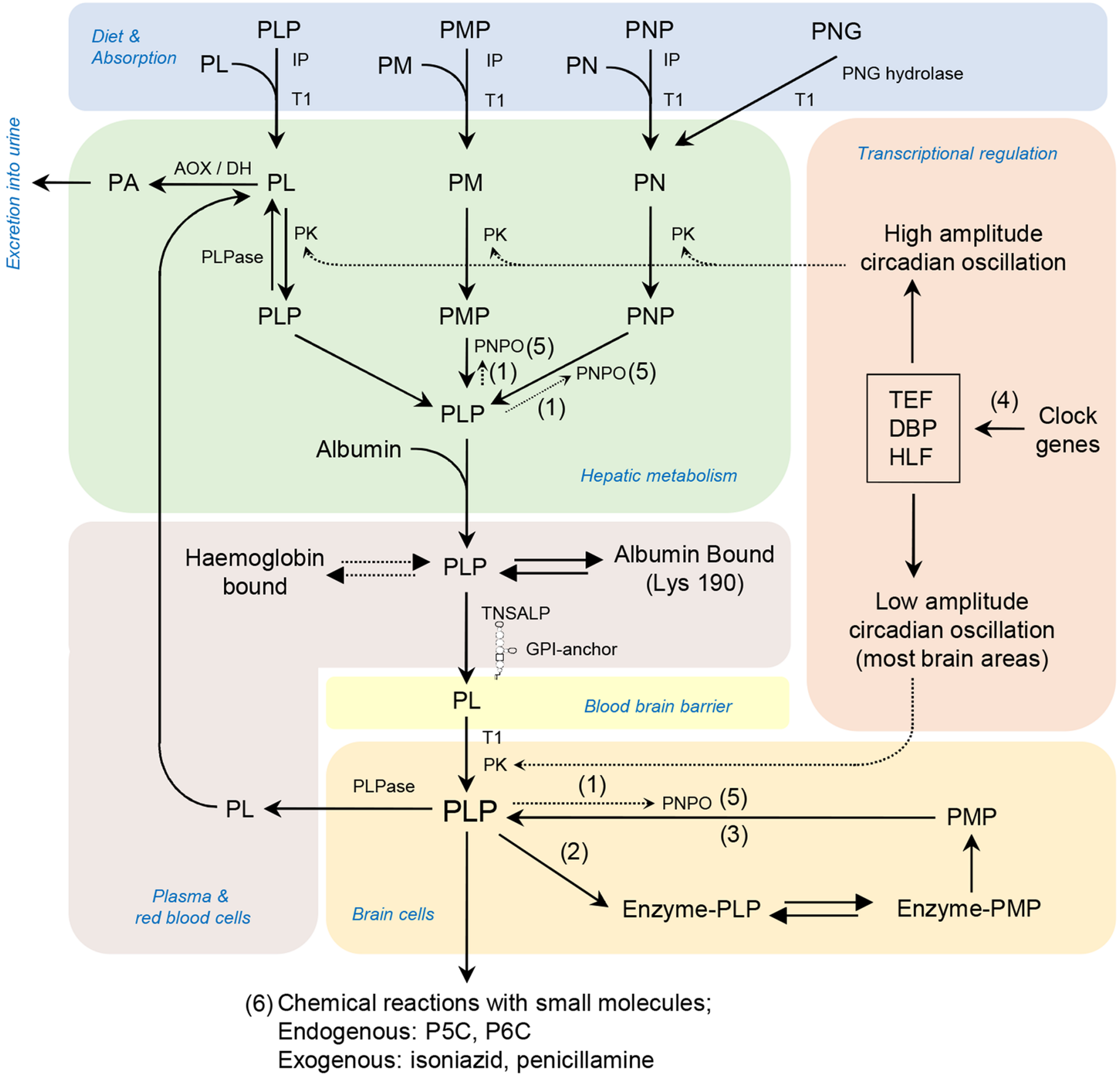
	Age	B_6 [dose]	PLP	PL	PA	PN	PNP	PMP	PM
Ref range	1y – 18 y[1]	None	11 - 34	16 - 56	0.09 - 3	< 0.03[2]	nm	< 5.4[2]	0.3 - 0.9
Subject 4	6 d[3]	PN [100 mg/day]	**<4**[4]	nm	nm	nm	nm	nm	nm
Subject 5	17 m	None[5]	**6, 5**	**15, 15**	nd	0.05, 0.08	0.9, 0.8	nd	0.1, 0.2
Subject 6	20 m	PN [40 mg/kg/day]	43	nm	nm	nm	nm	nm	nm

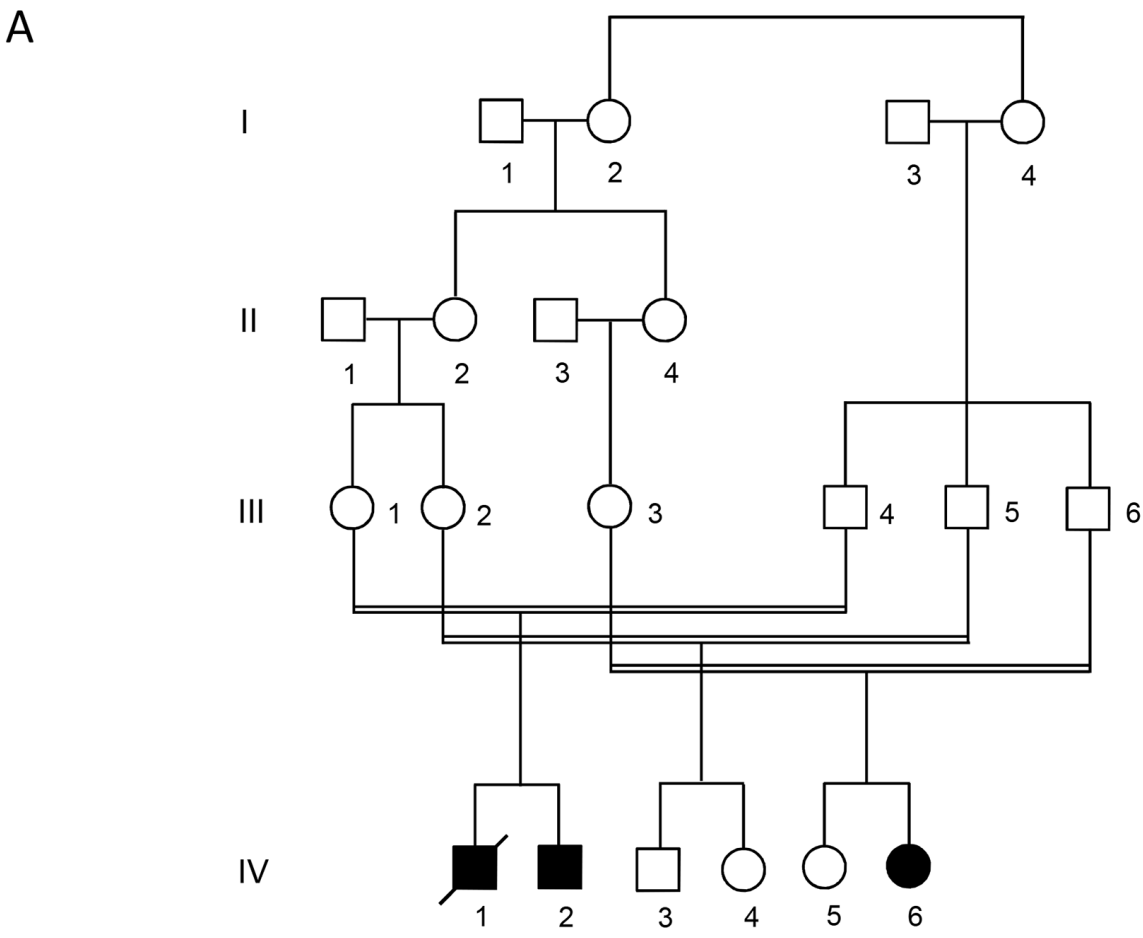
Data was not available for Subjects 1-3 and 7. nd, not detected; nm, not measured; y, years; d, day; m, months; Ref, reference; PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine; PLP, pyridoxal 5'-phosphate; PNP, pyridoxine 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PA, 4-pyridoxic acid. All units nmol/L, except for PNP which is stated in 'concentration units'. Values outside the reference range are shown in bold. [1]Albersen *et al.*, 2015[17]; [2]Limit of quantification for PN/PMP (Albersen *et al.*, 2012)[18]; [3]Measurement taken 2 days after starting pyridoxine; [4]Reference range for local lab (26 – 69); [5]Subject received tapered PLP withdrawal trial. CSF collected 48 hours after last dose. Duplicate measurements of same sample given for Subject 5.

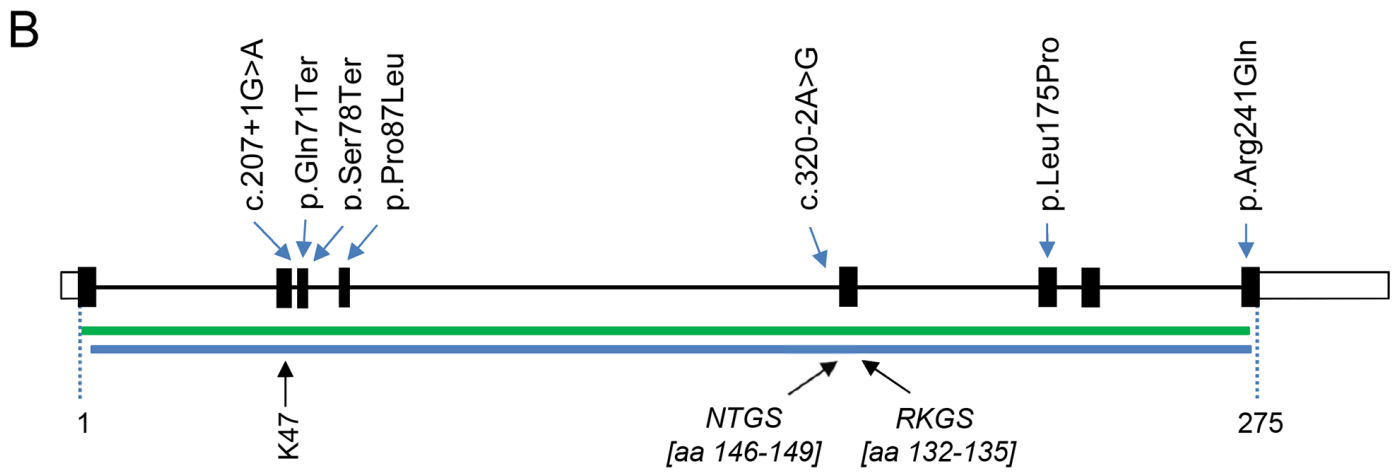
Table 5. CSF Monoamine Metabolite, Pterin and Folate Results of PROSC-Deficient Subjects

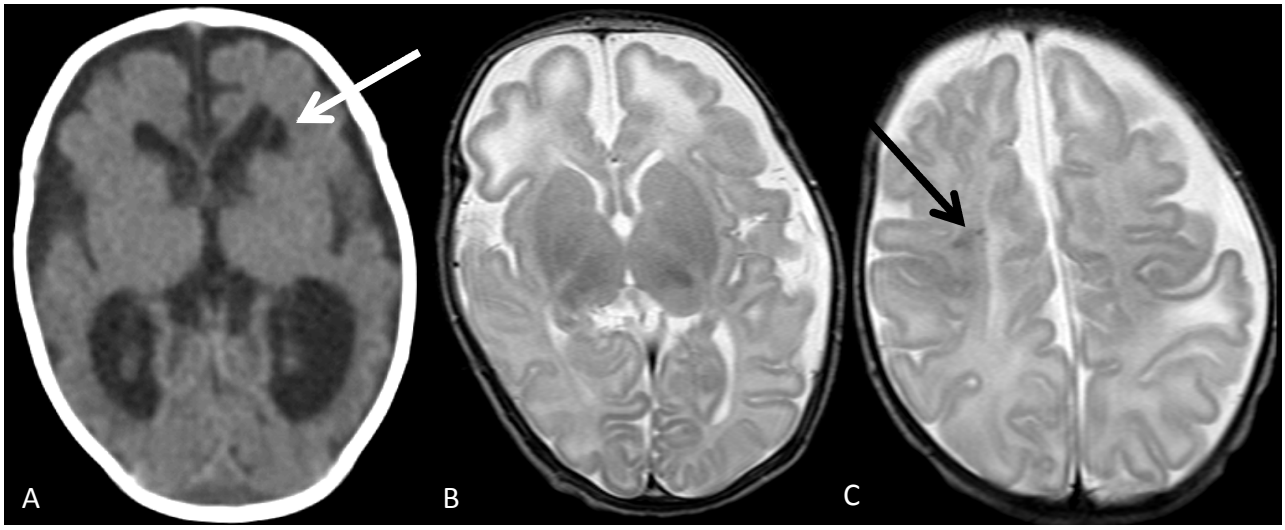
	Subject 2	Subject 4	Subject 5**	Subject 5**	Subject 6
	19 m	6 d	21 d	17 m	20 m
B_6 supplementation	Yes*	No	No	No	Yes
Homovanillic acid	755 (295 - 932)	**310** (324 - 1098)	886 (484 -1446)	770 (364 - 870)	556 (154 - 867)
5-Hydroxyindoleacetic acid	223 (114 - 336)	376 (199 - 608)	538 (302 - 952)	**387** (155 - 359)	158 (89 - 367)
3-Ortho-Methyldopa	46 (<300)	**586** (<300)	**508** (0 - 310)	**84** (0 - 50)	nm
L-Dopa	22 (< 25)	nm	**200** (0 - 15)	<2 (0 -15)	nm
5-Hydroxytryptophan	**105** (< 10)	nm	**32** (0 - 20)	**28** (0 -15)	nm
5-MTHF	89 (64 -182)	120 (72 - 305)	97 (62 - 287)	113 (37 - 214)	nm
Tetrahydrobiopterin	**10** (15 - 40)	71 (27 - 105)	**12** (25 - 121)	35 (20 - 61)	nm
7,8-Dihydrobiopterin	12 (12 - 30)	**17** (0.4 - 13.9)	8 (0 - 18)	4 (0 - 18)	nm
Total Neopterin	nm	**124** (7 - 65)	17 (6 - 59)	6 (5 - 53)	nm

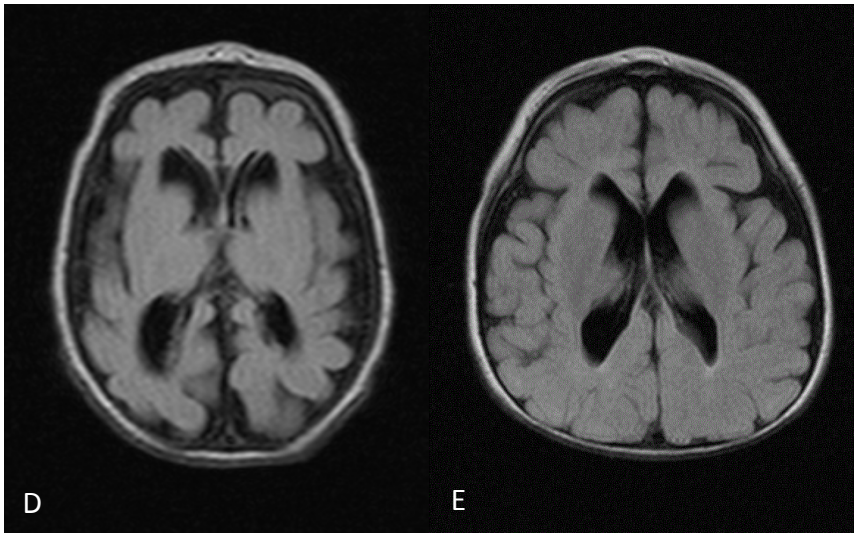
Results expressed as nmol/l. d = day, m = month, 5-MTHF = 5-methyl-tetrahydrofolate *Treated prophylactically. Subjects 2, 4 and 5 display some biochemical features of aromatic L-amino acid decarboxylase (AADC) deficiency; elevated concentrations of 3-ortho-methydopa, L-dopa and 5-hydroxytryptophan, low homovanillic acid. Subject 5 also had raised urinary vanillactic acid on several occasions although this normalised (additional details case history). **Plasma L-aromatic amino acid decarboxylase (AADC) activity markedly decreased at 1 m (12 pmol/ml/min; reference range: 47 - 119), marginally decreased at 7 m (44 pmol/ml/min; reference range; 47-119) and normal at 17 m (50 pmol/ml/min; reference range; 33-79); no mutations were found in *AADC*. Values highlighted in bold are outside of the reference range. Age related reference range given in parentheses.


Diet & Absorption
PLP
PMP
PNP
PNG
PL
PM
PN
IP
T1
PNG hydrolase
Excretion into urine
PA
AOX / DH
PLPase
PK
PNPO (5)
(1)
Albumin
Hepatic metabolism
Transcriptional regulation
High amplitude circadian oscillation
TEF
DBP
HLF
(4)
Clock genes
Low amplitude circadian oscillation (most brain areas)
Haemoglobin bound
Albumin Bound (Lys 190)
TNSALP
GPI-anchor
Blood brain barrier
(2)
(3)
Enzyme-PLP
Enzyme-PMP
Plasma & red blood cells
Brain cells
(6) Chemical reactions with small molecules;
Endogenous: P5C, P6C
Exogenous: isoniazid, penicillamine

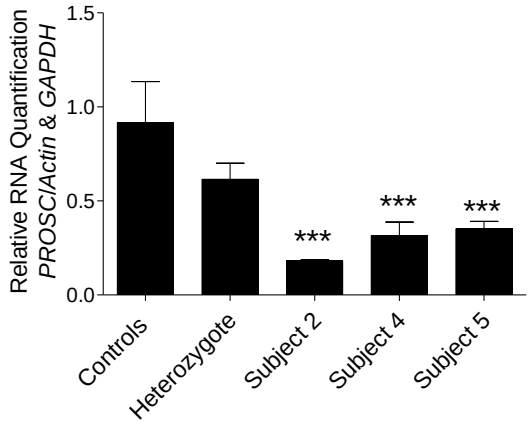

A
I
1
2
3
4
II
1
2
3
4
III
1
2
3
4
5
6
IV
1
2
3
4
5
6


B
c.207+1G>A
p.Gln71Ter
p.Ser78Ter
p.Pro87Leu
c.320-2A>G
p.Leu175Pro
p.Arg241Gln
1
K47
NTGS
[aa 146-149]
RKGS
[aa 132-135]
275

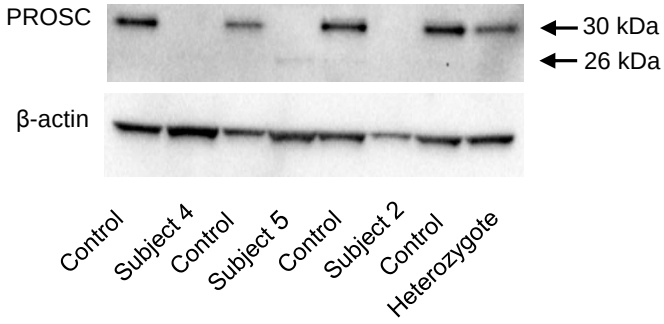

A
B
C


D
E

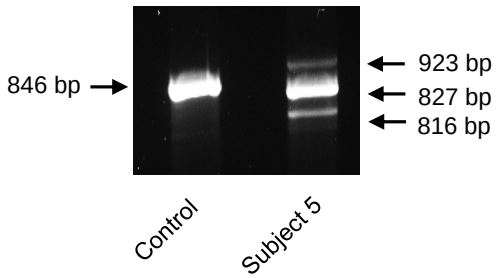
A


Relative RNA Quantification
PROSC/Actin & GAPDH
1.5
1.0
0.5
0.0
***
***
***
Controls
Heterozygote
Subject 2
Subject 4
Subject 5

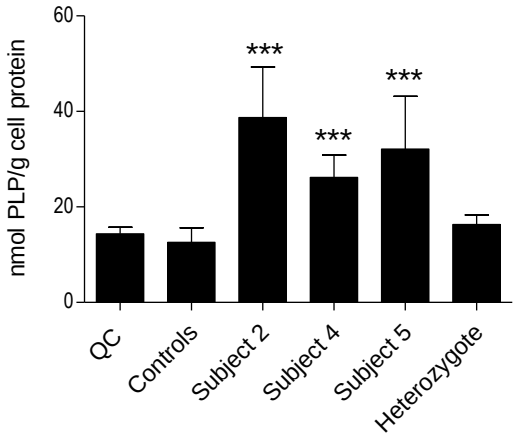
B

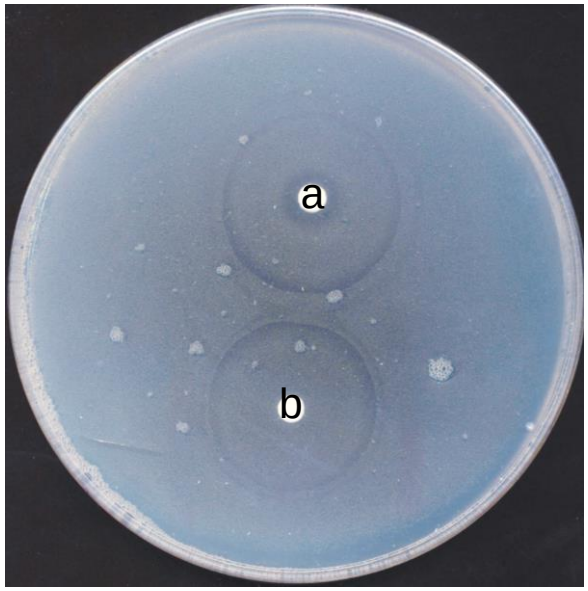

PROSC
β-actin
30 kDa
26 kDa
Control
Subject 4
Control
Subject 5
Control
Subject 2
Control
Heterozygote

C

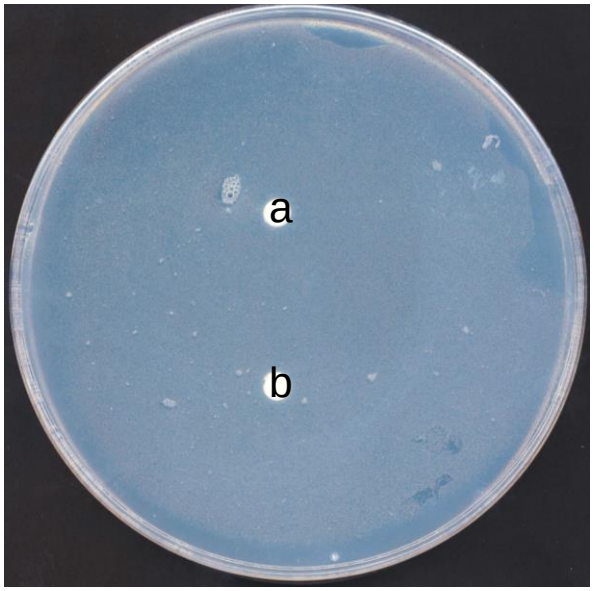

846 bp
923 bp
827 bp
816 bp
Control
Subject 5

D

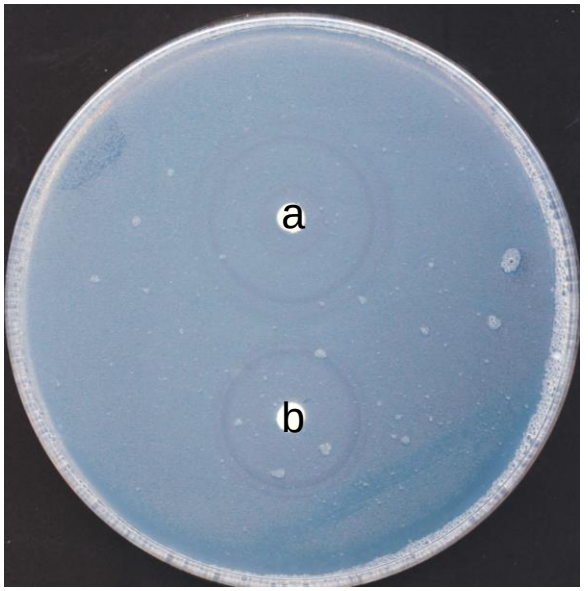

nmol PLP/g cell protein
60
40
20
0
***
***
***
QC
Controls
Subject 2
Subject 4
Subject 5
Heterozygote



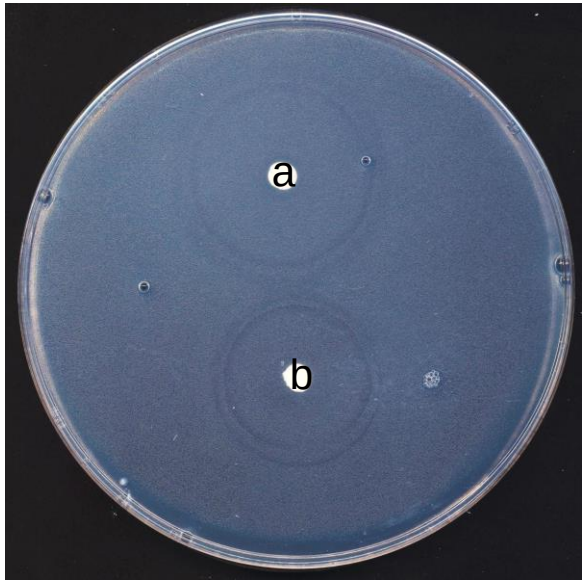
Δ*yggS*



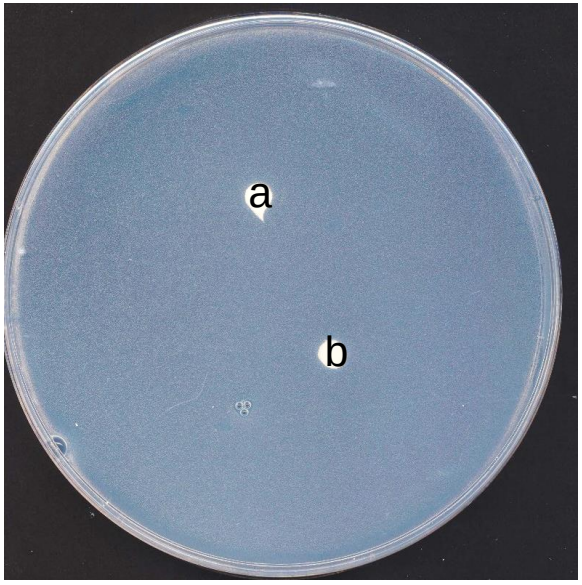
WT



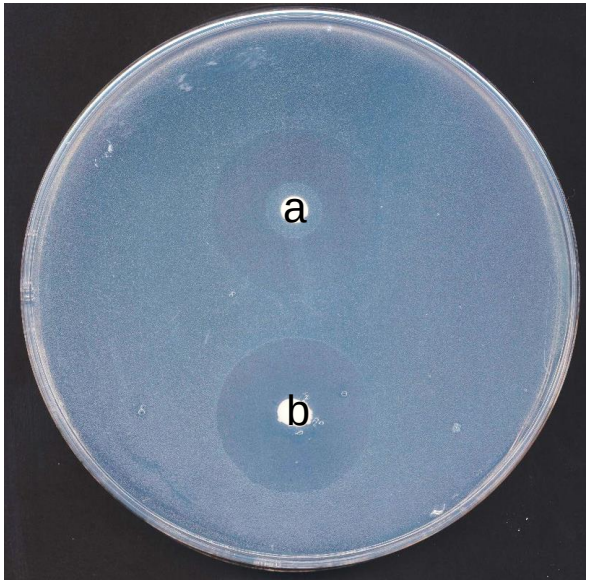
p.Ser78Ter



p.Leu175Pro



p.Pro87Leu



p.Arg241Gln

Clinical Histories

Subject 1 (Fig. 2A. IV.1) was born prematurely at 32 weeks gestation with an Apgar score of 5,10,10. He was small for gestational age with a birth weight (BW) of 1420 g, length of 36 cm and a head circumference (HC) of 27.5 cm (25-50th centile). He had anemia at birth which required blood transfusions and he also developed metabolic acidosis that required buffering; blood lactate was elevated. Myoclonic seizures started immediately after birth. EEGs showed only reduced background activity. Treatment with phenobarbital had no effect. At 5 days, 50 mg pyridoxine hydrochloride was given i.v. and led to immediate cessation of seizures, muscular hypotonia and apnea with respiratory acidosis requiring continuous positive airway pressure (CPAP). He did not receive any further pyridoxine treatment because of the pronounced reaction. At 1 week, he developed necrotizing enterocolitis that was treated surgically with resection of the transverse and descending colon and formation of a colostomy. The EEG deteriorated after the operation with a burst-suppression pattern and frequent subclinical seizures. He also developed more pronounced clinical seizures - tonic seizures, myoclonic jerks and epileptic grimacing, not controlled by phenobarbital or vigabatrin. A brain CT at 2 months showed underdevelopment of the brain with broad gyri and shallow sulci and a cyst adjacent to the left frontal horn. At 3 months the colostomy was closed. After this the seizures increased and were not controlled by midazolam, phenobarbital, lignocaine hydrochloride, valproate and betamethasone. He was therefore intubated and sedated with sodium thiopental. He initially became seizure-free but the seizures returned when the sedation was tapered. At 4½ months, he was again given i.v. pyridoxine HCl 50mg; there was immediate freedom from seizures and the EEG recordings normalized within 3 min. However, he had developed cardiac failure and was felt to have a very poor prognosis; a decision was made to terminate the ventilator treatment and he died.

Subject 2 is the younger brother of Subject 1 (Figure 2A; IV.2); his mother was treated with 100 mg pyridoxine HCl daily throughout pregnancy. She felt rhythmic jerks from the fetus during the last 5 weeks of gestation. BW was 2940 g, length 48 cm and HC 33 cm (2nd – 9th

centile). The Apgar score was 8, 9, 10. Clinical examination at birth was normal except for hypospadias. An EEG examination was normal. At 16 hours, the background activity became abnormal and he developed a seizure originating from the right hemisphere with clinical manifestations of irritability, myoclonic jerks, muscular hypertonia and apnoea. Treatment with pyridoxine HCl 100 mg i.v. led to immediate seizure freedom and normalization of the EEG. Treatment was continued with oral pyridoxine HCl 90 mg daily. His psychomotor development has been delayed. He walked unsupported at 19 months and started to say single words at 2 ½ years. Neuropsychological testing at 6 years of age showed moderate learning disability. He is now 7 years of age and of normal weight and height but has a head circumference at – 3 S.D. Clinical somatic and neurological examinations are normal except for mild fine and gross motor dyspraxia and dysarthric speech. He has 3-4 seizures per year, either generalized tonic-clonic seizures during sleep or myoclonic seizures during febrile infections, and is currently treated with clobazam 10 mg and pyridoxine HCl 400 mg/day. Recent EEG examinations have been normal. MRIs of the brain, performed at 3½ months and 23 months of age, were essentially normal.

Subject 3 is a cousin of Subjects 1 and 2 (Fig. 2A; IV.6) and is the second child born to healthy consanguineous Syrian parents. The pregnancy was normal. She was born at 36^{+6} weeks gestation. BW was 2720 g (25^{th}- 50^{th} centile), length 45 cm (9^{th}-25^{th} centile) and HC 32 cm (9^{th} centile). The Apgar score was 8, 8, 9. In the first few hours of life she had episodic dyspnoea and apnoea and at 6 hours she had a seizure with generalized stiffness, chewing and cyanosis. EEG showed a burst suppression pattern with seizure activity. Intravenous treatment with pyridoxine HCl 100 mg led to immediate freedom from seizures. The EEG background activity became severely suppressed with very low amplitude that gradually normalized and the seizure activity disappeared. She was then treated with oral pyridoxine HCl 80 mg and folinic acid 7.5 mg, once daily (od). At 3 months of age she had several generalized clonic seizures. Clobazam and carbamazepine were added and oral pyridoxine HCl was switched to oral pyridoxal phosphate 70 mg qds. She has since then had monthly

seizures, mostly in relation to febrile infections. Her psychomotor development has been delayed. At 3 years she had a developmental age around 6 months. She gave a social smile, had monosyllabic babbling, grasped with her whole hands, moved objects with her hands to her mouth, and was able to roll over, but not to sit unsupported. Clinical examination revealed her HC was at -4 SD. Muscle tone and tendon reflexes were normal. MRI of the brain at 26 months showed wide ventricles and extracerebral spaces with severely reduced periventricular white matter and a thin corpus callosum.

Subject 4 was the second child born to consanguineous British Asian parents; her brother is well. During the pregnancy, oligohydramnios was noted at 28 weeks (but subsequently resolved), and mother felt abnormal fetal movements. The birth at 36^{+3} weeks gestation was complicated by meconium staining of the liquor but no other signs of fetal distress. BW was 3.20 kg (<10th centile) and Apgar scores were 7, 8, 8. However, the infant collapsed at 10 minutes and developed a mixed respiratory and metabolic acidosis with a blood lactate of 21 mM. She required intubation and ventilation. She was jittery and hypertonic, and developed a pyrexia. She had abdominal distension with dilated loops of bowel on an abdominal X-ray. She was anemic and had a transfusion. Seizures, evident from 20 hours included jaw twitching, eye blinking and hiccups. Investigations revealed only a slightly low plasma magnesium with normal calcium. Seizures were resistant to phenobarbitone, phenytoin, a clonazepam infusion and levetiracetam; a bolus of midazolam produced temporary relief. An EEG on day 2 showed a burst suppression pattern. Pyridoxine (100 mg/d) was given i.v. from day 4 while the infant was still being ventilated. Seizures ceased rapidly and clonazepam and midazolam could be weaned over the next 3 days; levetiracetam was continued. Four days after starting pyridoxine treatment, she could be extubated and pyridoxine treatment continued via a nasogastric tube. An MRI scan at 10 days showed global underdevelopment of the brain and also white matter edema with asymmetrical hemorrhages in the deep white matter. The latter changes had resolved by 2 months. When urine analysis indicated normal urinary excretion of α-aminoadipic

semialdehyde (AASA) and a diagnosis of pyridox(am)ine phosphate oxidase deficiency was suspected, treatment was changed to pyridoxal 5'-phosphate (25 mg tds) by the oral/nasogastric route. This treatment has been continued at 45 mg/kg/d ever since. Treatment with levetiracetam was also continued and later attempts to discontinue this anticonvulsant led to recurrence of seizures. Seizure control on PLP and levetiracetam was as good as, or perhaps even better than, on pyridoxine and levetiracetam. Unfortunately the infant has shown significant developmental delay; she was able to roll over and sit with support at 13 months. Her HC, which had been on the 90th centile at 3 weeks, fell to between the 25th and 50th centiles at 14 months. She is now 3 years 6 months old and has clear delay in language and social communication skills (non-verbal) and still experiences infrequent seizures which occur in clusters often with fever. An MRI scan at 2 years showed white matter signal change and white matter volume loss (T2 weighted images).

Subject 5 was the second child born to non-consanguineous German parents; her brother is well. Pregnancy ultrasounds suggested intrauterine growth retardation and oligohydramnios. Severe oligohydramnios was present at 35 weeks and a cardiotocogram (CTG) showed pathological fetal tachycardia so she was delivered by emergency caesarean section. BW was 2.13 kg (10th centile). Birth HC was 30cm (9th centile UK-WHO). Apgar scores were 8, 8 and 9. Mild dysmorphic features were noted - upslanting palpebral fissures, mild telecanthus, a wide nose, prominent nasal bridge, broad face, and full cheeks. In the first hours of life she was noted to be irritable, tachypnoeic at times, bradypnoeic with a mild respiratory acidosis at others. She was treated with CPAP. She was tachycardic and pyrexial. At 12 hours, she had a bout of hyperexcitability and myoclonic jerks were seen. She had a metabolic acidosis with raised blood lactate (8-19 mM) and CSF lactate (9.8 mM). Seizures continued and included tonic elevation of her arms, other spasms, lip smacking and facial grimacing. An EEG showed a burst suppression pattern as well as multifocal spikes. At 2 days she was given pyridoxine 100 mg i.v. followed by 20 mg per day orally along with phenobarbitone. She had some bradypnoea but seizures ceased for 7 days and the EEG

improved. After 7 days, pyridoxine was discontinued but seizures recurred after 2 days. During the 2nd and 3rd weeks of life seizures were partially controlled by phenobarbitone and levetirecetam. Oral pyridoxine (20mg daily) was reintroduced at 4 weeks and improved seizure control. At 6 weeks PLP was introduced at a dose of 10 mg/kg tds. This led to resolution of the multifocal spikes on the EEG. Treatment with PLP was continued throughout the first 17 months at a dose of 40 mg/kg/d together with levetiracetam. The neonatal MRI scan showed large ventricles, broad gyri and shallow sulci, reduced white matter bulk, periventricular germinolytic cysts and a slim thoracic spinal cord. A repeat scan at 7 months showed reduced brain volume and reduced white matter bulk but age-appropriate myelination. Further MRI scans at 1 year 6 months, and at 3 years 11 months, showed massive brain atrophy, due to underdevelopment of white matter. At 17 months a tapered PLP withdrawal led to recurrence of seizures within 24 hours in the form of myoclonic jerks. Subsequently treatment with PLP (10 mg/kg tds) and levetiracetam was continued with good seizure control. Seizures sometimes occurred with febrile illness, taking the form of tonic seizures and simple partial motor seizures. Assessment at 5 years 4 months showed severe psychomotor delay (speech and understanding 12.5 months, expressive speech <11 months, perception <11 months, gross and fine motor skills <11 months). Her HC had fallen from the 9th centile to -4.4SD, with no head growth since 3 years 6 months. Furthermore growth failure had occurred with a height 3.4SD below the mean.

Subject 6 was the product of the second pregnancy of first cousin British Indian parents. The first pregnancy unfortunately resulted in intrauterine death at 22 weeks with evidence of infection and facial deformity. Subject 6 was born at 39 weeks gestation following evidence of fetal distress (a suboptimal CTG and meconium-stained liquor). BW was 3.25kg and birth HC 33cm (9th centile). Apgar scores were 9 at 1 minute and 9 at 5 minutes. He was irritable and hypertonic and at 2 hours started to have seizures (probably tonic although there was a clonic phase too). He developed respiratory distress at 16 hours. At 24 hours of age he had his first major seizure, which mother describes as jerky movements of both arms and

legs lasting 5 minutes; he had abnormal tone with back arching. At 48 hours he had a further seizure, for which he was given a trial of pyridoxine (200mg i.v.) and the seizures stopped immediately. Effective control continued with reduction of seizures to 1 per week. At 4 days, an EEG showed that there were variable periods of low amplitude burst suppression lasting from 10 – 12 seconds, however, all subsequent EEGs have shown no evidence of interictal epileptiform activity. Treatment was changed from oral pyridoxine to oral PLP (40 mg/kg/d ÷ 4) at 5 months. This resulted in improved seizure control but even with additional anticonvulsant therapy (phenobarbitone and leviteracetam) he had seizures once a month. A trial of PLP withdrawal at 11 months led to recurrence of seizures within a few hours and PLP was restarted at 40 mg/kg/d. Subsequent seizures have been generalized tonic-clonic seizures: typically a 5-10 minute seizure, which mother describes as both arms and legs having jerking movements and being unresponsive for 5 minutes prior to arching his back and his eyes rolling for a further five minutes. Seizures are often associated with fever.

Subject 7, an Italian boy, is now 16 years old. His parents are not consanguineous. The pregnancy was uneventful and he was born at 39 weeks gestation with normal BW (3.05 kg), HC (34cm; 25^{th}-50^{th} centile) and Apgar scores (9, 9, 10). There were no problems in the early neonatal period but he developed generalised tonic clonic seizures at 1 month. An EEG showed good background activity but multifocal abnormalities. He was treated sequentially with carbamazepine plus clobazam, clobazam plus nitrazepam (NZP), and NZP plus felbamate (FBM). He showed partial seizure control with these anticonvulsants. At the age of 23 months, after many recurrent partial and generalized seizures over a few days, he was given a trial of pyridoxine at a dose of 300 mg per day which resulted in complete cessation of seizures. Since the age of 8 years he has only received pyridoxine with all other treatments having been stopped. He is currently receiving a dose of 250 mg/d. He has had very good seizure control with only occasional breakthrough seizures with febrile illnesses. IQ measurements indicated a score of 76 at 3 years (Griffiths), 65 at 9 years (Raven's progressive matrices) and 60 with good adaptive skills at 17 years (Raven's progressive

matrices). He was able to attend normal school and now leads a normal life. His brain MRI is normal.

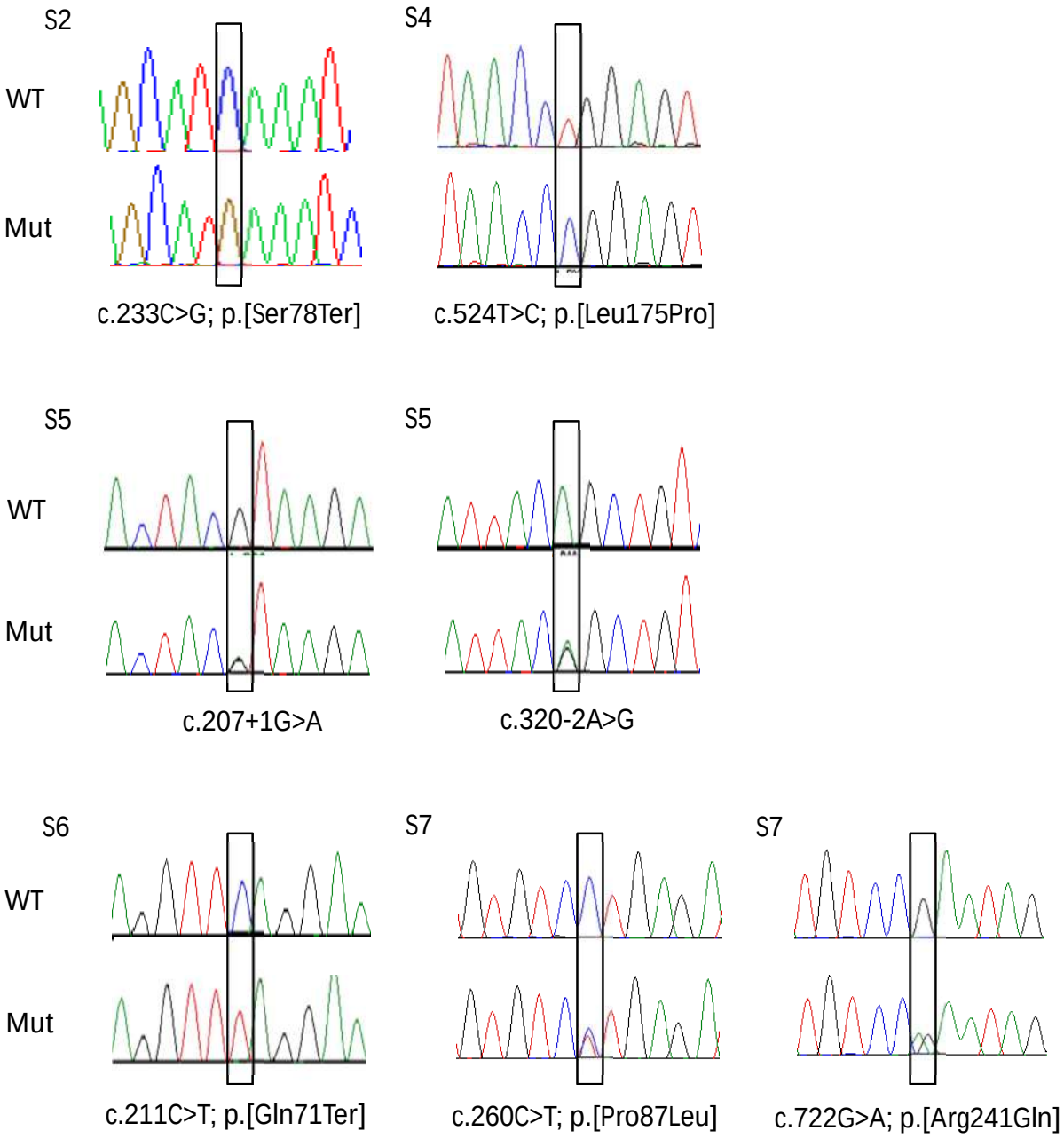


Figure S1. Sequence Chromatograms. For each affected subject, the top chromatogram shows the wild-type (WT) *PROSC* sequence and the chromatogram below the *PROSC* mutation (Mut) identified in the affected individual. Parental studies for Subjects 1-3, 4, and 6 demonstrated that both parents are heterozygous carriers of the identified mutation. Parental studies for Subject 5 revealed that c.207+1G>A and c.320-2A>G were carried on the maternal and paternal allele, respectively. In the case of Subject 7, c.260C>T and c.722G>A were carried on the maternal and paternal allele, respectively. S = subject.

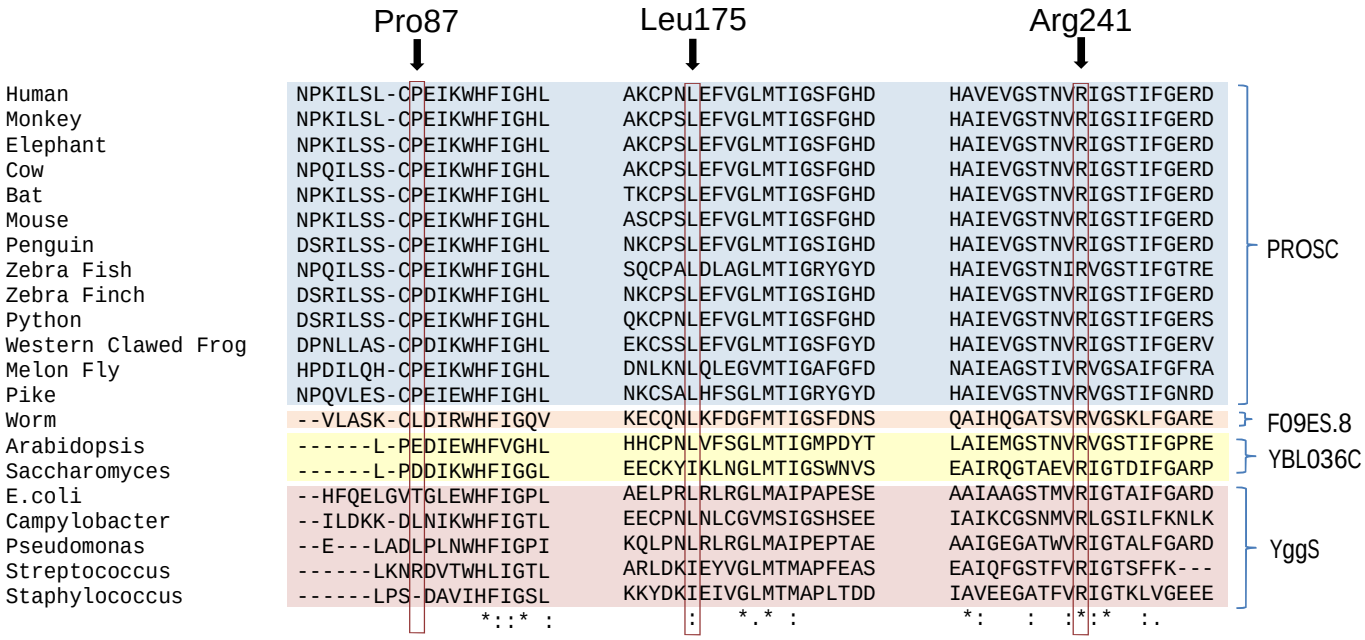


Figure S2. Sequence alignment of PROSC homologues. Residues identical to human PROSC are marked with an asterisk (*). Conservation between amino acids of strongly and weakly similar properties is indicated by a colon (:) and a period (.), respectively. Amino acids affected in humans by missense mutations are highlighted by a red box. Leu175 and Arg241 are conserved across PROSC and other members of the COG03.25 family (F09ES.8, YBL036C and YggS). Pro87 is only conserved across PROSC. PROSC is highly conserved among divergent species. The overall sequence identity for the entire amino acid sequence between; human and mouse is 86.5 %, human and E. coli is 35.9 %, human and Saccharomyces is 40.3 %. PROSC (higher organisms), F09ES.8 (worm), YBL036C (arabidopsis and yeast) and YggS (bacteria) sequences are highlighted in blue, orange, yellow and pink, respectively.

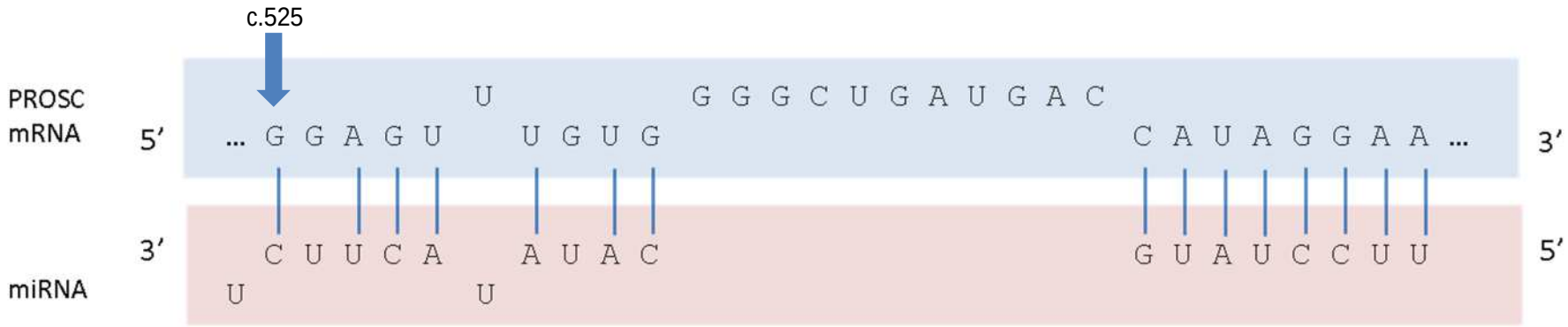


Figure S3. Leu175 is Part of the microRNA Binding Site of hsa-miR-202-5p. Western blot analysis of mutant p.Leu175Pro PROSC (Subject 4) revealed that no protein was detectable for this variant. Prediction analysis of *PROSC* using the MicroT-CDS web based programme (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) (Paraskevopoulou *et al.*, 2013) revealed that p.Leu175 (c.524T) is involved in an 8mer binding site at position 524-552 in the coding sequence of *PROSC* to which the microRNA (miRNA) hsa-miR-202-5p binds. miRNA sites located in the coding sequence (CDS) have been shown to be effective in inducing translation inhibition. Generally imperfect complementarity between miRNAs and their target sites occurs with extensive mismatched bulges especially in the central region (Standart & Jackson, 2007) as depicted. Leu175Pro may enhance the affinity of the *PROSC* transcript for the hsa-miR-202-5p (Amato *et al.*, 2013). MicroRNA (miRNA) sequence is in pink, *PROSC* mRNA target sites in blue. Position of miRNA binding site on chromosome 8:37632927-376632955.

Figure S4. Predicted PROSC Amino Acid Sequence for cDNA Splice Variants Detected in Subject 5. cDNA was generated from RNA extracted from fibroblasts of control individuals and Subject 5. Figure 3 in the main text shows that only one product (846bp) was evident for the control samples whilst three bands (923bp, 827bp and 816bp cDNA products) were evident for Subject 5. The products were cloned into TOPO-2.1 and sequenced. The amino acid sequences shown were predicted from the nucleotide sequences obtained. Protein P5.1 (corresponding to the 923bp cDNA band) and P5.3 (816bp band) were shown to originate from c.207+1G>A and P5.2 (827bp band) from c.320-2A>G. Alternating exons highlighted in black and blue. Amino acid highlighted in red indicates residue overlap splice site. Amino acids depicted in green highlight the consequence of c.207+1G>A. The predicted molecular masses of wild type and Subject 5 PROSC products are as indicated in parentheses. Immunogen sequence of antibody (used in Fig 3b in the paper) highlighted in wild-type sequence (bold/underlined). The antibody would not be expected to detect P5.1 (8.1 kDa) as the antibody site has been deleted. It also does not detect P5.2 (29.2 kDa) probably because the first nine amino acids of the recognition sequence have been deleted (Fig 3b). The 26.4 kDa product is however visible (Fig 3b) as expected. An alternative antibody was used that targeted the N' terminus of the protein however this was found to be very nonspecific.

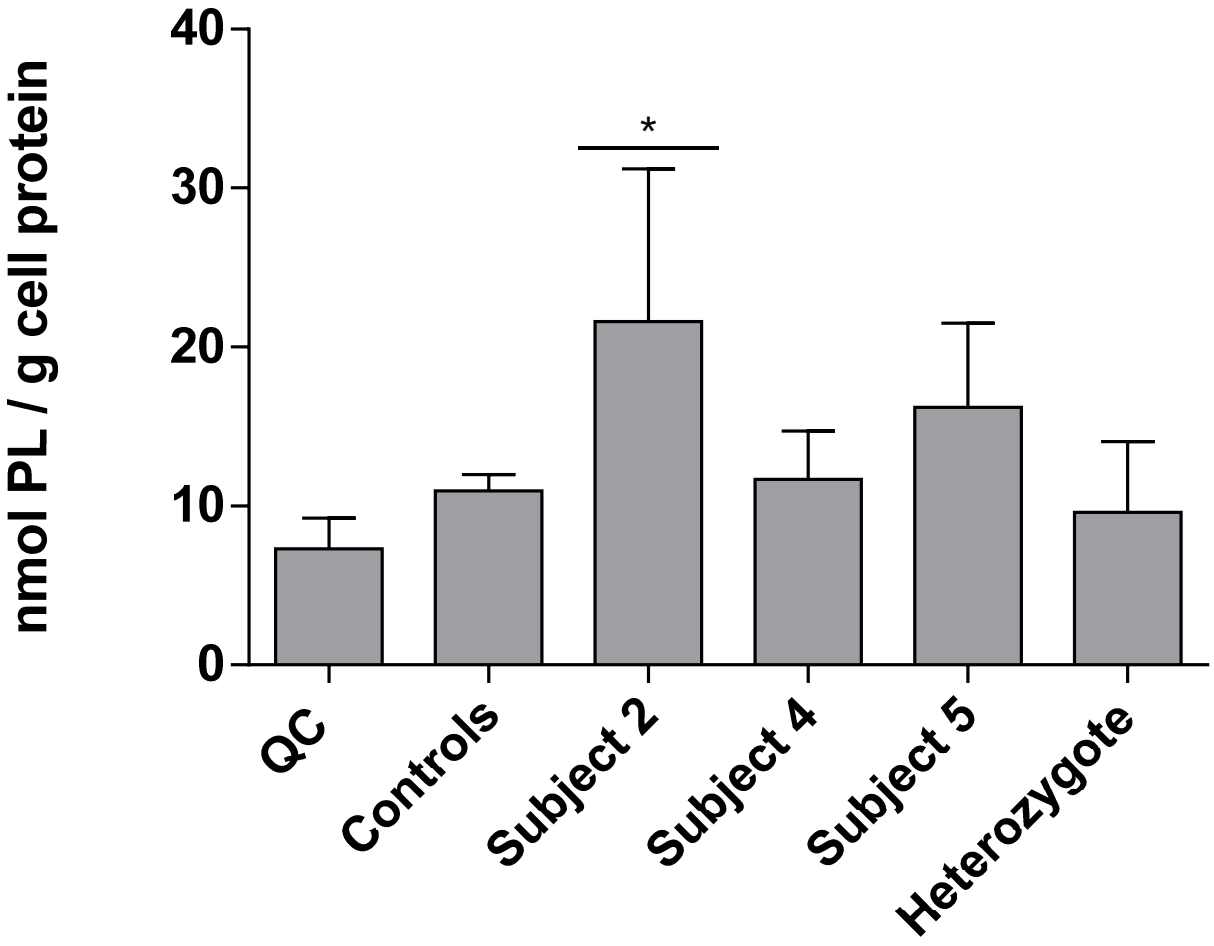


Figure S5. Quantification of Total Pyridoxal (PL) in Fibroblasts from Controls and Subjects 2, 4 and 5 and from the Mother of Subject 2 (Heterozygous for Ser78Ter). PL was analysed by LC-MS/MS, quantified relative to a known amount of internal standard [D_3-PL] and expressed as a ratio to cell protein. Data are presented as means ± 1 s.d.. Statistical analysis was performed using Student's two tailed t test; Subject 2 is statistically different to controls (p=0.0234). No statistical difference between Controls and Subjects 4 and 5 and Heterozygote. QC = quality control sample

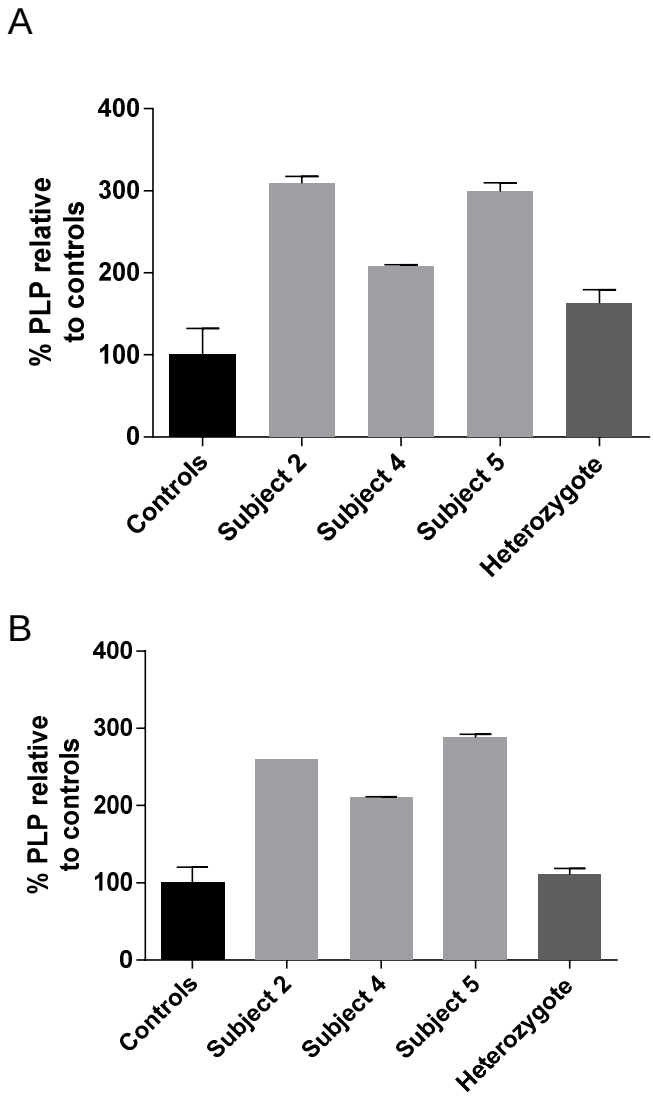


Figure S6. Amount of PLP Present in Low and High Molecular Weight Fractions of Control and PROSC-Deficient Fibroblasts.

(A) <3 kDa fraction and (B) > 3 kDa fraction. Controls; duplicate measurements of n= 3. Samples from subjects with PROSC deficiency; duplicate measurements of n = 2. Error bars show SEM. PROSC-deficient cells significantly different to controls; $p < 0.001$.

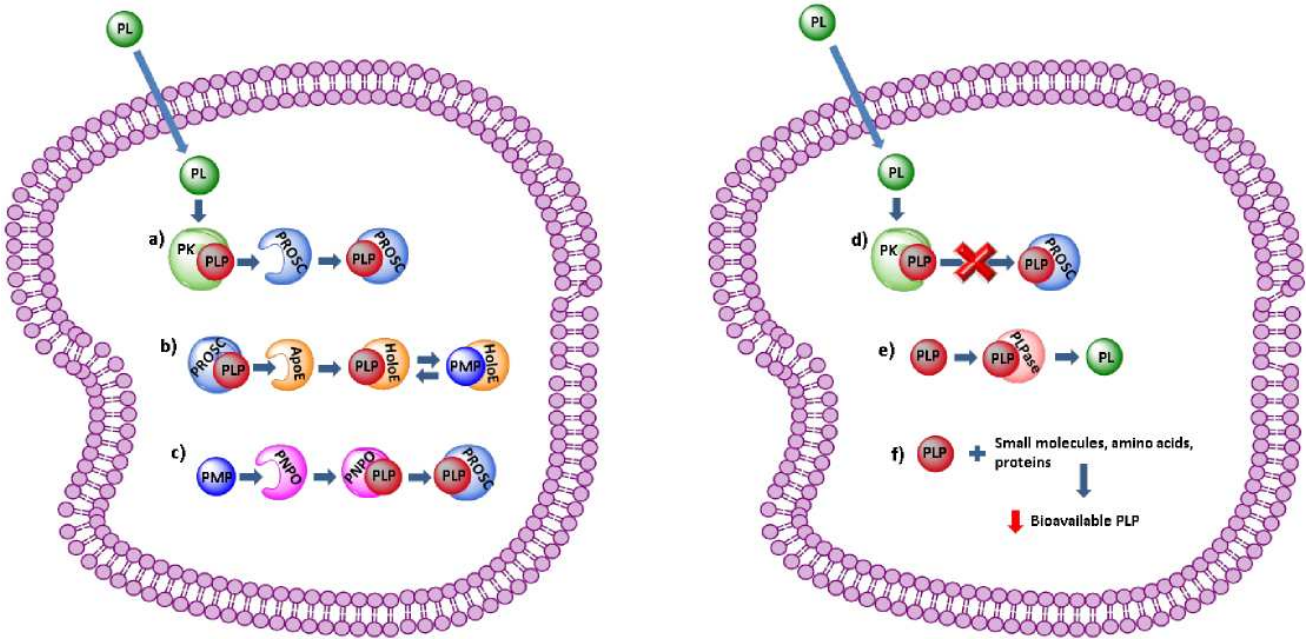


Figure S7. **PROSC Protects PLP and Regulates Intracellular PLP Concentrations**

Proposed disease mechanism. Pyridoxal (PL) enters the cell and is (a) phosphorylated by pyridoxal kinase and PLP is transferred to the PLP-binding site within the β-barrel of PROSC thereby maintaining free PLP at low concentrations preventing inappropriate reactions with other reactive molecules and protecting it from dephosphorylation by intracellular phosphatases. (b) PROSC transfers PLP to B_6-dependent apo-enzymes resulting in formation of a functional holo-enzyme. During the enzyme reaction catalysed by the B_6-dependent holoenzyme PMP may be formed and released (c) PNPO oxidises PMP to PLP prior to transferring it to PROSC. (d) When PROSC is dysfunctional (indicated by a red X) it cannot protect PLP resulting in (e) PLP being de-phosphorylated by endogenous cellular phosphatases and the amount of PL increasing or (f) PLP reacting with small molecules and proteins other than PLP apo-enzymes. Whilst the amount of total bound PLP in the cell is high when supplemented with supraphysiological concentrations of B_6, under normal conditions the amount of PLP available to B_6 apo-enzymes is insufficient. In PROSC-deficient individuals, this presents as B_6-dependent epilepsy.

Primer	Sequence (5' → 3')	Amplicon size (bp)
PROSCEx1F	AGCTAAGCGCGAGAAGGCGCTCCATTTCCAGGTTCG	379
PROSCEx1R	GCTTTACCGCTCAACCGTTCGTGACGACCCATTCCC	
PROSCEx2F	AGCTAAGCGCGAGAAGGCCAAGTTGAGATCAATGCCTGTC	410
PROSCEx3R	GCTTTACCGCTCAACCGTTACAACTCAACCACCAGGATG	
PROSCEx4F	AGCTAAGCGCGAGAAGGCCAGAGAGTGGATGTGAAAGGTG	364
PROSCEx4R	GCTTTACCGCTCAACCGTTTGGTGAATTCATTCTGTTCTTG	
PROSCEx5F	AGCTAAGCGCGAGAAGGCTGTTTTTCTTTTCCTGGCATA	370
PROSCEx5R	GCTTTACCGCTCAACCGTTGGCCTGTGATCCTGTGATTT	
PROSCEx6F	AGCTAAGCGCGAGAAGGCGTTCTCTTGTTGGGGGTCAC	361
PROSCEx6R	GCTTTACCGCTCAACCGTTAGGCAGTGTCTGGGATGACT	
PROSCEx7F	AGCTAAGCGCGAGAAGGCAACCGTTGTCAGAGAAGTTCAG	285
PROSCEx7R	GCTTTACCGCTCAACCGTTTTCCAAGGCTTCTACAGCAAAG	
PROSCEx8F	AGCTAAGCGCGAGAAGGCCCCGTCCATAGAAGGCTC	664
PROSCEx8R	GCTTTACCGCTCAACCGTTGACGACGTAGTAGGAGTTTGTAGG	
ForwardSeq	AGCTAAGCGCGAGAAGGC	na
ReverseSeq	GCTTTACCGCTCAACCGTT	na

Table S1. Tailed Primer Sequences and PCR Conditions Used for the Amplification of Human *PROSC*. ID of transcript amplified ENST00000328195. Tailed primers were used to facilitate downstream sequencing. 'Tail' sequence is highlighted in red and was incorporated into the PCR product during amplification. PCR was performed using Taq DNA polymerase (Life Technologies) and 1.5 mM $MgCl_2$. Exons 2 and 3 were amplified as a single amplicon. Cycling conditions: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, annealing temperature 58°C for 30 seconds, 72°C for 30 seconds; final cycle 72°C for 10 minutes. PCR products were sequenced using primers based on the 'tails' that had been incorporated (called Forward Seq and ReverseSeq). na = not applicable

Primer	Sequence (5' → 3')	Amplicon size (bp)
PROSCcDNAF	GGGGATGTGGAGAGCTGG	379
PROSCcDNAR	CAGTATTCCCTGGCTCAGTG	

Table S2. Primer Sequences and Conditions Used for Amplification of Full-length Human *PROSC* cDNA. Cycling conditions were 96°C for 2 minutes, followed by 35 cycles of 20 seconds at 95°C, 10 seconds at 50°C, 3 minutes at 60°C.

Primer	Sequence (5' → 3')
PROSCcDNA1F	GCTAGTGGCGGTCAGCAAAACC
PROSCcDNA2F	GGTTAAAGGTTATGGTCCAGAT
PROSCcDNA3F	GGACAAGTGCGCAGCAGACGTGAA
PROSCcDNA4F	GGGCTGGATGGCTGGGAGATCC
PROSCcDNA5F	AGCATGAAGAGATTGGGGACAGCC
PROSCcDNA6F	CATGCCCATGCTCAGCTCAACC
PROSCcDNA7F	CCATTGCCTGAAGCAAGCTTCC

Table S3. Primer Sequences and Conditions Used for Sequencing of Full-length Plasmidic Human *PROSC* cDNA. Sequencing reaction conditions were 96°C for 2 min, followed by 35 cycles of 20 sec at 96°C, 10 sec at 50°C, 3 min at 60°C.

Time (min)	Mobile Phase A (3.7% Acetic Acid & 0.01%HFBA)	Mobile Phase B (100% MeOH)	Flow Rate (ml/min)	Curve
0.00	97.5	2.5	0.4	0
0.40	97.5	2.5	0.4	0
3.75	50.0	50.0	0.4	6
3.76	0.1	99.9	0.4	11
4.26	97.5	2.5	0.4	1
5.00	97.5	2.5	0.4	1

Table S4. Mobile Phase Composition and HPLC Gradient Profile for the Separation of B_6 Vitamers on a HSS T3 Column

Analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Cone Voltage (V)	Collision Energy (V)
PMP	0.87	249.04	134.05	27	22
PNP	1.00	250.04	134.04	27	16
PLP	1.00	248.00	150.01	27	16
D_2-PLP	1.00	250.00	152.01	27	16
PA	1.09	184.06	147.99	18	18
D_2-PA	1.09	186.06	149.99	18	18
PL	1.12	168.10	150.50	21	12
D_3-PL	1.12	171.10	153.05	21	12
PM	1.12	169.12	134.04	22	20
D_3-PM	1.12	172.12	137.04	22	20
PN	1.36	170.09	134.04	27	19
D_2-PN	1.36	172.09	136.04	27	19

Table S5. The Mass Spectral Specifications for Detection of B_6 Vitamers in Plasma, CSF and Fibroblasts and their Deuterated Internal Standards. All are detected in positive ion mode.

Primer	Sequence (5' → 3')
PROSCSer78XF	CAGGAACTGCTAGAAAAAGCATGAAATCCCAAAATTCTGTC
PROSCSer78XR	GACAGAATTTTGGGATTTCATGCTTTTTCTAGCAGTTCCTG
ProsC_L175P_FW	CCAAGTGTCCTAACCCGGAGTTTGTGGGGCTG
ProsC_L175P_RV	CGTTTATGTGCTCCACGATGGCTATGGTCTCT
ProsC_R241Q_FW	GATCTACAAATGTCCAAATAGGAAGCACGAT
ProsC_R241Q_RV	CTACTTCAACCGCATGCTGGAAA
ProsC_P87L_FW	ATTCTGTCTTTGTGTCTTGAGATCAAATGGCAC
ProsC_P87L_RV	TTTGGGATTTGATGCTTTTTCTAGCAGTTCC

Table S6. Primers used for Site-directed Mutagenesis to Generate *PROSC* Missense and Nonsense Mutations.

gene	Genomic position on chrosome 8	ref/ alt allele	dbSNP number	Predicted effect on protein
NKX3-1				
NKX2-6				
STC1				
ADAM28	24211331	G/A	rs7814768	missense
ADAMDEC1	24261526	A/G	rs3765124	missense
ADAM7				
NEFM				
NEFL				
DOCK5	25234858	A/G	rs2659585	missense
GNRH1	25280706	G/G	rs35542850	missense
KCTD9				
CDCA2	25364331	G/A	rs4872318	missense
EBF2				
PPP2R2A				
BNIP3L				
PNMA2				
DPYSL2				
ADRA1A				
STMN4				
TRIM35				
PTK2B	27308585	A/C	rs751019	missense
CHRNA2				
EPHX2				
CLU				
CCDC25				
SCARA3				
ESCO2				
SCARA5				
PBK				
NUGGC	27918057	T/C	rs7817227	missense
NUGGC	27925185	G/A	rs74462442	missense
NUGGC	27927849	C/G	rs6998705	missense
ELP3				
PNOC				
FBXO16				
FZD3				
ZNF395				
EXTL3				
INTS9				
HMBOX1				
KIFI3B				
DUSP4				
SARAF				
LEPROTL1				
MBOAT4				
DCTN6	30034655	A/G	Not reported previously	missense
RBPMS				
GTF2E2				
GSR				
SMIM18				
UBXN8	30608976	C/T	rs2911690	missense
PPP2CB				

TEX15				
PURG				
WRN				
NRG1	32453358	G/A	rs3924999	missense
NRG1	32613983	T/C	rs10503929	missense
FUT10				
MAK16	33356074	A/G	rs6468171	missense
TTI2	33369944	T/C	rs2304748	missense
DUSP26				
RNF122				
UNC5D				
KCNU1	36767024	T/C	rs28608091	missense
ZNF703				
ERLIN2				
ADGRA2				
PROSC	37623254	C/G	Not reported previously	Stop-gain
RAB11FIP1	37728017	A/G	rs7817179	missense
GOT1L1				
BRF2				
ADRB3				
EIF4EBP1				
STAR				
LSM1				
ASH2L				
BAG4				
DDHD2				
PLPP5				
WHSC1L1				

Table S7. Protein-coding Genes in the Homozygous Region at Chromosome 8p21.2-p11.23 (bp 23,451,893-38,236,890 GRCh37/hg19) Identified by Homozygosity Mapping and Single Nucleotide Variants (SNVs) identified by Whole Exome Sequencing Shared Between Subjects 2 and 3. All SNVs identified in the region described were homozygous. Only non-synonymous coding variants are described.

			CSF amino acid concentration (μM)			Plasma amino acid concentration (μM)		
Amino acid	Substrate of Enzyme	PLP-dependent reaction	Subject 4 (Age 6 d)	Subject 5 (Age 21 d)	Subject 5 (Age 17 m)	Subject 4 (Age 1 d)	Subject 5 (Age 21 d)	Subject 5 (Age 5 m)
			B_6*	No B_6	No B_6	No B_6	No B_6	No B_6
Alanine	Alanine aminotransferase	Yes[1]	Not available	**49.3** (28.5-38.0)	**48.6** (23.0-39.5)	386 (112 – 686)	395 (131-460)	**524** (100-439)
Valine[2]	Branched chain amino acid transaminase	Yes	Not available	**32.3** (18.2-25.0)	19.6 (14.8-22.5)	110 (96 – 566)	**185** (80-120)	139 (60-294)
Isoleucine[2]	Branched chain amino acid transaminase	Yes	Not available	**19.8** (5.1-11.2)	8.9 (4.6-8.6)	**20** (26-159)	55 (26-80)	40 (28-95)
Leucine[2]	Branched chain amino acid transaminase	Yes	Not available	**26.7** (14.1-22.9)	**8.2** (11.0-18.7)	59 (50 – 264)	126 (46-160)	56 (45-160)
Glycine	Glycine cleavage enzyme	Yes	**19** (2-15)	**11.0** (5.9-8.5)	**48.6** (23.0-39.5)	**590** (81-303)	284 (224-514)	264 (60-380)
Phenylalanine[2]	Phenylalanine hydroxlase	No[3]	Not available	14.9 (8.3-15.9)	**15.0** (7.0-13.2)	60 (34 – 110)	65 (38-120)	56 (23-75)
Proline	Proline Oxidase	No[1]	Not available	**4.8** (0-0.1)	0 (0-0.1)	**523** (45-452)	249 (107-330)	154 (50-298)

Table S8. Abnormal Plasma and CSF Amino Acid Results of Samples from Subjects with *PROSC* Mutations and PLP Requirements of Enzymes Involved in their Metabolism [1]Raised alanine and proline could also be a consequence of mitochondrial dysfunction. Blood lactate was elevated in the neonatal period in 3/5 subjects and MRS of subject 5 at 7 months showed that there was persistence of lactate accumulation in the brain. [2]Essential amino acids; increases seen may reflect dietary intake and not necessarily the lack of availability of PLP [3]Product of reaction is tyrosine which is involved in a PLP-dependent reaction *Sample taken after subject had been supplemented with B_6 for 2 days; d = days; m = months. Values highlighted in bold are above and below the age-related reference range. Age related reference range given in parentheses.

	Tyrosine concentration (μM)	
Age	CSF	Plasma
2.5 m	**49** (1 - 22)	nm
4 m	**29** (1 - 22)	nm
7 m	**27** (1 - 22)	nm
17 m	11 (4 - 12)	46 (50 -125)
5 y	nm	**166** (50 - 125)

Table S9. Tyrosine Concentrations in CSF and Plasma for Subject 2 whilst Receiving B_6 Supplementation

m = months; y = years; nm = not measured. Elevated tyrosine levels not evident in samples analysed prior to supplementation with vitamin B_6. Values highlighted in bold above the age-related reference range. Age related reference range given in parentheses.

Age	Homocarnosine concentration (μM)
21 d	**0** (3.9 - 7.7)
17 m	**0** (4.3 - 10.4)

Table S10. Homocarnosine Concentrations in CSF for Subject 5 whilst not Receiving B_6 Supplementation

d = days; m = months. Abnormal homocarnosine levels highlighted in bold. Age related reference range given in parentheses. Homocarnosine was not measured in other PROSC-deficient subjects.

	Age	B_6 [dose]	PLP	PL	PA	PN	PNP	PMP	PM
Control range*		None	15 - 75	nm	nm	nm	nm	nm	nm
Subject 2*	1 d**	PN [50 mg BD]	**174**	nm	nm	nm	nm	nm	nm
	1 w	PN [50 mg BD]	**> 300**	nm	nm	nm	nm	nm	nm
	11 m	PN [150 mg BD]	**4371**	nm	nm	nm	nm	nm	nm
Control range*		None	7 - 233	8 - 129	4 - 171	nd	nm	nd	nd
Subject 5*	2 y 7 m	PLP [36 mg/kg/day]	**1030**	**2280**	**5020**	nd	nm	nd	nd
Control range***	4.3 y – 16 y	None	46 - 321	5 - 18	16 - 139	nd - 0.6	nd	nd - 9	nd
Subject 2	6 y	PN [200 mg BD]	**2626**	**2559**	**5522**	**2**	nd	nd	nd
Subject 3	3 y	PLP [70 mg QDS]	**2769**	**796**	**2043**	0.5	nd	nd	nd
Subject 4	6 m	PLP [45 mg/kg/day]	**2166**	**1695**	**700**	nd	nd	nd	nd
PNPO****	2 y	PLP [30 mg/kg/d]	**580**	**427**	**793**	**575**	**43**	**18**	**193**
PNPO****	10 y	PLP [30 mg/kg/d]	**633**	**5798**	**7926**	**599**	**77**	**101**	**2731**
PDE****	10 y	PN [100 mg BD]	**24**	**6351**	**5904**	**60**	nd	**15**	**108**
PDE****	12 y	PN [100 mg BD]	**11**	**6476**	**5069**	**7**	nd	**24**	**144**
PDE****	8 y	PN [100 mg BD]	**588**	**198**	**239**	0.4	nd	nd	nd
PDE****	8 y	PN [100 mg BD]	**603**	**203**	**321**	0.4	nd	nd	nd

Table S11. Plasma B_6 Vitamer Profiles for Subjects with PROSC Deficiency, PNPO Deficiency and Pyridoxine Dependent Epilepsy due to Mutations in *ALDH7A1* (PDE) nd; not detected, nm; not measured. PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine; PLP, pyridoxal 5'-phosphate; PNP, pyridoxine 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PA, 4-pyridoxic acid. All units nmol/L, except for PNP which is stated in 'concentration units'. Values outside the reference range are shown in bold. BD = twice a day; QDS = four times a day. *Samples measured in local laboratory; **received PN prophylactically; ***Samples measured at UCL using method and reference range as described in Footitt *et al.*, 2013; ****Data from Footitt *et al.*, 2013. Comparisons between PROSC deficient subjects and individuals with PNPO and PDE deficiencies receiving comparable supraphysiological doses of B_6 was only made for individuals measured in the laboratories at UCL and the National Hospital, London; these labs partook in a quality control scheme.

Subject	Alkaline Phosphatase activity (U/L)	WES analysis
2	394 (70 - 282); 171 (129 - 506)	yes; no variants detected
3	-	yes; no variants detected
4	233 (137 - 264)	not performed
5	156 (35 - 398); 358 (122 - 334); 351 (122 - 334); 194 (105 - 310); 171 (105 - 310)	not performed
6	207 (35 - 398); 308 (122 - 334); 334 (122 - 334)	not performed
7	166 (150 - 300); 147 (150 - 300)	not performed

Table S12. No Evidence of Hypophosphatasia Due to Mutations in Tissue Non-Specific Alkaline Phosphatase in Subjects with PROSC Deficiency Relevant age-related range for the individual subjects in parentheses.

	1mg/ml pyridoxine		0.1mg/ml pyridoxine	
	1st zone of inhibition (cm)	2nd zone of inhibition (cm)	1st zone of inhibition (cm)	2nd zone of inhibition (cm)
Δ*yggS*	2.25 ± 0.10 *(n=3)*	2.60 ± 0.10 *(n=3)*	1.88 ± 0.20 *(n=3)*	0.78 ± 1.36 *(n=3)*
Wild-type *PROSC*	No zone *(n=3)*	No zone *(n=3)*	No zone *(n=3)*	No zone *(n=3)*
Ser78Ter	2.29 ± 0.11 *(n=4)*	2.58 ± 0.10 *(n=4)*	1.89 ± 0.16 *(n=4)*	2.23 ± 0.19 *(n=3)*
Leu175Pro	2.22 ± 0.22 *(n=6)*	2.45 ± 0.28 *(n=5)*	1.92 ± 0.12 *(n=5)*	2.30 ± 0.18 *(n=4)*
Pro87Leu	No zone *(n=3)*	No zone *(n=3)*	No zone *(n=3)*	No zone *(n=3)*
Arg241Gln[#]	4.45 ± 0.43 *(n=6)*	see legend	3.53 ± 0.44 *(n=6)*	see legend

Table S13. Quantification of Zone of Inhibition (Inner Radius) and Zone of Inhibition (Outer Radius) for *E.coli* Complementation Studies

The sensitivity to pyridoxine is defined as the distance from the disc, which has been impregnated with 20 μL of pyridoxine, on the media where the Δ*yggS* strain does not grow. 1mg/ml pyridoxine; disc a in Figure 6; 0.1mg/ml pyridoxine; disc b in Figure 6. n = number of replicates in parentheses; [#] only 1 circle of inhibition observed after complementation of Δ*yggS* with Arg241Gln. No circle of inhibition was evident for Pro87Leu and wild-type PROSC as they were able to complement the Δ*yggS* background and prevent the pyridoxine sensitivity at both concentrations of pyridoxine used.

SUPPLEMENTAL REFERENCES

Amato F, Seia M, Giordano S, Elce A, Zarrilli F, Castaldo G, Tomaiuolo R. Gene mutation in microRNA target sites of CFTR gene: a novel pathogenetic mechanism in cystic fibrosis? *PLoS One.* **8**(3):e60448 (2013).

Footitt EJ, Clayton PT, Mills K, Heales SJ, Neergheen V, Oppenheim M, Mills PB. Measurement of plasma B6 vitamer profiles in children with inborn errors of vitamin B6 metabolism using an LC-MS/MS method. *J Inherit Metab Dis.* **36**, 139-145 (2013).

Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, Filippidis C, Dalamagas T, Hatzigeorgiou AG. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res. 41(Web Server issue):W169-73 (2013).

Standart N, Jackson RJ. MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. *Genes Dev.* **21**, 1975-1982 (2007).