Supplementary material

Clinical Patient Descriptions

note: MRI findings detailed in Fig. 1 and Supplementary Table 1

Case vignettes with phenotypic features not previously described

Fatal mitochondrial encephalopathy

Patient 4

This girl of Dutch non-consanguineous origin, who died at 2 weeks of age, was the first of DCDA twins, born via spontaneous vaginal delivery at 36+6 weeks gestation (birth weight: 2215 g (10^{th} percentile), head circumference 30.7 cm (-2.5 SD corrected for gestational age) and APGAR 6/7/8). The family history was negative for epilepsy or developmental problems. Her twin sib is healthy with normal development. Directly postpartum, spontaneous breathing was insufficient, requiring assisted ventilation for 5 minutes followed by CPAP with oxygen. Spontaneous ventilation was restored. Glucose was 4.5 mmol/L but rapidly decreased to <0.6 mmol/L one hour postpartum and remained between 0.8 and 1.8 for four hours despite extreme supplementation dosages; at 6 hours postpartum normoglycemia was first reached (6.6 mmol/L). Initial lactate was 19 mmol/L, CK 3137 U/L (normalized to 232 U/L at day 9). A brain ultrasound on postnatal day 1 showed a dilation of the ventricular system, intraventricular bulkheads, an abnormal gyral pattern and abnormal white matter.

Neurologically the girl showed strong motor unrest and progressive axial hyperextension upon light touch. Seizures probably started at day 1 postnatally (but could not be proven by EEG at that time) and escalated at day 3 (irritability, nystagmus, tonic spasms of the face, thorax and arms, later on also tachycardia, hypertension, apneas, and desaturations, followed by crying and grimacing). The first EEG (postnatal day 1) showed a diffusely abnormal and excessively discontinuous pattern. Upon external stimulation, there was sharp polymorphic and asynchronous activity in the central areas and sometimes more generalized. EEGs on day 4 and 7 displayed a similar background, progressively frequent BIRDS (brief ictal rhythmic discharges) and progressive episodes of rhythmic sharp activity compatible with electrographic neonatal seizures, sometimes without clinical correlate. Over the course of two weeks several AEDs were trialed, all leading to unsustained (minutes to one hour) and only partial seizure control. Because of the severe structural brain abnormalities on ultrasound and MRI, pyridoxine treatment was not considered. Phenobarbital loading doses up to 40 mg/kg were followed by 5 mg/kg/day. Levetiracetam was given twice daily (60 mg/kg) after 2 loading dosages of 20 mg/kg. Continuous intravenous midazolam (0.8 mg/kg/hour) with several additional loading doses had a similar effect. Finally, repeated doses of oral chloral hydrate (50 mg/kg) accomplished a good clinical response for

several hours. However, seizures became intractable and on day 16, after elevating midazolam and adding morphine for comfort control the girl died of respiratory depression and bradycardia. Permission for restricted brain autopsy was granted by the parents (only one slice). Histopathological examination showed focal abnormalities, mainly of the white matter consistent with hypoxic-ischemic injury. WES open exome trio-analysis came back negative at first. However, re-analysis of open exome data revealed a compound heterozygous mutation in *PLPBP*.

Patient 5

This female neonate was the first child to consanguineous parents (second cousins) of Cree First Nation ancestry. The pregnancy was unremarkable, but a Caesarean delivery was performed at term due to a non-reassuring fetal heart rate. Her birthweight was 3470 g (76^{th} percentile), head circumference was 35 cm (82^{nd} percentile) and height was 47cm (14^{th} percentile). The child briefly (10-15 seconds) received positive pressure ventilation for poor respiratory effort, being initially stable. Over the first hours of life, she developed progressive respiratory failure requiring intubation and transfer to a tertiary care NICU.
The admitting diagnosis was suspected birth asphyxia. Her neurological examination was notable for hypertonia, hyperreflexia, and abnormal movements (persistent flexion and clenching of her upper extremities). Clinical seizures were noted on the first day of life; EEG was markedly abnormal with a burst-suppression pattern, and she was given a neurological diagnosis of early infantile epileptic encephalopathy (Ohtahara syndrome). Routine lab studies were notable for a persistently increased lactate level in blood (range 1.5 - 11.2 mmol/L) and cerebrospinal fluid (5.6 mmol/L).

The patient was successfully extubated post-transport, however her seizures proved to be refractory. Seizures were managed, to the extent possible, with an intravenous midazolam infusion (150 µg/kg/hour), followed by an escalating series of up to six simultaneous anticonvulsant agents, and high-dose prednisone. Empiric therapy with biotin and thiamine produced no obvious benefit (pyridoxine was not tried). Seizures and apneic episodes persisted, becoming increasingly frequent despite these treatments. At eight weeks of age, she acutely deteriorated with recurrent apneas, acute renal failure, and hemodynamic compromise, and care was recognized to be futile, and withdrawn.

The patient's clinical presentation and imaging were considered most consistent with a mitochondrial disorder. Plasma amino acids were notable for hyperglycinemia (943-1010 µmol/L; reference interval 81-436) with corresponding high glycine in CSF (28 µmol/L; reference interval 3-23); of note, alanine and proline concentrations in blood and CSF were normal. Acylcarnitine profile was normal. Urine organic acids showed increased excretion of lactic, pyruvic, and 2-hydroxybutyric acids, consistent with lactic

acidosis. Muscle biopsy was refused; cultured skin fibroblasts showed an elevated lactate-to-pyruvate ratio (41.7 +/- 7.13; reference interval 9.6-26.5), normal activities of several enzymes (pyruvate dehydrogenase (PDH) native: 0.94±0.06nmoles/min/mg protein, ref 0.46-1.60; PDH: 1.32±0.06nmoles/min/mg protein, ref 0.87-2.33; pyruvate carboxylase: 0.51, ref 0.35-5.18; and respiratory complexes II-IV), and normal mitochondrial morphology and inner membrane potential. Extracellular flux testing showed an apparent reduction of carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP)-stimulated spare respiratory capacity.

Genetic investigations which were normal or inconclusive in the patient included oligonucleotide microarray, mtDNA point mutation panel, and an NGS-based nuclear mitochondrial gene panel (Mitome200, Baylor College). She was enrolled into a local research program and an NGS-based panel of 4,813 genes associated with any clinical phenotype (Illumina TruSight One) was performed and negative. Whole-exome sequencing of the proband and both parents was performed as described (Beaulieu *et al.*, 2014; Hamilton *et al.*, 2016), identifying a homozygous frameshift mutation in NM_007198(*PLPBP*):c.370_373del (p.Asp124Lysfs*2). Absence of PLPHP protein expression was confirmed by immunoblot of fibroblast lysates from the patient and controls (Supplementary Fig. 1).

Movement disorder without epilepsy

Patient 7

This boy, currently 23 months old, was born at term to non-consanguineous parents who originate from a small town in Guatemala. He was born with bilateral syndactyly of the third and fourth fingers. His birth weight was 3.317 kg (47.6th percentile, Z = -0.06), head circumference was 33 cm (12.5th percentile, Z = -1.15) and APGAR 9, 9. He initially presented at 2 months of age for abnormal movements: flexor posturing with arm abduction, tonic posturing of his UE and LE with internal rotation of his arms, jerking of the left arm and upward eye deviation with each event. There were no recognized triggers of these events, and they clustered for minutes to over an hour. He did not present with clear epilepsy. His initial routine EEG was read as disorganized background and bursts of higher-amplitude activity, with several spike and slow wave complexes followed by electro-decrement with clinical correlate of subtle twitch. Although this EEG did not meet criteria for hypsarrhythmia, there was high clinical suspicion for infantile spasms with emerging hypsarrhythmia on EEG; he was thus treated for infantile spasms with high dose prednisolone. CSF analysis of cell count, chemistry and culture were unremarkable.

His parents discontinued prednisolone on the 8th day of treatment due to side effects of irritability,

diarrhea, persistence and worsening of his abnormal movements. An inpatient video EEG captured non-epileptic opisthotonic-like events (back arching, sometimes twisting at the trunk, occasional arm stiffening) and oculogyric crises. These movements did not correlate with electrographic changes on the EEG suggestive of seizures or spasms and were determined non-epileptic. He would become extremely tachycardic with the events. Cardiac and GI workups were negative. His movements did not respond to lorazepam (0.1 mg/kg), though reduced in frequency during levetiracetam treatment (30 mg/kg twice daily), and parenteral hydration. Biochemical labs revealed a profile suggestive of aromatic L-amino acid decarboxylase (AADC) deficiency (see details below) and he was started on recommended treatment for this disorder: PN 50 mg BID, PLP 60 mg TID and Sinemet 0.4 mL TID (approximately 1 mg/kg/day based on levodopa component) at 2.5 months of age with complete resolution of symptoms on this regimen. Once *PLPBP* mutation was identified, Sinemet was later discontinued (at age of 8 months) and he is currently on a mixed regimen of PN (23 mg/kg/day div BID) and PLP (30 mg/kg/day div TID). Following initiation of treatment, all subsequent EEGs have been normal.

Early developmental milestones were achieved within the normal age range; more recently, asymmetric delays were identified. At the age of 20 months, his vision and hearing grossly intact and motor neurological exam was normal: normal bulk, full strength at all extremities at distal and proximal muscles in BUE and BLE, no hypotonia, sits, crawls, walks independently, stands flat footed; sensation: intact grossly at all extremities; coordination: no tremor, reaches for objects with both hands, transfers objects between hands, pincer grasp, using hands equally; reflexes: 2+ bilateral bicep and BR, symmetric brisk 2+ at patella, symmetric 2 at ankles, no clonus. At 23 months of age, significant delays were noted in expressive and receptive language skills with preservation of gross and fine motor development.

Biochemical investigations: Lactate remained normal when checked on hospital readmission (1.7 mM, reference range: <2.0 mM). However, during a third admission, he was confirmed to have elevated lactate (8.46) and metabolic acidosis on VBG with elevated anion gap (23) as well as hyperglycemia (325) in the setting of repeated opisthotonic events. His UA showed 4+ glucose and 1+ ketones. His hyperglycemia and elevated lactate corrected quickly following a NS bolus. CRP and ammonia were normal.

Urine organic acids resulted positive for presence of vanillactic acid, vanilpyruvic acid, and n-acetyl-vanilalanine; also, minor elevations of lactic, malic, 2-ketoglutaric, and n-acetylaspartic acids. This profile is typical of aromatic AADC deficiency, an enzyme necessary for synthesis of neurotransmitters (DA, Epi, NE, 5HT). The rest of the metabolic workup (including plasma amino acids, acylcarnitine profiling) was negative. Confirmatory testing with AADC enzyme assay revealed partial enzymatic activity (18.84 pmol/min/mL), suspicious for carrier status of the condition but not complete AADC enzyme deficiency. Biochemical analysis of CSF at age of 2 months (before B6 treatment) showed

normal levels of glucose (71 mg/dL, reference range: >40 mg/dL) but elevated protein concentration (75 mg/dL, reference range: <45 mg/dL) which normalized after B6 treatment (25 mg/dL at age of 2.5 months). Pre-B6 treatment CSF metabolomics (at age of 2 months) revealed several minor elevations of (Z score): 3-methoxytyrosine (4.2), palmitoyl-GPA 16:0 (3.7), alpha-ketoglutarate (3.2), adenosine (2.6), 2-aminooctanoate (2.6) and tryptophan (2.5).

Clinical whole-exome sequencing (WES) on the proband identified a homozygous variant in the *PLPBP* (c.280 A>T, p.Ile94Phe in exon 4). A homoplasmic variant in MT-ND1 was also described. A dopamine-related disorders gene panel identified a heterozygous pathogenic splice variant in *DBH* (c.339+2T>C). No variants were detected in *DDC*, the gene that encodes AADC. Sinemet (Levodopa/carbidopa) treatment was successfully discontinued after WES resulted, further supporting PLPHP dysfunction, rather than AADC deficiency, as disease-causing.

Folinic acid responsive seizures

Patient 1

This Omani boy, now 3 years and 10 months old, was born to a G3P3 mother. His parents are first cousins, who are healthy with normal learning abilities and there is a family history of similar disease in a younger newly born sibling. Antenatally, the mother experienced increased fetal movements. He was born at term, cried immediately and APGAR scores were 9 / 10. His birth weight was 2.95 kg (10^{th} percentile), length: 49 cm (50^{th} percentile) and HC: 35 cm (50^{th} percentile).

Seizures were first observed on the 5^{th} day of life, presenting with decreased consciousness, uprolling of the eyes and tonic-clonic movements of the body; each episode lasted 10-15 minutes and recurred every few minutes. He then developed myoclonic seizures. He was treated with phenytoin, phenobarbitone and midazolam infusion without clinical response; subsequently he was started on clonazepam and topiramate with initial reduction of seizures but subsequent relapse. His EEG showed burst suppression.

At 5 weeks of age, he was started on oral pyridoxine (25 mg BD) with immediate effect; he was sedated yet hemodynamically stable. All anti-epileptics were discontinued after the first dose of pyridoxine because of excessive sleepiness. He continued to be sleepy (remained sleepy for almost 72 hours) and therefore pyridoxine was withheld for 48 hours then restarted at 5 mg BD with gradual increment to 25 mg BD. EEG was repeated, and it showed marked improvement with no burst suppression on the lower pyridoxine dose. At the age of one year, seizures relapsed in the form of generalized tonic-clonic seizures but were brief (lasting one to two minutes) and infrequent (once or twice per month) and mostly occurred

during febrile illness. He was started on levetiracetam but there was no response, so it was tapered and discontinued.

At the age of 2 years and 4 months, the dose of pyridoxine was increased to 120 mg BD p.o. (= 24 mg/kg/d) that is increased to TID during febrile illness and his seizures were controlled for around 2 months but then he was admitted again with *status epilepticus*. Pyridoxine was thus substituted by PLP starting at a dose of 200 mg TID (= 42 mg/kg/d) which was then increased to PLP 300 mg TID (=58mg/kg/day) with no notable improvement of his seizure control. Subsequently, folinic acid 15 mg BID (= 2mg/kg/day) was added to his PLP regimen, this combination resulted in the best seizure control during his entire course of treatment. He was last seen at the age of 3 years and 11 months in February 2018, where his parents reported marked reduction in the frequency of his seizures. They reported only 2 brief episodes in 3 months period, mainly fever provoked.

He suffered global developmental delay, of a moderate to severe degree: When he was assessed at 2.5 years of age, his developmental age was around 12-18 months. Speech and language developmental age is around 7-8 months of age (he had 4 syllable babbles, had 1 word (unspecific) – Mama-, could not do head shaking for "No," was not able to babble monologs, could know his own name). He is hyperactive and was diagnosed with autism spectrum disorder (ASD). After improving his seizure control following folinic acid supplementation, his development improved, and he started to gain some milestones. He is currently walking without support and steadily and is able to run and climb the stairs. He can say around 10 words with meaning, he obeys commands and can scribble. He has also become less hyperactive. Physical exam revealed no dysmorphic features with biometry on the 50^{th} centile; systemic exam was also unremarkable without organomegaly. Motor neurological examination showed no focal deficits.

Biochemical investigation at the age of 15 months showed high-normal urinary α-aminoadipic semialdehyde (α-AASA, 0.19 µmol/l, reference range: 0-0.2 µmol/l) but plasma pipecolic acid was within the reference range. Antiquitin deficiency was subsequently ruled out by Sanger sequencing of *ALDH7A1*. Plasma amino acids were measured twice and were normal while blood lactate was high-normal (1.7 mmol/l, reference range: 0.5-2.2 mmol/l) (tests were done at the age of 6 weeks).

Whole-exome sequencing performed in this proband identified a pathogenic homozygous missense variant in *PLPBP*: chr8(GRCh37): g.37630300C>T; NM_007198: c.347C>T; p.Thr116Ile. In addition to this variant, this patient was found have another rare variant of unknown significance: chr8(GRCh37): g.37635617C>G; NM_007198: c.823C>G; p.His275Asp (homozygous missense). The variant affects last amino acid in the protein and two of the *in silico* prediction tools described in Supplementary Table 2 proposed a benign/non-functional effect for this variant.

Other cases according to phenotypic severity:

Severe phenotypes

Patient 3

This girl from Curacao Island in the Dutch Antilles (African/Creole descent), now 5 years and 2 months old, was born at 37+5 weeks of gestation after an emergency caesarian section because of fetal distress. She is the first and only living child of possibly consanguineous parents. The pregnancy was complicated by a vanishing twin at 9 weeks of gestation. APGAR scores were 8/9. There was meconium in the amniotic fluid. Umbilical cord blood gas had pH 7.00. Birth weight was 2422 grams (5-10th centile / -1,91 SD), birth length was 47 cm (25th centile/ -1.23SD) and head circumference was 30 cm (<2nd centile / -4,01 SD). Her fontanel was small. After birth, she needed CPAP for breathing difficulties and she had trouble keeping her temperature. She received antibiotics because of suspicion of a perinatal infection. Blood lactate was 13.2 mmol/L (reference range: <2.2 mmol/L); blood gas at day 0: pH 7.15 (normal range: 7.35-7.45); pCO_2 4.2 kPa (normal range: 4.7-6.4 kPa); pO2 7.5 kPa (normal range: 10.0-13.3 kPa); HCO_3 11 mmol/L (normal range: 22-29 mmol/L); and base excess -17.3 mmol/l (normal range: -3 - +3 mmol/L). Lactate was between 4.2 and 8.8 mmol/L on days 1-5 (normal range =<2.2 mmol/L). Blood glucose was normal, creatine kinase was 6593 U/L (normal range: <600 U/L) at day 1 and went down to 460 U/L at day 5.

On day 1, she had clinically evident tonic seizures and an abnormal cerebral function monitoring (CFM)[1]. On the third day she manifested tonic-clonic seizures despite phenobarbitone (20+10 mg/kg), clonazepam (for a seizure, dose not known) and midazolam until 0.2 mg/kg/hour. During the first days she was sometimes quiet but could be hyperkinetic with somewhat shaking movements. She had dysregulation of muscle tone. Head circumference was at -4SD.

She was variable hypo- and hypertonic. EEG at day 5 was in keeping with encephalopathy showing a discontinuous pattern, and a tendency to burst suppression. Epileptiform discharges in the form of sharp waves were frequently observed during the burst but without clear clinical correlate. The liver projected two centimeters below the costal margins.

[1] CFM is a device used for continuous recording and monitoring of fluctuations in the amplitude of EEG, usually using one or two pairs of electrodes. In newborns, it is exploited for detection of seizure activity, tracking the response to AEDs and determining prognosis (Azzopardi D. Clinical applications of cerebral function monitoring in neonates. Semin Fetal Neonatal Med 2015; 20(3): 154-63).

At day 5, PLP was started orally (40 mg 3 dd = 48mg/kg/d) after which the convulsions vanished, and blood lactate started to normalize after day 6 (between 1.6 and 2.9 mmol/L).

At 2.5 months of age there were no clinical signs of epileptic activity, the EEG was normal, and the head circumference had shown catch-up growth to -2.5 SD. The PLP dose was lowered to 20 mg 3dd (15mg/kg/d) because of vomiting. At six months of age the development was still normal. There was a short possibly epileptic episode after which the dose of PLP was adjusted to 40 mg 3dd (16mg/kg/d).

At 10 months of age, she had 15-20 minute long tonic-clonic seizures shortly after stopping the PLP because *PNPO* Sanger analysis was normal as was urine concentration of α-AASA. The next days she had several epileptic insults and the EEG results were slower and less differentiated showing mild encephalopathic changes, but no overt epileptic phenomena. PLP was restarted (40 mg 3dd = 12 mg/kg/day) because of suspicion of a yet undetected pyridoxine/ PLP-responsive epilepsy, and again her clinical condition improved significantly. Levetiracetam was started at 20 mg/kg/day.

At 14 months of age she had an epileptic insult after sleep deprivation. The parents tried to reduce the PLP dose at 18 months of age, but she had another insult after that, so they restarted the medication. The girl has signs of pavor nocturnus from 20 months of age. She has had several epileptic insults that were mostly induced by viral infections/fever or sleep deprivation. At the age of 3 years and 10 months, her B6 therapy was switched to pyridoxine at 100 mg/day in one dose (= 5.9 mg/kg/day), because PN has less severe side effects on the long term. B6 vitamer conversion went smoothly and at the age of 4 years and 2 months, levetiracetam was gradually discontinued. This did not seem to affect frequency of seizures. She had a seizure about once every two months at this time, more severe than on the PLP regimen. Seizures occurred mostly during illness. At the same age (4 years and 2 months), her pyridoxine dose was leveled up to 150 mg/day (100 mg in the morning and 50 mg in the evening, = 8.8 mg/kg/day) and her seizures became less frequent. The dose was adjusted to 100 mg 2dd (= 9.0 mg/kg/day) at the age of 5 years. Midazolam is used during seizure attacks. In addition, she is also taking omeprazol (10mg BID) and macrogol (4g daily) to control her GE reflux and constipation, respectively.

Her development currently at age of 5 years is profoundly delayed; she could walk independently at 35 months of age, but she is autistic and does not speak. She has strabism. Physical examination at 3.2 years of age showed no overt dysmorphism except a slight upslant of the eyes and a slightly prominent forehead. Length at the 25th centile and head circumference at 2.5th centile / -2 SD. Neurologically, at 3 years and 8 months she hardly makes eye contact, follows her own lead. She has some stereotypic hand movements, and her hand motor skills are slightly clumsy, but not ataxic. She walks somewhat unstable with a wide based gait. The leg tonus seems slightly high, reflexes are vivid and no Babinski reflex.

Additional investigations included TORCH serology (negative). Metabolic screening of urine at day 3 before PLP therapy showed normal amino acid profile, a trace of sulfite, high lactate, and negative α-

AASA. In blood, carnitines, acylcarnitines and methylmalonic acid levels were normal, and plasma amino acids showed elevated glycine 915 µmol/L (normal range 197-487 µmol/L) and ornithine (197 µmol/l, normal range 42-170 µmol/L).

Pre-treatment CSF screening at day 3 revealed normal total protein (670 mg/L, normal range: 450-1090 mg/L); high lactate 4.4 mmol/l (normal range: 1.1-2.4 mmol/L); high pyruvate (0.23 mmol/L, normal range: 0.03-0.15 mmol/L) and normal glucose (4.2 mmol/L, normal range: 2.2-4.4 mmol/L). Amino acids showed slightly high values of glycine (25 µmol/L; normal range: 3-17 µmol/L), threonine, glutamine, and ornithine. Neurotransmitters and pterins were normal.

Chromosome micro-array showed a paternal 5p15.2 duplication of 433 kb (genes in the duplication interval are *ANKRD33B*, *DAP* and *CTNND2*) and a maternal 12q24.33 duplication of 370 kb (genes in the duplication interval are: *GPR133* and *LOC116437*). Both variants are likely benign. Whole-exome sequencing of the proband and both parents was performed as described (Dyment *et al.*, 2013), identifying a homozygous missense variant in the *PLPBP* gene: Chr8(GRCh37):g.37623143G>A; NM_007198.3:c.199G>A; p.(Glu67Lys).

Patient 6

This boy, now 4 years and 3 months old, was born to consanguineous parents (second cousins) from the UAE after a pregnancy complicated by possible fetal seizures in the late third trimester consisting of rapid movements. There is a family history of similar epileptic encephalopathy with infantile spasms in a sibling who died from pneumonia while being treated with steroids. He was born at term with birth weight of 2.8 kg. His head circumference measured at the age of 10 months was at the 10th percentile (44 cm) and has remained normocephalic. Apgar scores were not available but there were no reports of complications or need for resuscitation after delivery. He had irritability from the first day with possible seizures and clear diagnosis of seizures by day 4 of life. Initial seizures types were infantile spasms and rapid clonic seizures. Results of first EEG at 2 months are unknown. An EEG at 4 months showed multifocal epileptiform activity predominantly in the frontal and parietal regions. He had transient response with 2 weeks seizure free on prednisolone then the effect waned. He had no response to levetiracetam or vigabatrin. Around 6 months of age, pyridoxine 50 mg BID (approximately 6 mg/kg/day) was given, then cutting back to 25 mg BID resulted in complete control of spasms and clonic seizures, and his EEG normalized. Within 1-2 months, however, he developed new seizures, generalized tonic-clonic seizures with illness or fever, lasting up to 30 minutes in duration every 1-3 months. He has had improvement in duration and frequency of seizures with oxcarbazepine and early treatment with diazepam. His longest seizure free interval was approximately 3 months. He had a brief withdrawal of

PN for two days with recurrence of seizures and thus it was resumed at a dose of 50 mg BID. After PLPHP deficiency diagnosis was made, his PN dose was increased to 100 mg BID (12.8 mg/kg/day). He has been on this dose ongoing in addition to oxcarbazepine 420 mg BID (53.8 mg/kg/day).

Developmentally, he was severely delayed without achieving any milestones during the first 6 months prior to PN treatment; he was markedly hypotonic and made no eye contact. After treatment, he made excellent improvement in his development but still has mild motor delays and a diagnosis of ASD was made at 2 ½ years old. He sat independently by 12 months, walked by 2 years, and had a pincer on one hand by 2 ½ years. He repeats words but does not talk independently or communicate with gestures and his eye contact is limited. He has limited social reciprocity and joint attention. He has frequent stereotypies and self-stimulatory behaviors fitting the ASD.
At 2 years and 7 months of age, he was assessed on the Bayley Scales of Infant and Toddler Development, where his scores were: Cognitive Composite 70 (2^{nd} percentile), Language Composite 62 (1^{st} percentile), Motor Composite 85 (16^{th} percentile).

Patient 10

This girl, currently at 10 years and 6 months of age, was born from consanguineous (first cousins) parents of Kurdish descent. There is a family history of similar disease in the younger sister (patient 11). She was born at 38 weeks gestation via C-section due to fetal decelerations and meconium stained amniotic fluid. Ultrasound examination performed at 20 weeks of gestation was remarkable for cysts in the head, but these were not seen on repeat ultrasound at 28 weeks. Her APGAR scores were 8 and 9. After birth, she was irritable with a high-pitched cry, dysconjugate eye movements, and tonic posturing was seen early on.

Within the first day of life, she presented with seizures characterized by flexor spasms and eye deviations; oxygen desaturations were seen. She continued to exhibit irritability and seizure activity with segmental myoclonic jerks involving the upper trunk, eye deviation, crying, hiccupping and flexor spasms.

She was given a phenobarbital load during the first two days of life, with a mild response. An EEG after phenobarbital load showed discontinuous background rhythm with periods of quiescence, lasting up to 10 seconds, consistent with mild cerebral dysrhythmia. On day four of life, an overnight extended video EEG was pursued. At the beginning, near burst suppression pattern was seen, characterized by spike and slow wave and poly spike and slow wave complexes lasting up to 10 seconds. Relative periods of quiescence lasting up to 20 seconds were seen. During the burst of generalized paroxysmal discharges, she exhibited

periodic episodes of high pitched cry, flexor spasms with arm extension, with and without hiccupping and with and without eye bobbing, lip smacking and emesis.

A 50 mg dose of PN was given twice over a short period of time. After 5 minutes, the periodic episodes of high pitched cry, flexor spasm, and hiccupping stopped and there was a significant improvement in the EEG background rhythm. During wakefulness, the background rhythm was continuous with fair synchrony and symmetry for age. Background rhythm appeared to be discontinuous during quiet sleep. A moderate number of sharp waves were seen over the left central temporal and right temporal region. Phenobarbital and phenytoin were discontinued, and the child remained without seizures. She was discharged home at 11 days of age, was breast feeding well and taking 75 mg PN per day. Doses were given in the evening since the child became very sleepy as a result. At the age of 8 years and 2 months, the family took her off PN treatment for two weeks which led to uncontrollable seizures and was taken to the ED where her seizures could not be stopped until she was put back on PN.

In terms of her language development, she began babbling at approximately 12 months of age. She started to say "mama" and "dada" at 3 years of age. She currently has several hundred words, which tend to be more representative of objects, and can sometimes be difficult to recognize. She will string four or five words together to communicate. She can follow one-step, very familiar commands or one-step commands with gestures. She does know her body parts including more minor body parts such as teeth and elbows. She does identify many of her letters. She is able to play with other children mostly her siblings. She points when she wants something and is fully potty-trained.

A neurological exam found that she has hypotonia with joint laxity, mild dysmetria and is unable to balance on each foot for 3 seconds. She has a wide based gait with poor coordination but is able to navigate an iPad. She can walk up and down stairs by herself, hops on each leg independently. She can kick and throw a ball and ride a tricycle. She needs some help with dressing but can pull up pants and underpants on her own and can take off her coat. She needs some help with putting on a coat. She can use a spoon and a fork well but makes a lot of mess. She holds a pen well. She does not yet write letters or numbers but can trace them or do so if her family is using hand over hand. She does not yet draw items that others recognize.

For her seizures she now takes 100 mg PN BID (4.7 mg/kg/day). She also requires lamotrigine 50 mg BID (3.5 mg/kg/day) and clobazam 10 mg BID (0.75 mg/kg/day) for optimal seizure control. During illness however, she can have breakthrough seizures.

Biochemical investigations included normal urine organic acids and purines; blood lactate, acylcarnitines, amino acids (both after PN therapy); and a normal CSF amino acids (except for a slight increase in

alanine (43 nmol/ml)), folate/5MTHF, lactate, protein, glucose, BH4, neopterin, PLP, and neurotransmitter metabolites (5HIAA, HVA, 3-OMD).

Genetic investigations included normal 500Kb array CGH microarray, Prader-Willi/Angelman methylation studies, and Sanger analysis of *ALDH7A1* (heterozygous for non-pathogenic variant p.K411Q), and deletion/duplication analysis (negative), *CDKL5, SCN1A, SCN1B, GABRG2,* and *PCDH19* sequencing (all negative).

Patient 11

This girl, who is now 6 years and 10 months old, is the sister of patient 10. She was delivered via C-section after an unremarkable pregnancy. Her head circumference was in the 2nd percentile. On the first day of life, she had ophisthotonus, irritability, and eye deviation throughout the day (episodic, but up to 1 hour). This was not diagnosed as seizures until an EEG was performed at a few days of life. The EEG showed a discontinuous record with multifocal sharp waves (bilateral frontal/central/temporal). She was admitted to the NICA for two weeks due to meconium aspiration and seizures.

She was noted to have focal seizures (hemibody clonic activity with lateral eye deviation to either side) lasting 7 seconds to 5 minutes (average 2 minutes, 2-3 times a week), or generalized convulsions with whole body stiffening and neck extension, lasting more than 2 minutes, about twice a month. Her seizures typically occurred at night.

She had no initial response to AEDs. With initial PN administration, the EEG report describes persistence of sharp waves at moderate frequency, and her seizures did persist over several weeks and thus levetiracetam was added to her treatment. Subsequent additions of clobazam and lamotrigine have been helpful, but she still has seizures with fever. At the age of 4 years and 10 months, her PN dose was reduced to 50 mg BID and she suffered increase in frequency of seizures. She is currently on following medications: 100 mg PN BID (7.8 mg/kg/day), lamotrigine 37.5 mg BID (4.5 mg/kg/day) and clobazam 10 mg BID (1.25mg/kg/day).

Her neurological examination revealed mild dysmetria, and a wide based and ataxic gait. She is very hypotonic in the trunk, making mobility much more difficult. She continues to progress in gross and fine motor skills. Still cannot climb up or down stairs. She has separation anxiety and severe stranger anxiety. She knows a lot more words now than previously. She is interested in others, points at what she wants, but cries if approached by other children.

Biochemical investigations: A comprehensive metabolic panel (sodium, potassium, chloride, calcium, bicarbonates, glucose, BUN, creatinine, total protein, albumin, A/G ratio, total bilirubin, alkaline

phosphatase, GOT/AST, GPT/ALT) was screened four times (first one at age of 15 months) and resulted normal profiles in all.

Genetic investigations: GeneDx Infantile Epilepsy Panel (all negative): sequencing and deletion/duplication analysis of the following genes: *ADSL, ALDH7A1, ARX, ATP6AP2, CDKL5, CLN3, CLN5, CLN6, CLN8, CNTNAP2, CTSD, FOXG1, GABRG2, GAMT, KCNQ2, KCNQ3, MECP2, MFSD8, NRXN1, PCDH19, PNKP, PNPO, POLG, PPT1, SCN1A, SCN2A, SCN1B, SLC25A22, SLC2A1, SLC9A6, SPTAN1, STXBP1, TCF4, TPP1, TSC1, TSC2, UBE3A* and *ZEB2*.

Clinical whole exome sequencing ultimately discovered the *PLPBP* variant in patients 10 and 11 after reanalysis as the original analysis did not classify the "*PROSC*" gene that had not yet been described.

Mild phenotypes

Patient 2

This boy of Omani descent currently aged 13 years and 10 months was born to consanguineous parents (first cousins) at term without antenatal or postnatal complications. There is family history of 2 siblings' deaths; both were due to intractable seizures (at the age of 2-4 months). He has 5 living siblings (3 males and 2 females) that are all healthy. His birth head circumference was at the 10th percentile.

At the age of 7 days, brief, frequent seizures were noted with behavioral arrest progressing to tonic-clonic movements. He was tried on different AEDs but no response until pyridoxine was administered and subsequently seizures were controlled before the age of 1 month. Infrequent seizures occurred mainly during febrile illnesses, the most recent one at the age of 7-8 years. EEG reports are not available.

He is currently on pyridoxine 80 mg BID (= 5 mg/kg/day), increased during febrile illness to 80mg TID. Physical examination revealed no dysmorphic features, anthropometric measurements on the 10th centile, no systemic abnormalities and no organomegaly. Development in all domains and cognition are normal for age; he attends a regular grade 6 at school and has average school performance. His motor neurological exam was reported as normal. Biochemical screening revealed no detectable α-AASA in urine and Sanger sequencing of *ALDH7A1* was negative. Given the striking response to pyridoxine, no other investigations were carried.

Patient 8

This boy of Arab descent, now 8 years and 1 month old, was born at term via spontaneous vaginal delivery to a primigravida mother with insulin dependent diabetes. The parents are consanguineous with a family history of pyridoxine-dependent epilepsy. The patient is 1st cousins with patient 9.

His APGAR scores were 7 & 9 at 1 & 5 minutes, respectively. At birth he weighed 2.98 kg (50th percentile), was 55 cm tall (90th percentile) and his head circumference was 35cm (50th percentile). He was feeding well and active until the age of 1 week when he started to have episodes of myoclonic movements of the upper and lower limbs lasting for few seconds in clusters. He continued to have daily episodes. He was irritable and crying with disturbed sleep. The seizures became very frequent with time and at the age of 3 weeks he was admitted for the control of seizures. His initial EEG at the age of 3 weeks showed burst suppression. He was initially loaded with phenobarbitone but there was no response. He was then started on midazolam infusion and IV levetiracetam but he continued to have frequent seizures. At age of 25 days, a dose of 20 mg oral PN was tried and the seizures immediately stopped. He was sleepy for more than 10 hours for which he was shifted to PICU for observation. An EEG was repeated and it was normal. He was then gradually weaned off midazolam and levetiracetam. He was back to his normal activity. Phenobarbitone was also tapered and discontinued. He continued to be on PN only 40 mg BID with increasing the dose to TID during febrile illnesses. He remained seizure free since then except at the age of 5 years when he had a febrile illness and there was not enough PN at home to increase the dose. After that he had no more seizures. He is currently on pyridoxine 80 mg BD (6 mg/kg/day), increased during febrile illness to 80 mg TID = 8.8 mg/kg/day.

He achieved all his developmental milestones at an appropriate age. He is in grade 2 at school now with excellent performance. A physical examination found no dysmorphic features or neurocutaneous marks, no organomegally, and his motor neurological exam was normal (normal tone, power and DTR, planters are downgoing, normal cranial nerves examination, no cerebellar signs). His weight, height and head circumference are currently between the 50th and 75th percentiles.

Biochemical investigations found normal pipecolic acid levels and metabolic workup at the age of 3 weeks revealed raised blood lactate (4.5 & 3.4) but normal pH. Amino acids and acylcarnitines were unremarkable on tandem mass spectrometry in dried blood spots. Long-term EEG at the age of 4 years and 4 months resulted normal.

Patient 9

This boy of Arab descent, now 14 months old, was born at term by spontaneous vaginal delivery 37 weeks of gestation to a primigravida mother with no antenatal complications. The parents were consanguineous with a family history of pyridoxine-dependent epilepsy, the patient is the cousin of

patient 8. His APGAR scores were 9 and 10 at 1 and 5 minutes, respectively. His birth weight was 2.56kg (50^{th} percentile), he was 49cm tall (50^{th} percentile) and his head circumference was 33cm (50^{th} percentile). He was admitted to the SCBU soon after delivery with the impression of TTN (transient tachypnea of the newborn). He was in SCBU for 5 days during which he was treated for presumed sepsis and jaundice. After discharge on day 5 of life, the parents started to notice frequent episodes of tonic seizures. The episodes were brief and lasting for seconds only. He continued to be active and was feeding well. His first EEG at the age of 10 days showed burst suppression. He was started on PN at home by his uncle (father of patient 8). At hospital, he received 40 mg once and he became very sleepy but had no more seizures. Within 24 hours he became active and was again feeding well. He was discharged on oral PN. He had no other symptoms. He is currently on 20 mg PN BID (8.5 mg/kg/day), increased during febrile illness to 80 mg TID (= 12.5 mg/kg/day).

Physical examination revealed no dysmorphic features or neurocutaneous marks and his weight, height and head circumference are all now in the 10^{th}-50^{th} percentile. He has normal tone, power and cranial nerves examination but noted to have hyperreflexia in all limbs. Urinary aminoadipic semialdehyde was mildly elevated at 0.35 mmol/mol Creatinine (reference, 0-0.19). Urinary pipecolic acid concentration was normal at 0.12 mmol/mol Creatinine (reference, 0.01-1.54). Piperidine-6 carboxylic acid was normal at 0.37 mmol/mol Creatinine (reference 0-1.62). Amino acids and acylcarnitines were unremarkable on tandem mass spectrometry in dried blood spots.

Repeat EEG at 10 months was normal.

Unclassified severity

Patient 12

This African American girl, now 5 months old, was born via spontaneous vaginal delivery at 35 weeks of gestation to a 17-year-old G1 P0 female after an uncomplicated pregnancy. The parents were consanguineous. Her APGAR scores were 7 at 1 minute and 9 at 5 minutes and birth head circumference was 31 cm which is at 22^{nd} percentile. She presented shortly after birth with neonatal seizures. She started having repeated stereotyped episodes of extremity jerking and irregular respirations within the first few hours of life and evolved into super refractory neonatal seizures.

She did have initial period of seizure freedom after phenobarbital loading but relapsed within the first week of life. Her seizures failed multiple antiepileptic medications, including phenobarbital, phenytoin, topiramate, levetiracetam, clonazepam, vigabatrin, midazolam, lorazepam, leucovorin, and a single dose

of PN (100 mg IV) given early in the course. The VEEG background had no noted improvement after the first PN dose. She had focal seizures and myoclonic jerks followed by tonic posturing and initial EEG showed a burst suppression pattern followed by very frequent multifocal motor seizures as well as bilateral synchronous tonic seizures and/or myoclonic seizures with generalized epileptiform activity. After failing multiple conventional anticonvulsants, dextromethorphan was tried without success. She was placed on a 3:1 ketogenic diet and serine supplementation for low CSF serine. PLP was started at one month of age resulting in seizure freedom, significant improvement in EEG background activity and improvement in her neurologic exam. EEG background became continuous and no electrographic or clinical seizures were after PLP was started. Focal interictal epileptiform activity continued to be present but overall there was much improvement after initiation of PLP.

For seizure control, she is now taking 40 mg/kg/day of PLP divided q12h and 9 mg/kg/day of phenobarbital. She has been weaned from the ketogenic diet. On exam at age 2 months, she was microcephalic (z score -4.4), non-dysmorphic and was feeding well by mouth. She had conjugate eye movements and emerging visual fixation. She had normal axial tone and localized pain to extremity. Her deep tendon reflexes were 3+. No myoclonus was seen. Upon most recent check at age of 4.5 months, her EEG has 4-4.5 Hz background of normal voltage with no epileptiform activity. She is developing relatively well, has mild hypotonia but intact visual fixation and is a good oral feeder. Mother reports possible rare brief seizures but none noted in 24 hour EEG.

Her laboratory work-up revealed normal CBC, normal CMP, negative CRP, normal ammonia, initial elevated serum lactic acid which normalized within first 2 days, negative HSV PCR, negative TORCH titers, normal CSF lactic acid, normal CSF pyruvic acid, normal CSF glucose and normal CSF protein. Low CSF serine (30 nmol/mL, normal range: 44 - 136 nmol/mL) was noted, but other CSF amino acids were normal. Plasma amino acids checked at age of 6 days revealed elevated glycine (575 nmol/mL, reference range: 111 - 426 nmol/mL). Repeat plasma amino acids at 9 days of life showed normal glycine levels (370 nmol/mL). CSF glycine was normal at 3 weeks of age 23 nmol/ml (reference range: 5 - 115 nmol/mL). Acylcarnitine, urine organic acids and uric acid were all within reference intervals. Lymphocyte choriomeningitis AB IgG and IgM was negative. Pipecolic acid was 0.4 nmol/mL (normal range <6 nmol/mL). Urinary S-sulfocysteine was within limits. CSF neurotransmitters (5-hydroxyindoleacetic acid, HVA, 3-Omethyldopa) were all normal.

A microarray showed vast areas of homozygosity, totaling 20% of the genome. GeneDX Xome DxSlice on the proband revealed a pathogenic mutation in the gene *PLPBP* which results in pyridoxine-dependent seizures. In addition, 3 other homozygous variants were identified; a homozygous pathogenic variant in

CYP27A1 (c.1421G>A) and two homozygous variants of uncertain significance in *DENND5A* (c.1429A>G) and *VPS53* (c.997C>T).

Supplemental methods

Whole-exome and Sanger sequencing and *in silico* analysis

Patients 1 and 2

Whole exome sequencing (WES) was performed on patients 1 and 2 using the SureSelectXT Library Prep Kit and Illumina HiSeq 4000 (Macrogen, Korea). The data was analyzed using a semi-automated bioinformatics pipeline (Tarailo-Graovac *et al.*, 2016). Illumina sequencing reads were aligned to the human reference genome version hg19 using Bowtie2 aligner (Langmead and Salzberg, 2012) and local realignment was performed using Genome Analysis Toolkit (McKenna *et al.*, 2010), achieving mean coverage of 24x for both patients 1 and 2. Variants were called using SAMtools (Li *et al.*, 2009) and annotated using SnpEff (Cingolani *et al.*, 2012). Rare variants were identified using public databases, such as exome variant server (EVS), dbSNP v138 (Sherry *et al.*, 2001) and the Exome Aggregation Consortium (ExAC) database (Lek *et al.*, 2016), as well as our in-house database of more than 400 exomes and 40 genomes (UBC) and against an in-house database of 817 Saudi Arab exomes at Alfaisal University (Dr. Fowzan Alkuraya, personal communication). Manual inspection on variant quality was carried out with Integrative Genomics Viewer (IGV) (Robinson *et al.*, 2011).

Patient 3

Clinical child-parents whole-exome sequencing (trio-WES) was performed at the Department of Human Genetics at the Radboudumc (Nijmegen, The Netherlands), with examination of all known genes according to previously described WES methods (de Ligt *et al.*, 2012; Lelieveld *et al.*, 2016).

Patient 4

Whole-exome sequencing of the proband and both parents was performed as described (Dyment *et al.*, 2013).

Patient 5

Trio WES was performed using SureSelect Human All Exon Kit version 4 (Agilent) for target enrichment. The library was sequenced with 100bp paired-end reads on a HiSeq 2000 platform (Illumina), and bioinformatics analysis was carried out as described previously (Dyment *et al.*, 2013). Sanger sequencing showed the affected individual was homozygous for this variant, parents heterozygous.

Patients 6, 7, 10, 11 and 12

Using genomic DNA from the proband and parents if available, the exonic regions and flanking splice junctions of the genome were captured using the SureSelect Human All Exon V4 (50 Mb), the Clinical Research Exome kit (Agilent) or the IDT xGen Exome Research Panel v1.0. Massively parallel (NextGen) sequencing was done on an Illumina system with 100bp or greater paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19 and analyzed for sequence variants using a custom-developed analysis tool. Additional sequencing technology and variant interpretation protocol has been previously described (Tanaka *et al.*, 2015). The general assertion criteria for variant classification are publicly available on the GeneDx ClinVar submission page (http://www.ncbi.nlm.nih.gov/clinvar/submitters/26957/). Given patient 11 is a sibling of patient 10, the variants were diagnosed through targeted Sanger sequencing.

Patients 8 and 9

The *PLPBP* mutation in these cousin patients was identified by targeted Sanger sequencing.

***In silico* assessment of variants**

In silico variant effect predictions and scores from the 6 prediction algorithms (SIFT, Polyphen2 HDIV, MutationTaster, MutationAssessor, FATHMM MKL and PROVEAN) for all *PLPBP* single-nucleotide variants (SNVs) were retrieved from GenomeBrowse 2.1.2 (Golden Helix, USA) using its data track "dbNSFP Functional Predictions and Scores 3.0". The track curates and visualizes functional predictions and scores that are originally obtained from the dbNSFP database (Liu *et al.*, 2011, 2013). Only one tool (MutationTaster (Schwarz *et al.*, 2014)) provided prediction for the 4bp deletion mutation in patients 5 and 12 (obtained manually from http://www.mutationtaster.org). CADD scores (Kircher *et al.*, 2014) were queried individually.

Primary skin fibroblast culture

For patient 5, a skin biopsy was taken from which a fibroblast cell line was established at the Centre for Applied Genomics (Toronto, Canada) and maintained in HyClone DMEM media (GE Healthcare Life Sciences) supplemented with 10% FBS, Penicillin-Streptomycin (SV30010, GE Healthcare Life Sciences) and 2mm L-glutamine (SH3003401, Thermo Scientific).

Patient fibroblast protein analysis

Total protein from the patient and three control lines was extracted in RIPA buffer containing protease inhibitors (Sigma) and was run on SDS-PAGE (20µg) following standard protocols. Antibodies used

were rabbit anti-PROSC (Proteintech, 25154-1-AP, 1:5000); anti-β-tubulin (Abcam, ab6046, 1:20 000) and anti-GAPDH (ImmunoChemical, 200-901-BJ4, 1:10 000) were used as loading controls. HRP-linked anti-rabbit or anti-mouse IgG (1:2000) was used as secondary, and the Clarity ECL WB Substrate kit (BioRad) was used for protein detection using a ChemiDoc Touch Imaging System (BioRad).

Analysis of mitochondrial function in fibroblasts

A sample of the patient 5 fibroblast line was sent to the Mitochondrial Disease Laboratory (SickKids, Toronto). Measurements performed were pyruvate dehydrogenase (PDH) in its native and dichloroacetate activated forms, pyruvate carboxylase (PC), cytochrome oxidase, succinate cytochrome c reductase, and the cellular lactate/pyruvate ratio.

Oxygen consumption rate (OCR) was measured in patient and control fibroblasts using a Seahorse XF-24 Extracellular Flux Analyzer and V7 PS cell culture microplates (Agilent). Cells were seeded 50 000/well 24 hours before the assay, which followed the standard protocols of the XF Cell Mito Stress Test (Agilent). Data were normalized to protein concentration.

***PLPBP* targeting in HEK293 cells**

Two guide RNAs were designed in exon 2 of *PLPBP* (NM_007198) targeting the region downstream of the start codon using the CRISPR design website (http://crispr.mit.edu/). The guide RNA sequences were TTGCTGACCGCCACTAGCCG (Guide 1 on reverse strand; primers 1F 5' CACCGTTGCTGACCGCCACTAGCCG 3' and 1R 5' AAACCGGCTAGTGGCGGTCAGCAAC 3') and CATCCAGCCCCGGCTAGTGG (Guide 2 on forward strand; primers 2F 5' CACCGCATCCAGCCCCGGCTAGTGG 3' and 2R 5' AAACCCACTAGCCGGGGCTGGATG C 3'). Oligonucleotide guide sequences were cloned into the pSpCas9(BB)-2A-GFP plasmid (Addgene Plasmid 48138). The resulting plasmids were transfected into HEK293 cells and GFP positive cells were sorted two days after transfection. These cells were used for obtaining clonal cell lines. We obtained two clonal cell lines with predicted biallelic disease-causing mutations; Guide 1_B, homozygous for c.124_127delCTAG (L42Wfs*12) and Guide 2_C, homozygous for c.128_129ins131bp (A44Gfs*55).

PLPHP overexpression in HEK293 cells and sample preparation for immunofluorescence

HEK293 cells were seeded in 12-well plates containing coverslips and transfected with a plasmid encoding Myc-DDK-tagged *PLPBP* (Origene, RC200853, C-terminal) using TurboFect (Thermo Fisher)

following manufacturer specifications. Cells were fixed in pre-warmed 4% paraformaldehyde in PBS at room temperature for 10 minutes. After washing with PBS, coverslips were blocked for one hour with 1% BSA in PBS/0.3% Triton-X100. Primary antibodies (mouse anti-DDK monoclonal (Origene, TA50011, 1:100) and a rabbit polyclonal against human Tom20 (FL-145) (Santa Cruz sc-11415, 1:1000) were diluted in PBS containing 1% BSA and incubated for one hour. Secondary antibodies (Cy3-AffiniPure Goat anti-mouse IgG (H+L) (Jackson ImmunoResearch 115-165-003, 1:750) and Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Life Sciences A11034, 1:750)) were diluted in PBS containing 1% BSA and incubated for one hour. Cover slips were stained for five minutes in DAPI. Microscopy was performed using AxioObserver Z1 LSM800 63x/1.4 (Zeiss).

Quantification of B6 vitamers in plasma, leucocytes and cultured cells

Plasma samples from patient 4 (prior to treatment with any form of vitamin B6) and patient 3 (during treatment with PLP) were collected, shed from light and stored at -80°C. B6 vitamers PLP, pyridoxal (PL), PN, pyridoxamine (PM) and the degradation product 4-pyridoxic acid (PA) were quantified by LC-MSMS as previously described (van der Ham *et al.*, 2012; Mathis *et al.*, 2016). Pyridoxine-5'-phosphate (PNP) was not quantified due to plasma-related technical limitations of the method (ion suppression) and pyridoxamine-5'-phosphate (PMP) was not quantified as it is known to be highly unstable in plasma.

Fibroblasts from patient 5 and four controls, and HEK293 cells were cultured in DMEM GlutaMAX-I (Gibco, cat # 31966) containing 10% fetal bovine serum and 1% penicillin-streptomycin. B6 vitamers were extracted with tricholracetic acid (50g/L) and quantified with UPLC-MS/MS in biological triplicates as described by (van der Ham *et al.*, 2012).

Zebrafish genotyping

F1s were raised to adulthood and were fin-clipped for genotyping by HMA-PAGE. Fish with candidate variants causing frameshift mutations were backcrossed to WT fish to further reduce the chance of off-target effects, generating F2 heterozygotes. F3 larvae from the crossing of F2 heterozygotes were genotyped by extracting DNA from 3-4 days post-fertilization (dpf) larval fins and HMA-PAGE was used following previously described protocols (Pena *et al.*, 2017; Kosuta *et al.*, 2018). Primers used: *plpbp-F 5'* GCACTCTGGCTATGTGGAGA 3'; *plpbp*-R 5' AGCTGTCACTCATCCCTCGT 3'. Because differentiating homozygous mutants and homozygous WT genotypes requires two rounds of HMA-PAGE, and since no suitable primers could be identified for a reliable multiplex PCR strategy that would clearly identify homozygous mutants, two separate F2 mutant lines were crossed to generate compound

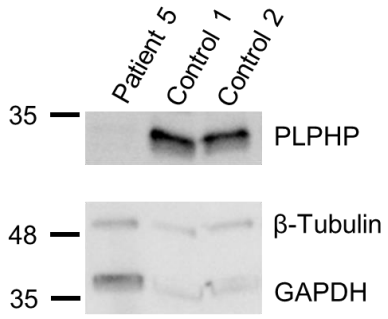
heterozygous F3 offspring which facilitated genotyping by HMA-PAGE in a high-throughput manner (Supplementary Figs. 4 and 5). A pilot study was performed to show no difference in phenotype or survival between the compound heterozygous and homozygous mutant lines (Supplementary Fig. 6).

Western blotting for zebrafish larvae

Pools of 4 larvae were collected at 11dpf and total soluble proteins were extracted following previously established protocols (Pena *et al.*, 2017). 40µg of protein from each sample was separated by SDS-PAGE using BioRad 4-20% pre-cast stain-free gels and blotted on low fluorescence PVDF (BioRad). Antibodies used were rabbit Anti-PROSC (Proteintech, 25154-1-AP, 1:5000) and HRP-linked anti-rabbit IgG (1:2000) was used as secondary. The Clarity ECL WB Substrate kit (BioRad) was used for protein detection using a ChemiDoc Touch Imaging System (BioRad) and proteins were quantified against stain-free total protein.

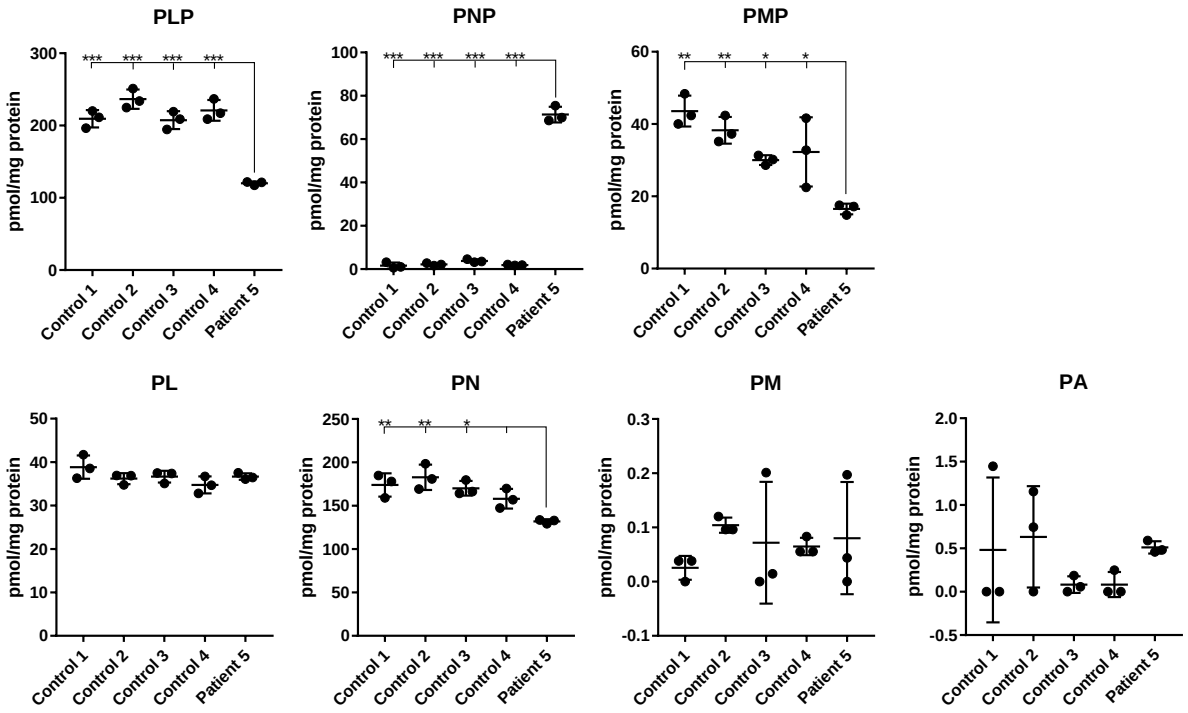
Supplemental results

Patient 5's fibroblasts do not express PLPHP



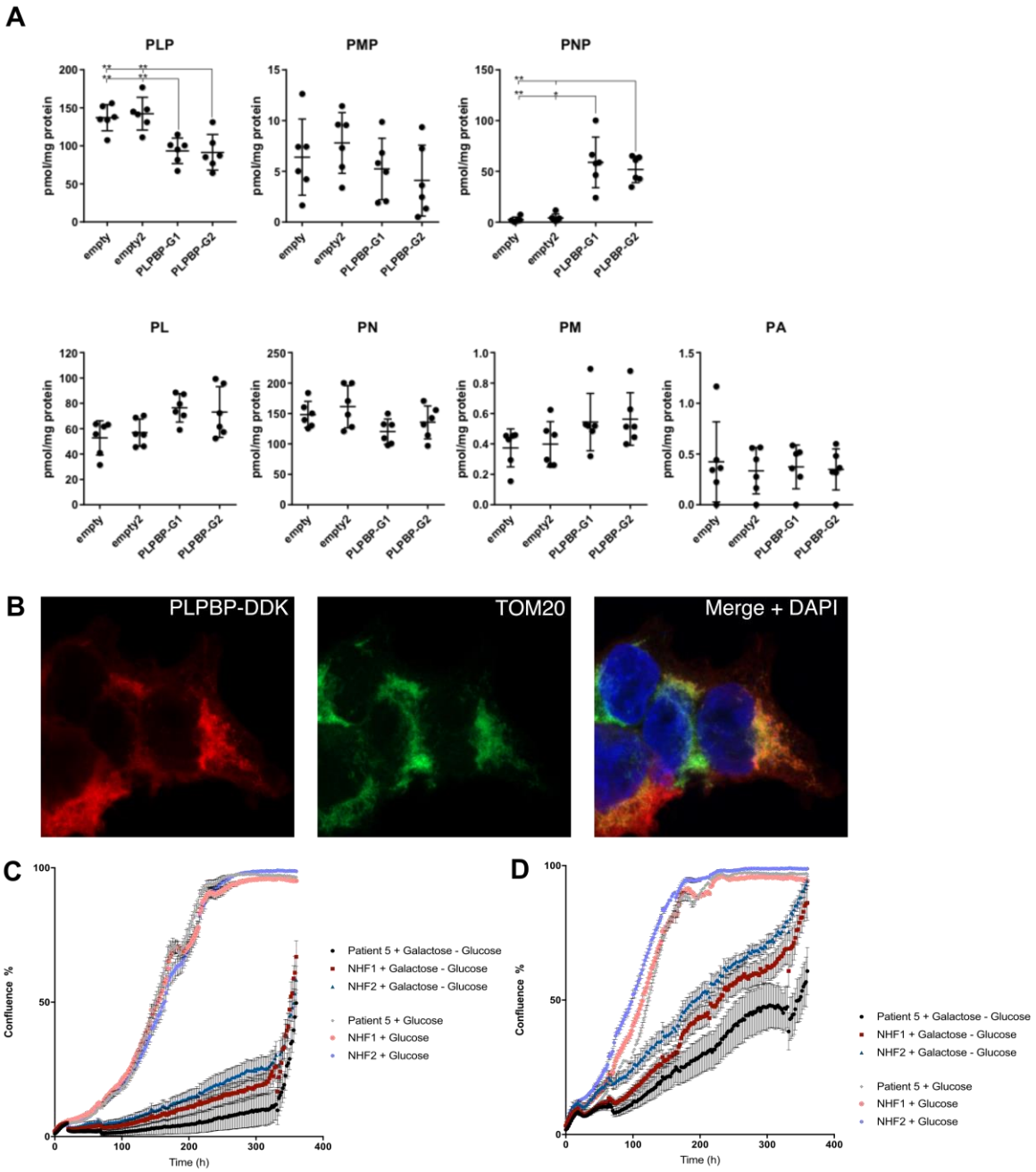
Supplementary Figure 1: western blot of fibroblast lysates (20µg protein) showing that patient 5 is deficient for PLPHP.

Patient 5's fibroblasts show an altered B6 vitamer profile



Supplementary Figure 2: B6 vitamer profiles in cultured fibroblasts from four control subjects and patient 5. Data are n=3 biological replicates per group. PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5'-phosphate; PN, pyridoxine; PNP, pyridoxine 5'-phosphate. ANOVA ***p<0.001, **p<0.01, *p<0.05.

HEK293 cells deficient for PLPHP show altered B6 vitamer profiles and expression of DDK-tagged PLPHP shows mitochondrial localization



Supplementary Figure 3: (A) B6 vitamer profiles in control (WT+empty vector) nd PLPHP-deficient HEK293 cells (PLPHP-KO: PLPBP-G1 and PLPBP-G2). Data are from n=6 independent experiments (each consisting of 3 biological replicates per group), ±SD. **(B)** HEK293T cells overexpressing PLPHP with a C-terminal Myc-DDK tag shows co-localization of PLPHP with Tom20, a mitochondrial marker. Incucyte analysis of cell growth in 5mM galactose or 25mM glucose as carbon source seeded at 500 cells per well (C) or 1000 cells per well (D) in 96 well plates. DMEM no glucose + 1mM sodium pyruvate was used as base media for C and D. Abbreviations: PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; PNP, pyridoxine 5'-phosphate; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PA, pyridoxic acid. **p<0.01, *p<0.05.

Supplementary Table 1: Detailed MRI findings

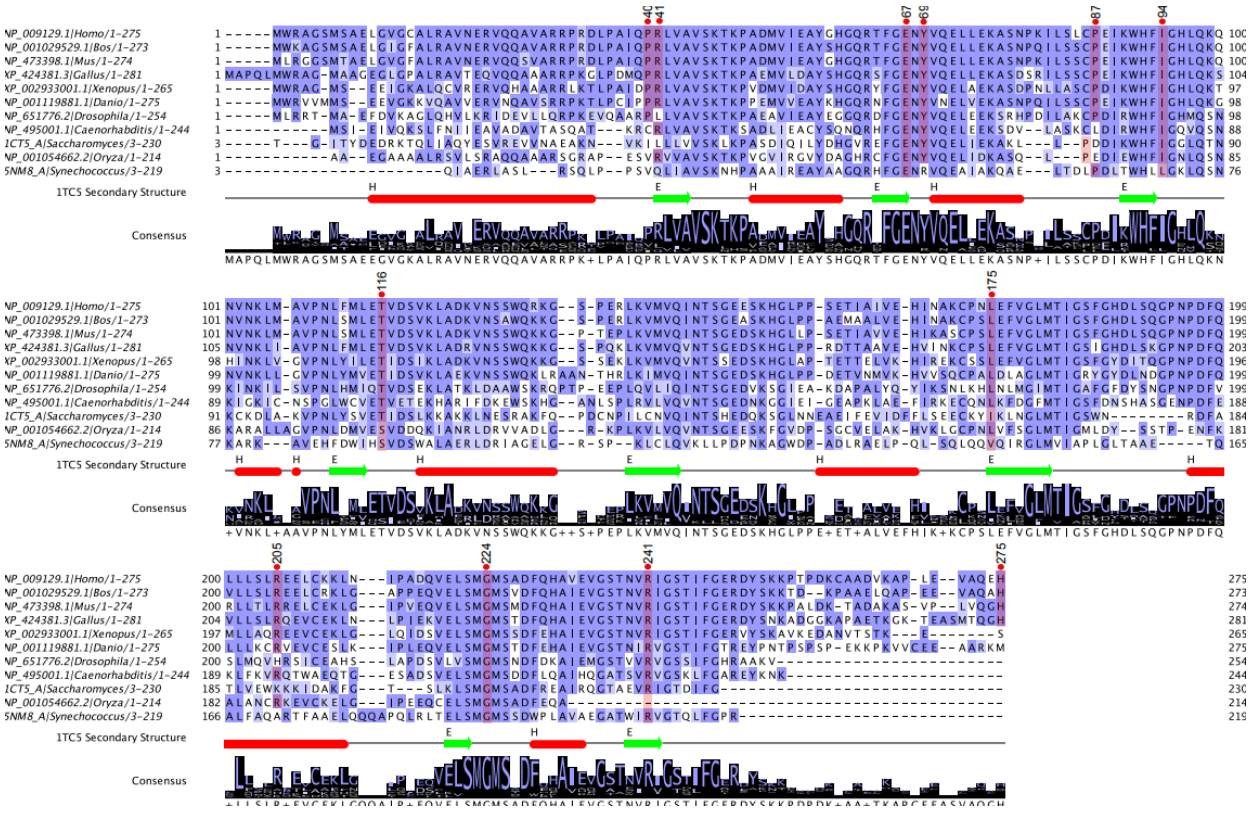
Patient ID	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12
MRI age	6 weeks, 8.5 months, 3.5 y	Not performed	10 days	1 day	6 days	8 months	12 weeks, 3.5 months,	MRI not available for review	MRI not available for review	6 years	2 years	MRI not available for review
WM abnormalities	Very mild T2-hyperintense and T1-hypointense changes in periventricular WM at age 6 weeks		Yes, T2 hyperintense and T1 hypointense, swollen aspect	Yes, T2 hyperintense and T1 hypointense, swollen aspect, subcortical cystic degeneration	Yes, T2 hyperintense and T1 hypointense, swollen aspect, subcortical cystic degeneration	No	no			Mild T2-hyperintensity in the posterior periventricular white matter	Faint T2-hyperintensity in the posterior periventricular white matter	
Cortex abnormalities	no		Simplified gyral pattern	Simplified gyral pattern	Simplified gyral pattern	no	no			no	no	
Basal ganglia abnormalities	no		no	no	no	no	no			no	no	
Thalamus abnormalities	no		no	no	no	no	no			no	no	
Cerebellar involvement	no		no	T2-hyperintense signal of the hilus of the dentate nucleus	no	no	no			no	no	
Cysts anterior horn	no		++	++	+ (L>R)	no	no			no	no	
CC abnormalities	no		Thin CC	Thin CC		no	no			Pronounced isthmus	no	
Other abnormalities	Age 8 months: mild communicating hydrocephalus with prominent external CSF spaces. Age 3.5 y: normal MRI			Lactate doublet at MR spectroscopy (basal ganglia)	Cavum septi pellucidi; small lactate doublet at MR spectroscopy (basal ganglia)	MRI normal	MRI normal	MRI at age 4 weeks reported as normal.	MRI at age 10 months reported as normal.			MRI at ages of 2 days and 3 weeks: Diffuse broadening of the gyri in both cerebral hemispheres, mild dilatation of the lateral and third ventricles with multiple intraventricular septations. There are blood products in the left lateral ventricule.

Supplementary Table 2: List of *PLPBP* variants found in our cohort of 12 patients. All variants are expressed as found in PLPHP (NP_009129.1). Variant effect is predicted is based on 7 *in silico* prediction tools (SIFT, Polyphen2 HDIV, MutationTaster, MutationAssessor, FATHMM MKL, PROVEAN and CADD). DUET uses as input the structural model developed for the human PLPHP to predict if a given amino acid change is stabilizing or destabilizing (ΔΔG). * Not modelled due to lack of this residue in yeast model used as template. NA: not available; NR: not reported.

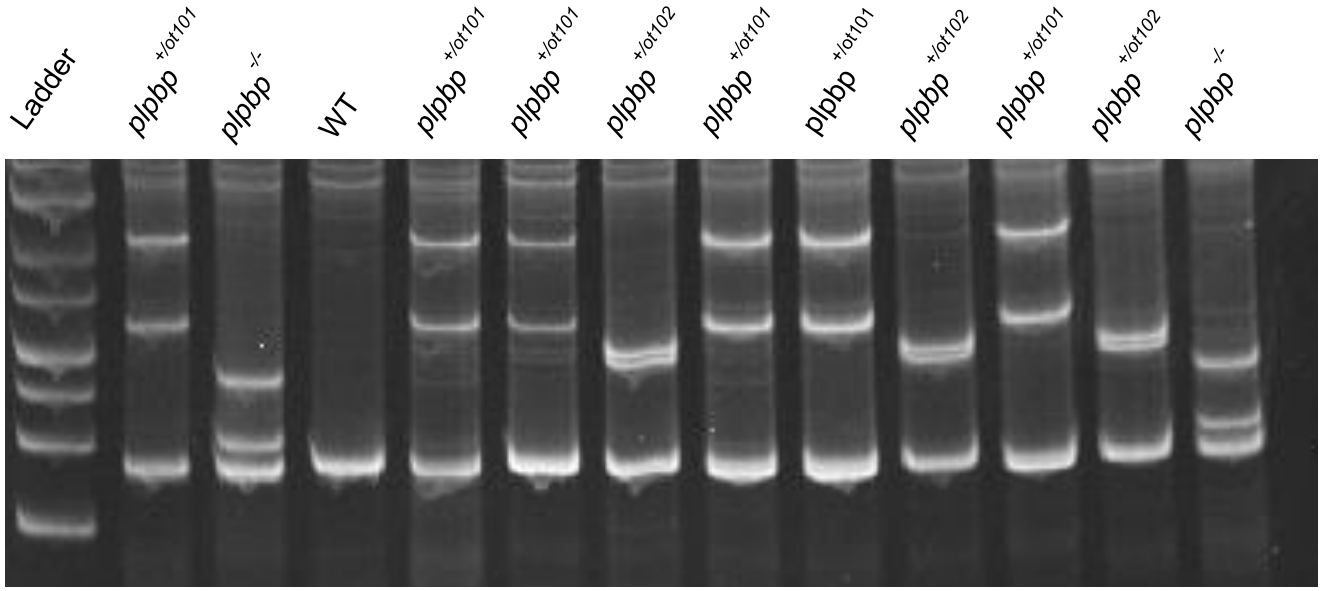
Variant annotation			**Detailed *in silico* predictions [predicted effect (score)]**							
Genomic (GRCh37)	**cDNA and protein**	**Variant frequency (gnomAD)**	**DUET Predicted Stability Change (ΔΔG):**	**SIFT**	**Polyphen 2 HDIV**	**MutationTaster**	**MutationAssessor**	**FATHM M MKL**	**PROVEAN**	**CADD score**
chr8: g.37630300 C>T	NM_007198: c.347C>T; p.Thr116Ile	NR	0.123 Kcal/mol (Stabilizing)	Damaging (0.003)	Probably damaging (1)	Damaging (1)	Functional (high) (3.855)	Damaging (0.98019)	Damaging (-5.56)	29.20
chr8: g.37635617 C>G	NM_007198: c.823C>G; p.His275Asp	NR	Not modeled*	Damaging (0.017)	Benign (0.361)	Damaging (0.918861)	Non-functional (low) (1.1)	Damaging (0.96396)	Neutral (-0.71)	23.3
chr8: g.37623066 G>A	NM_007198: c.122G>A; p.Arg41Gln	$4.06*10^{-6}$	-0.265 Kcal/mol (Destabilizing)	Damaging (0.04)	Probably damaging (0.978)	Damaging (1)	Non-functional (low) (1.795)	Damaging (0.99714)	Damaging (-2.73)	28.7
Chr8: g.37623143 G>A	NM_007198.3: c.199G>A; p.Glu67Lys	$4.06*10^{-6}$	-2.127 Kcal/mol (Destabilizing)	Damaging (0)	Probably damaging (1)	Damaging (1)	Functional (high) (4.1)	Damaging (0.99824)	Damaging (-3.96)	35
Chr8: g.37630271 A>G	NM_007198.3: c.320-2A>G splicing	$1.08*10^{-5}$	-	NA	NA	Damaging (1)	NA	Damaging (0.99207)	NA	24.7
Chr8: g.37633509 G>C	NM_007198.3: c.671G>C; p.Gly224Ala	NR	-0.966 Kcal/mol (Destabilizing)	Damaging (0)	Probably damaging (0.999)	Damaging (1)	Functional (high) (4.07)	Damaging (0.99191)	Damaging (-5.69)	27.7
Chr8: g.37630323_37630326del	NM_007198: c.370_373del; (p.Asp124Lysfs*2)	NR	-	NA	NA	Damaging (1)	NA	NA	NA	NA
chr8: g.37623834 A>T	NM_007198: c.280A>T; p.Ile94Phe	NR	-1.398 Kcal/mol (Destabilizing)	Damaging (0.001)	Probably damaging (1)	Damaging (1)	Functional (high) (4.43)	Damaging (0.99692)	Damaging (-3.96)	29.6

Supplementary Table 3: concentrations of B6 vitamers in plasma from 2 patients affected with PLPHP deficiency. Concentrations are expressed in nM.

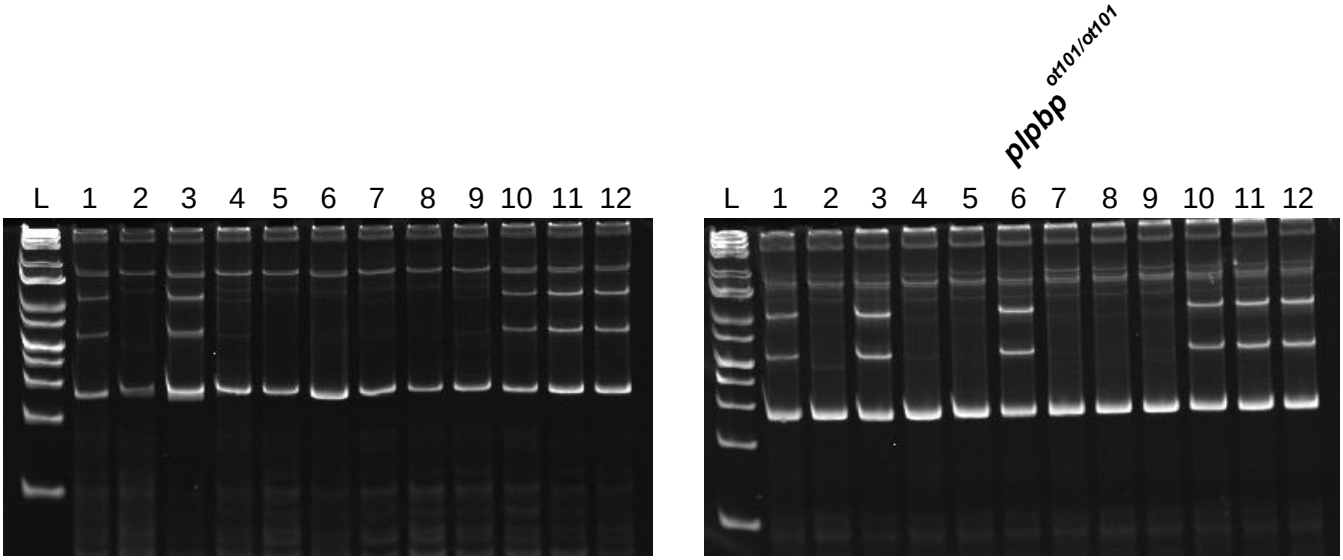
	PL	PM	PN	PA	PLP
Patient 4	39	<2.7	0,1	130	1,1
Patient 3, treated	276	<2.7	0,1	365	685
Reference interval, untreated (Mathis *et al.*, 2016)	6.6-54	<2.7	<1	6.7-84	16-269



Supplementary Figure 5: Protein sequence alignment of PLPHP orthologues from several species (RefSeq identifiers shown in the sequence labels). Residues found mutated in patients are highlighted in red (missense mutations). Secondary structure as in the yeast orthologue (PDB 1CT5) is shown under the alignment. Consensus sequence is also shown. Image produced using Jalview (Waterhouse *et al.*, 2009) .

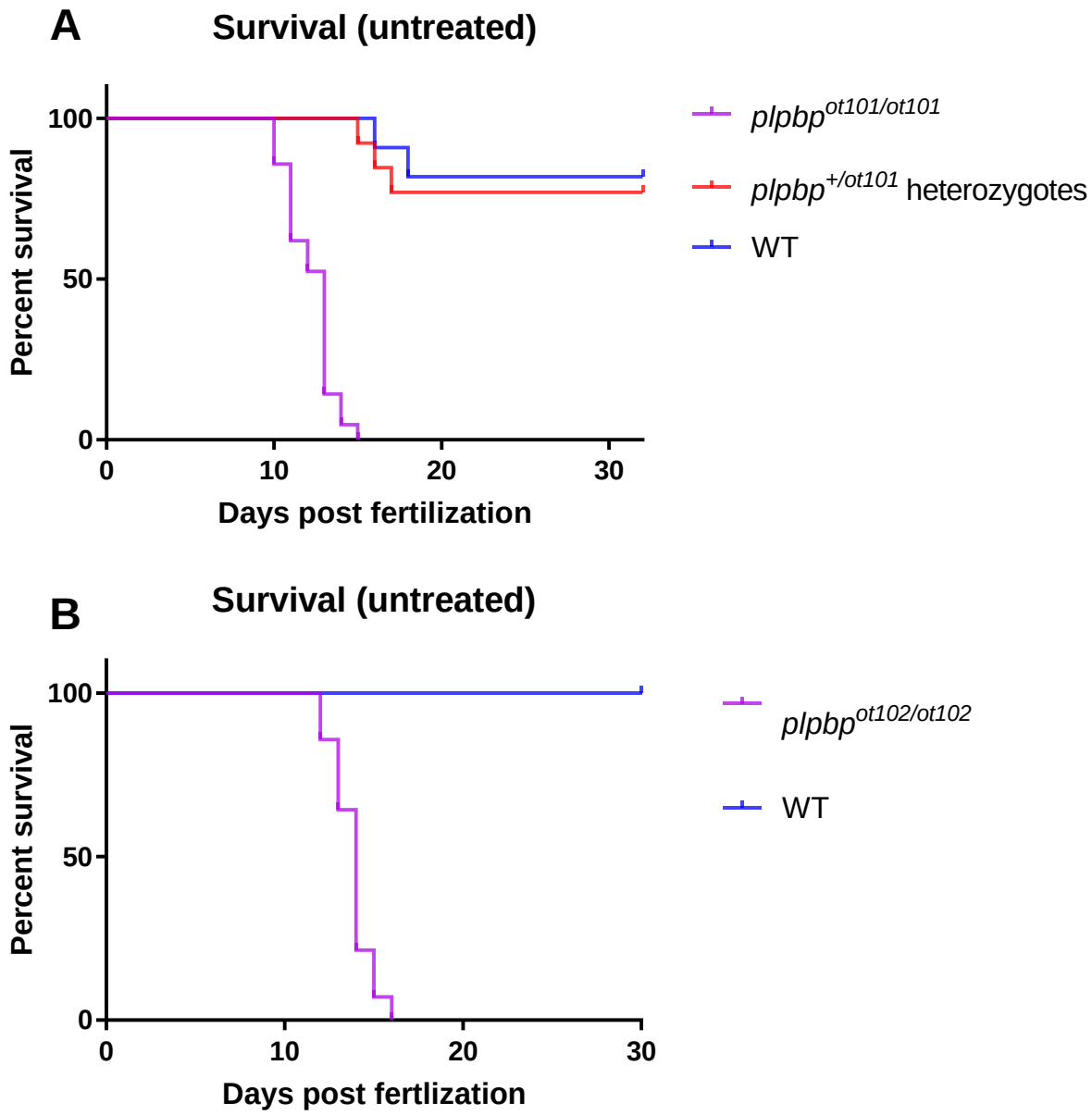


Supplementary Figure 6: Example HMA-PAGE gel showing the four genotypes: compound heterozygous mutants (*plpbp*$^{-/-}$), heterozygotes (*plpbp*$^{+/ot101}$ and *plpbp*$^{+/ot102}$), and WT. Each genotype can easily be distinguished from a single gel run by denaturing the PCR products and running on a PAGE gel, forming heteroduplexes in non-homozygous genotypes.

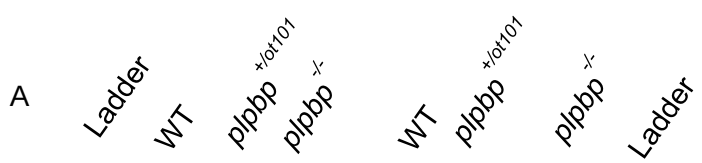


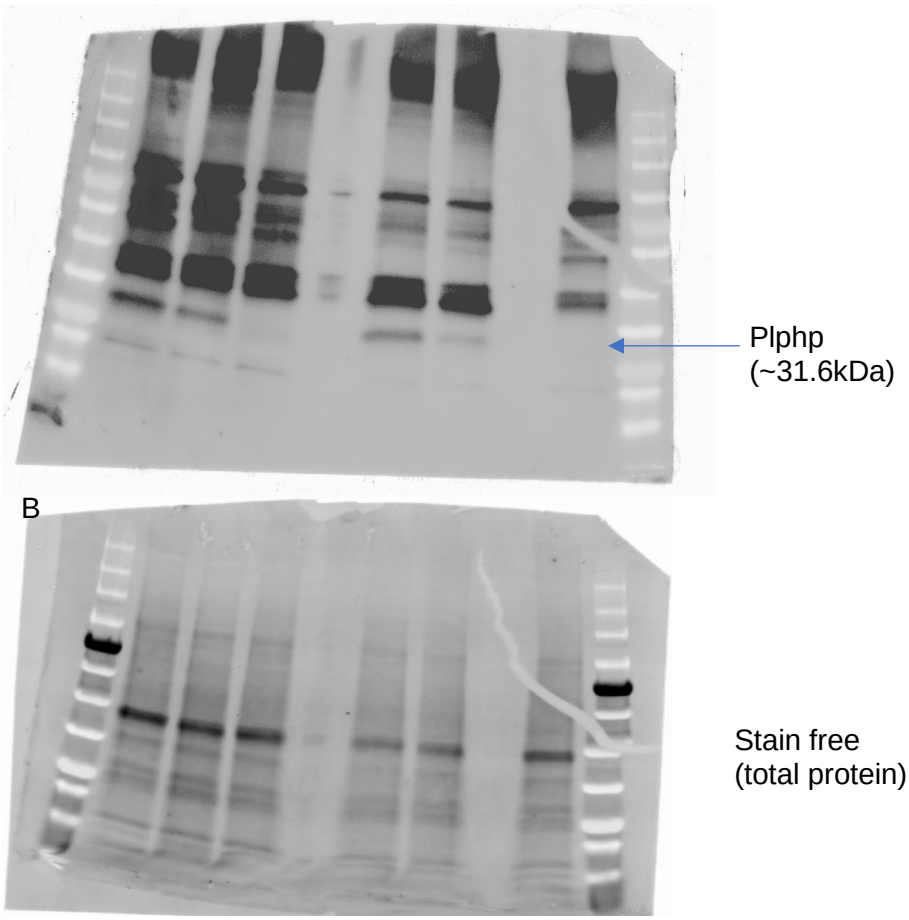
Gel 1 Gel 2: PCR product mixed with WT

Supplementary Figure 7: HMA-PAGE gels from a crossing of *plpbp*$^{+/ot101}$ heterozygous F2s. Since homozygous mutant genotypes do not form heteroduplexes, a second round of HMA-PAGE must be run by mixing the PCR products with PCR product from known WT fish (right), to distinguish mutants from WT larvae. Given the need for rapid genotyping, a compound heterozygous mutant model was used for most experiments, however homozygous mutants were shown to have the same phenotype (see Supplementary Figure 8).

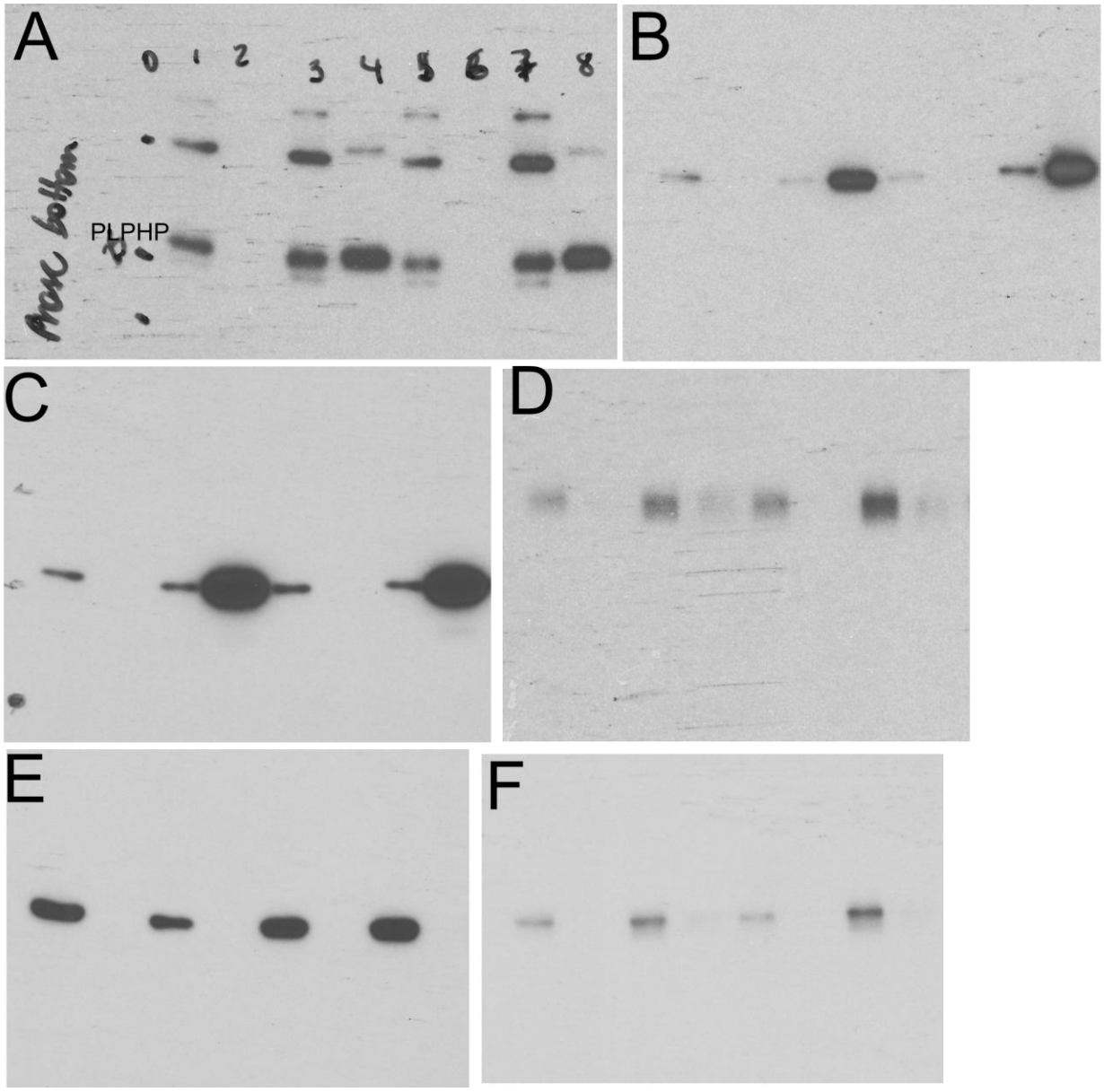


Supplementary Figure 8: Mutant zebrafish larvae homozygous for either the 4bp (A) or 2bp (B) frameshift mutations show a similar survival pattern as the compound heterozygous larvae *plpbp*$^{ot101p/ot102}$ (Fig. 4C). Additionally, these larvae start seizing by 10dpf/11dpf. We thus did subsequent experiments with compound heterozygotes due to ease of genotyping (Supplementary Fig. 6 and 7).





Supplementary Figure 9: whole image of WB showing no Plphp protein (~31.6kDa) detected in *plpbp*$^{-/-}$ larvae. The left three lanes represent pools of four larvae, whereas the lanes on the right are individual larvae. (A) chemiluminescent blot (B) stain free blot.



Supplementary Figure 10: uncropped western blots of HeLa cells (wild type (lanes 1,2, 5,6) and HA-tagged mitochondria (lanes 3, 4, 7,8), ran as whole cell lysates (lanes 1, 3, 5, 7) or immunoprecipitated with anti-HA (lanes 2, 4, 6, 8). Blots show (A) PLPHP, (B) SHMT2, (C) VDAC, (D) LAMP2, (E) GAPDH, and (F) GOLGIN-97.

Supplemental References

Azzopardi D. Clinical applications of cerebral function monitoring in neonates. Semin Fetal Neonatal Med 2015; 20(3): 154-63.

Beaulieu CL, Majewski J, Schwartzentruber J, Samuels ME, Fernandez BA, Bernier FP, *et al.* FORGE Canada Consortium: outcomes of a 2-year national rare-disease gene-discovery project. Am J Hum Genet 2014; 94(6): 809-17.

Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, *et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 2012; 6(2): 80-92.

de Ligt J, Willemsen MH, van Bon BW, Kleefstra T, Yntema HG, Kroes T, *et al.* Diagnostic exome sequencing in persons with severe intellectual disability. N Engl J Med 2012; 367(20): 1921-9.

Dyment DA, Smith AC, Alcantara D, Schwartzentruber JA, Basel-Vanagaite L, Curry CJ, *et al.* Mutations in PIK3R1 cause SHORT syndrome. Am J Hum Genet 2013; 93(1): 158-66.

Hamilton A, Tetreault M, Dyment DA, Zou R, Kernohan K, Geraghty MT, *et al.* Concordance between whole-exome sequencing and clinical Sanger sequencing: implications for patient care. Mol Genet Genomic Med 2016; 4(5): 504-12.

Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet 2014; 46(3): 310-5.

Kosuta C, Daniel K, Johnstone DL, Mongeon K, Ban K, LeBlanc S, *et al.* High-throughput DNA Extraction and Genotyping of 3dpf Zebrafish Larvae by Fin Clipping. J Vis Exp 2018(136).

Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012; 9(4): 357-9.

Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, *et al.* Analysis of protein-coding genetic variation in 60,706 humans. Nature 2016; 536(7616): 285-91.

Lelieveld SH, Reijnders MR, Pfundt R, Yntema HG, Kamsteeg EJ, de Vries P, *et al.* Meta-analysis of 2,104 trios provides support for 10 new genes for intellectual disability. Nat Neurosci 2016; 19(9): 1194-6.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, *et al.* The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009; 25(16): 2078-9.

Liu X, Jian X, Boerwinkle E. dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions. Hum Mutat 2011; 32(8): 894-9.

Liu X, Jian X, Boerwinkle E. dbNSFP v2.0: a database of human non-synonymous SNVs and their functional predictions and annotations. Hum Mutat 2013; 34(9): E2393-402.

Mathis D, Abela L, Albersen M, Burer C, Crowther L, Beese K, *et al.* The value of plasma vitamin B6 profiles in early onset epileptic encephalopathies. J Inherit Metab Dis 2016; 39(5): 733-41.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, *et al.* The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010; 20(9): 1297-303.

Pena IA, Roussel Y, Daniel K, Mongeon K, Johnstone D, Mendes HW, *et al.* Pyridoxine-Dependent Epilepsy in Zebrafish Caused by Aldh7a1 Deficiency. Genetics 2017; 207(4): 1501-18.

Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, *et al.* Integrative genomics viewer. Nat Biotechnol 2011; 29(1): 24-6.

Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods 2014; 11(4): 361-2.

Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, *et al.* dbSNP: the NCBI database of genetic variation. Nucleic Acids Res 2001; 29(1): 308-11.

Tanaka AJ, Cho MT, Millan F, Juusola J, Retterer K, Joshi C, *et al.* Mutations in SPATA5 Are Associated with Microcephaly, Intellectual Disability, Seizures, and Hearing Loss. Am J Hum Genet 2015; 97(3): 457-64.

Tarailo-Graovac M, Shyr C, Ross CJ, Horvath GA, Salvarinova R, Ye XC, *et al.* Exome Sequencing and the Management of Neurometabolic Disorders. N Engl J Med 2016; 374(23): 2246-55.

van der Ham M, Albersen M, de Koning TJ, Visser G, Middendorp A, Bosma M, *et al.* Quantification of vitamin B6 vitamers in human cerebrospinal fluid by ultra performance liquid chromatography-tandem mass spectrometry. Anal Chim Acta 2012; 712: 108-14.

Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics 2009; 25(9): 1189-91.