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Supplemental Data

Biallelic Mutations in *LIPT2* Cause a Mitochondrial Lipoylation Defect Associated with Severe Neonatal Encephalopathy

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Supplemental Note: Case Reports

Individual 1 (P1), a boy, was the first child of non-consanguineous parents of French (mother) and Ivory Coast (father) origin. He was born after an uneventful pregnancy and spontaneous delivery at term with normal birth parameters (body weight 3,750 g, length 51 cm, head circumference 35 cm). Apgar scores were 9/10/10. From birth on, he presented with truncal hypotonia, spastic tetraparesis, and dystonia. Brain MRI showed marked supra-tentorial cortical atrophy with ventricular dilatation as well as bi-frontal white matter abnormalities and delayed myelination (Figure 1A middle). MRS spectroscopy with a long TE (144) showed a lactate peak (Figure 1B). This clinical and radiological presentation led us to suspect a mitochondrial disease. At age 2.5 years, the affected individual was bed- and wheelchair-bound with no head control. He could neither sit unaided nor speak or understand simple orders. He was otherwise fully conscious and alert, and he could smile at times and follow with his eyes. His weight was -2.5 SD, his length was at the median, and his head circumference (HC) was -4 SD. Major swallowing difficulties led to gastrostomy. At the age of 3 years, he developed complex epilepsy treated by oxcarbazepine (Trileptal®), clobazam (Urbanyl®), and topiramate (Epitomax®). Electroencephalography at the age of 7 years showed in the awake state, an absence of occipital alpha rhythm, polyrhythmic background activity with diffuse alpha, theta and rare delta rhythms, multifocal spikes and sharp waves, sometimes in bursts or rhythmic sequences, with right posterior predominance (Figure S3A). Cardiac ultrasonography, audiological and ophthalmological examination were normal. At the age of 7 years and 9 months, the ventricular dilatation and sub-tentorial cortical atrophy were increased. No episodes of metabolic decompensation have been documented.

Affected individual 2 (P2) and 3 (P3) are born from healthy parents of German origin. P2, a boy, was born at the 39th week of gestation by caesarean section due to a gestational diabetes of the mother (BMI 40). Apgar scores were 9/10/10 and birth parameters within normal range (body weight 3,180 g, length 51 cm, head circumference 38 cm). Lactate concentration was 3.7 mmol/L initially and increased to values between 8 and 10, in a single measurement up to 111 mmol/L (N<1.8 mmol/L). The child showed severe muscular hypotonia. Newborn reflexes could only partially be induced. Brain sonography showed mildly enlarged lateral ventricles at the third day of life (data not shown), which was confirmed by brain MRI performed at 4 weeks showing also periventricular cystic changes. According to

sonographic investigations these changes were progressive. EEG performed at the age of 7 days showed neither awake-sleep differentiation nor physiological grapho-elements, background activity was persistently low-voltaged with amplitudes under 15 μV (Figure S3B). Myoclonic twitches were observed during sleep, however without EEG correlate. Otoacoustic emission was pathologic on both sides. Newborn screening did not reveal any abnormalities. A ketogenic diet was started, which resulted in a decrease of lactate concentrations but also lead to a weight loss and worsening of his condition. The child died at the age of 2 months.

Affected individual 3 (P3), the older sister of P2, was born spontaneously at 34 weeks of gestation. Apgar scores were 5/5/9, birth weight 2,400 g. She showed problems in respiratory adaptation and was ventilated by continuous positive airway pressure (CPAP) for 3 hours. Severe lactic acidosis up to 17 mmol/L was observed and further on respiratory decompensation and signs suspicious of epilepsy treated by phenobarbital. EEG at the (chronological) age of 3 weeks was abnormal lacking physiological grapho-elements and sleep-wake differentiation, showing reduced amplitudes (below 20 μV), however on phenobarbital treatment. There was no pattern typical for epilepsy. EEG at the age of 5 months was still pathological without physiological elements, however showing a richer background activity. At the age of 6 months multifocal spikes and sharp waves were noticed (Figure S3 C, D and E). Brain sonography at one week was suspicious for intraventricular hemorrhage with hypoechogenic areas adjacent to the 3rd and 4th ventricle (data not shown). At the age of 3 weeks micro- and macrocystic changes of the parietal brain parenchyma on both sides were noted. Brain MRI showed enlarged lateral ventricles and formation of cysts in the cortex and white matter of the whole cerebral structures. Furthermore, gyration of the cerebral hemispheres was decreased (Figure 1A bottom). Echocardiography showed a mild septum hypertrophy but normal ventricular function. The child died at the age of 7 months.

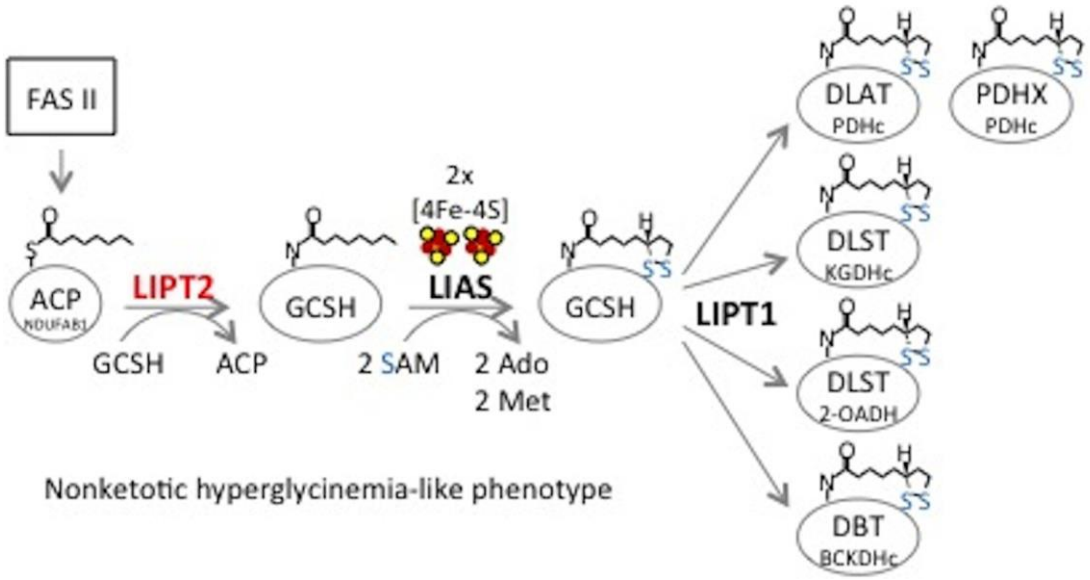

Figure S1. Synthesis of the lipoate cofactor in mitochondria.

Mitochondrial fatty acid synthesis (FAS II) forms octanoic acid bound to the mitochondrial acylcarrier protein (ACP) encoded by *NDUFAB1*. LIPT2 transfers the octanoic acid residue to the glycine cleavage H protein (GCSH). Lipoate is formed by the action of lipoic acid synthetase (LIAS), which uses S-adenosylmethionine (SAM) as a substrate and depends on two [4Fe-4S] iron-sulfur cluster cofactors. Finally LIPT1 transfers the lipoate residue from GCSH to E2 subunits of either pyruvate dehydrogenase complex (PDHc; pyruvate oxidation), α-oxoglutarate dehydrogenase complex (KGDHc; Krebs cycle), 2-oxoadipate dehydrogenase (2-OADH; lysine degradation), and branched-chain ketoacid dehydrogenase complex (BCKDHc; leucine, isoleucine, valine catabolism). Furthermore lipoate is transferred to the PDHX subunit of PDHc.

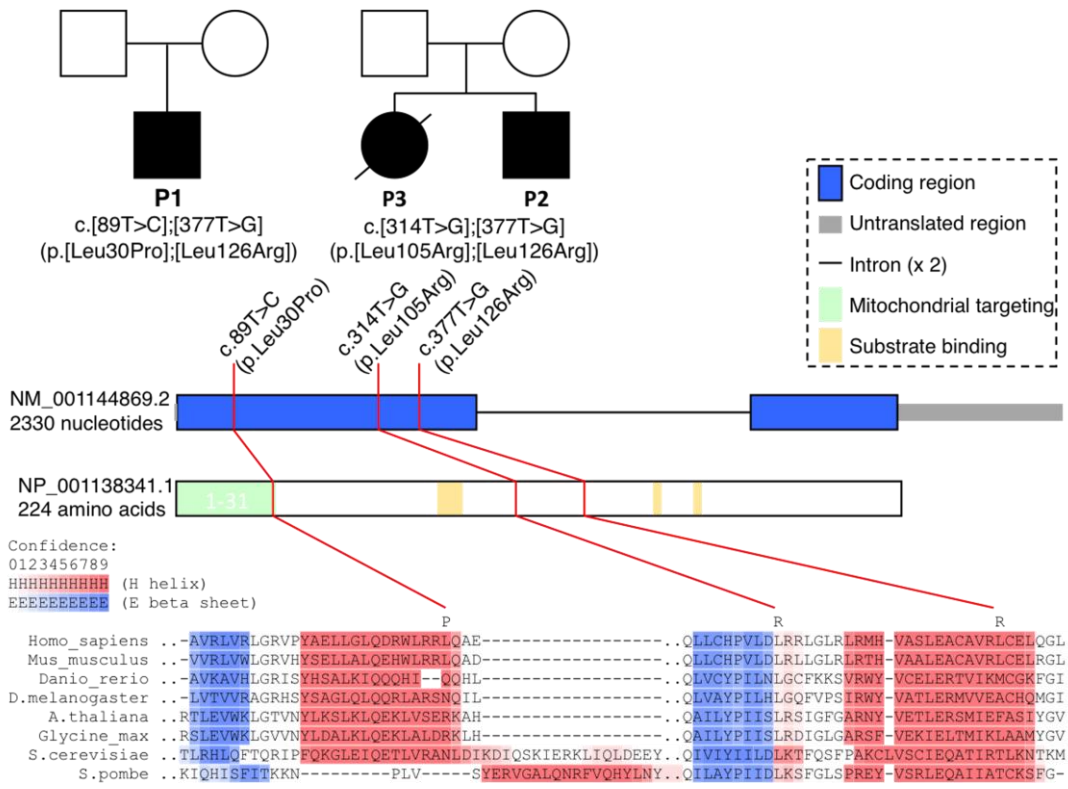

Figure S2. Pedigrees of affected individuals with LIPT2 deficiency and localization of mutations.

Three missense mutations have been identified showing different degree of phylogenetic conservation. Alignment was performed by ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Secondary structure prediction was performed by Ali2D (https://toolkit.tuebingen.mpg.de/ali2d/)

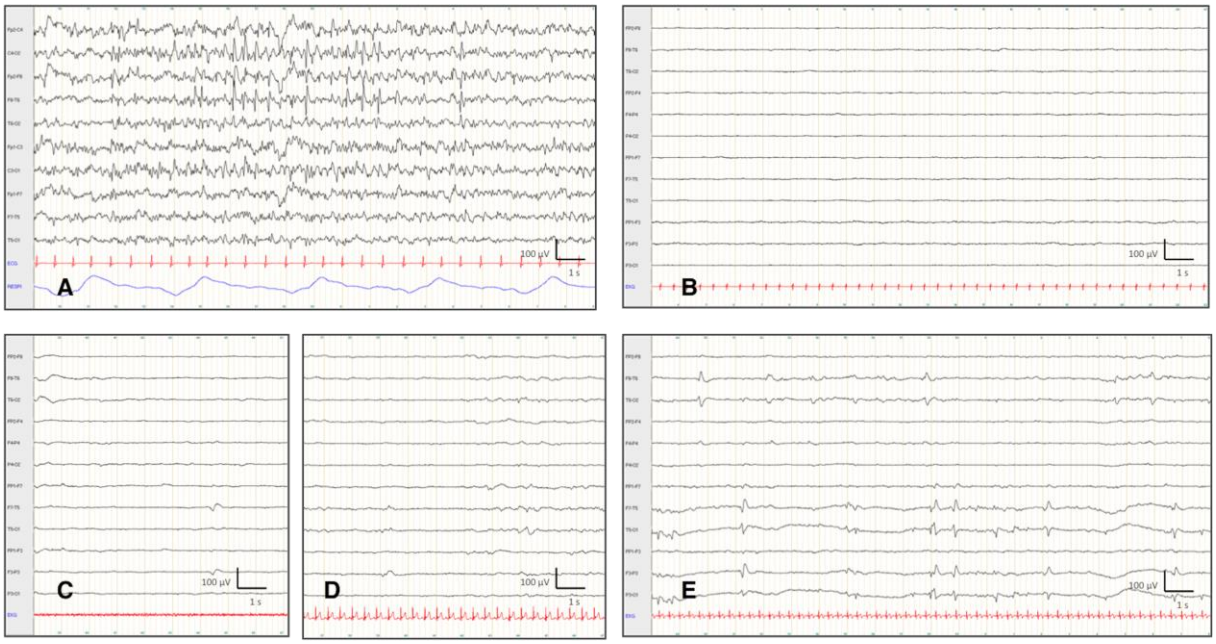

Figure S3: Electroencephalography in individuals with LIPT2 deficiency

A: P1 at the age of 7 years. Awake state. Polyrhythmic background activity with diffuse alpha, theta and rare delta rhythms, multifocal spikes and sharp waves, sometimes in bursts or rhythmic sequences, with right posterior predominance. **B: P2 at the age of 7 days.** Clinically quiet sleep. Background activity undifferentiated compared to awake state and active sleep, low-voltaged with amplitudes under 15 µV. **C: P3 at the age of 3 weeks (chronological age).** Clinically quiet sleep. Background activity undifferentiated compared to awake state and active sleep, low-voltaged with amplitudes under 20 µV. **D: P3 at the age of 5 months (chronological age).** Clinically sleep. Low-voltage activity, no physiological sleep figures. **E: P3 at the age of 6 months (chronological age).** Awake state. Low-voltage background activity, multifocal low amplitude spikes and sharp waves with left parieto-temporal predominance.

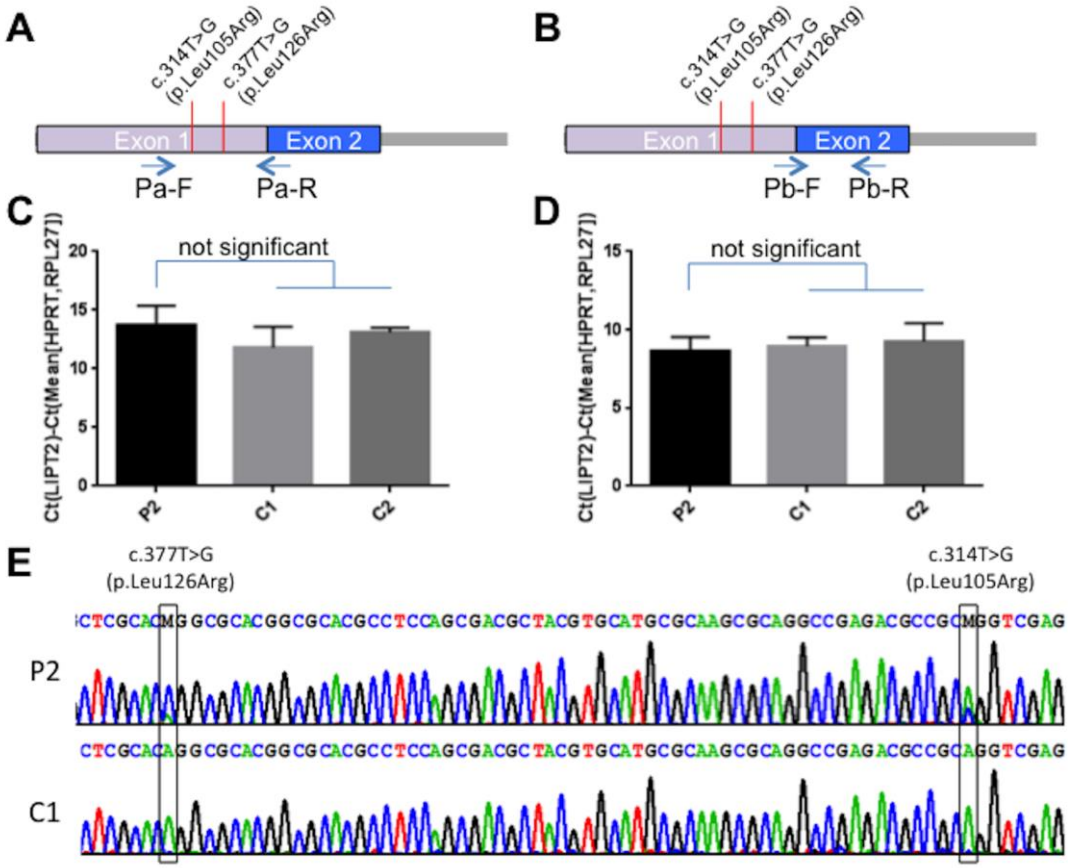

Figure S4. Expression analysis of *LIPT2* in fibroblasts of P2.

Quantitative real-time PCR of *LIPT2* cDNA was performed with the following set of primers: (A, C) Pa-F (forward), 5‘-GGCCTGGCCACCTTCCAC-3‘ and Pa-R (reverse), 5‘-CCACAGCGGACTCCGATC-3‘ as well as (B, D) Pb-F (forward), 5‘-TCTGCGCGATCGGAGTCC-3‘ and Pb-R (reverse), 5‘-GTGGCATTACTTCTTCCACGG-3‘. Each experiment was done in duplicate and was repeated once (4 data points for each measurement). Delta Ct (cycle threshold) values were calculated in relation to two housekeeping genes (*HPRT*, *RPL27*). No significant differences in expression of *LIPT2* between P2 and two controls (C1, C2) was found when analyzed by one-way ANOVA. The *LIPT2* PCR product (A) was analyzed by Sanger sequencing with the reverse primer (E) and revealed the presence of both variants c.377T>G (p.Leu126Arg) and c.314T>G (p.Leu105Arg), the latter in a bit lower amount.

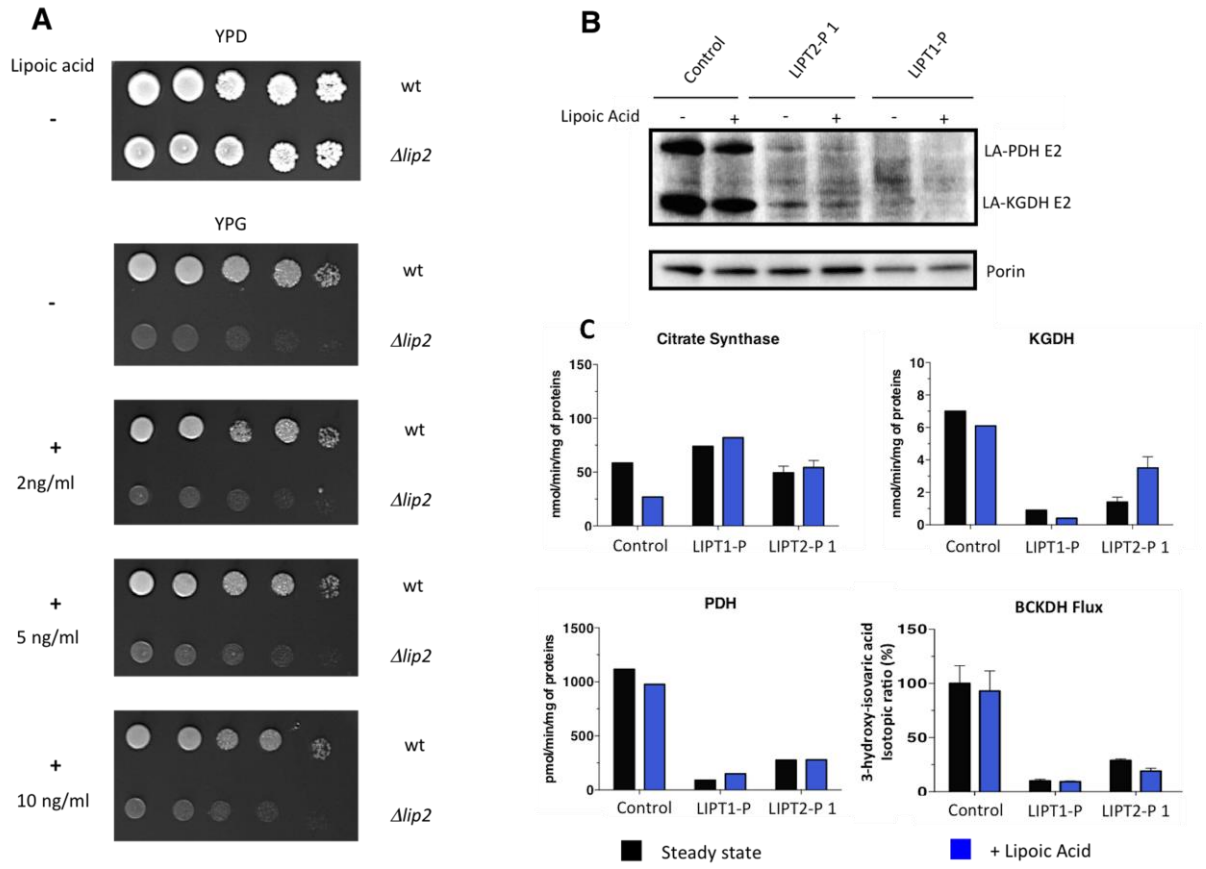

Figure S5: Effects of lipoic acid supplementation on Δ*lip2 (LIPT2 ortholog)*-deleted yeast strain and fibroblasts.

A: Δ*lip2*-deleted yeast strain failed to grow on YPG medium at 28 °C. Growth was not restored after lipoic acid supplementation. **B, C:** Fibroblasts were supplemented with 100 µM lipoic acid during three weeks. Lipoylation (studied in triplicates; anti-lipoic acid : ab58724, Abcam®, 1:1600; anti-porin antibody: ab14734, Abcam®, 1:1000) **(B)** and activities of α-oxoacid dehydrogenases (presented as means +/- SD) **(C)** were evaluated in basal conditions and after lipoic acid supplementation. They did not improve after treatment.

Table S1. Main clinical features in affected individuals.

	P1	P2	P3
Age of onset	Neonatal	Neonatal	Neonatal
Current age	10 years-old	Deceased at the age of 2 months, without any development	Deceased at the age of 7 months, without any development
Clinical and biological findings at presentation	Truncal hypotonia Spastic tetraparesis Dystonia Epilepsy Microcephaly Delayed psychomotor development Hyperlactatemia	Severe muscular hypotonia Lactic acidosis	Respiratory distress Lactic acidosis
MRI findings	Supra-tentorial cortical atrophy Ventricular dilatation Bifrontal white matter abnormalities Delayed myelination Progressive thalamus and putamen hyperintensities	Enlarged lateral ventricules Periventricular cystic changes	Enlarged lateral ventricules Cortical and white matter cystic abnormalities Decreased gyrification of the cerebral hemispheres

Table S2. Relevant plasma, urinary and CSF metabolites in LIPT2 affected individuals.

Reference intervals for each individual are provided as the ranges of the age-matched reference population. In P2, investigations were performed under ketogenic diet.

LIPT2-Individuals	**P1**					**P2**	**P3**
Age	**2.5 y**	**5 y**		**8 y**		**Neonatal period**	
Blood (µmol/L)							
Glutamate N (P1, 2.5y): 2-118 N (P1, ≥ 5y): 19-95 N (P2): 30-110 N (P3): 27-168	80	34	39	128	194	161	109
Glutamine N (P1, 2.5y): 334-666 N (P1, ≥ 5y): 368-692 N (P2): 370-958 N (P3): 279-1071	726	679	645	793	784	552	520
Proline N (P1, 2.5y): 61-285 N (P1, ≥ 5y): 92-240 N (P2): 107-330 N (P3): 82-367	224	160	217	238	269	202	459
Alanine N (P1, 2.5y): 134-301 N (P1, ≥ 5y): 148-412 N (P2): 131-460 N (P3): 144-450	542	239	285	284	365	243	781
Glycine N (P1, 2.5y): 149-301 N (P1, ≥ 5y): 134-314 N (P2): 224-514 N (P3): 138-381	419	372	503	571	595	250	456
Valine N (P1, 2.5y): 158-310 N (P1, ≥ 5y): 179-327 N (P2): 80-210 N (P3): 62-238	127	100	107	110	111	167	55
Isoleucine N (P1, 2.5y): 37-89 N (P1, ≥ 5y): 47-86 N (P2): 26-80 N (P3): 18-79	33	30	20	25	30	48	14

Leucine N (P1, 2.5y): 68-168 N (P1, ≥ 5y): 86-182 N (P2): 46-160 N (P3): 59-138	57	55	48	46	52	85	28
Lysine N (P1, 2.5y): 113-269 N (P1, ≥ 5y): 90-262 N (P2): 92-310 N (P3): 68-286	223	153	178	189	205	145	162
Blood (mmol/L)							
Lactate N < 1.8 (P1, P3)	4.5					3.7-111	5-17
Pyruvate N < 0.14 (P1) N: 0.06-0.1 (P3)	0.07						0.167
CSF (mmol/L)							
Lactate N < 2.2	1.4						3.1
Pyruvate N < 0.14	0.07						0.167
Urine (µmol/mmol creatinine)							
Lactate N (P1) < 76 N (P3) < 217	94						30160
Pyruvate N: ND							3329
α-oxoglutarate N (P1) < 79 N (P3) < 87	78						465
Succinate N < 97	25						
Fumarate N < 10	4						
3-hydroxybutyrate N < 385						1068	
Glutarate N < 8						39	
2-hydroxyglutarate N < 30						204	
3-hydroxyglutarate N < 8						22	
2-methyl-2,3-dihydroxybutyrate N < 8						28	
Uracil N < 29						57	

Table S3: Enzyme activities in fibroblasts from affected individual LIPT2-P1, LIPT2-P2, LIPT2-P3 and controls.

α-oxoacid dehydrogenases activities measured in fibroblasts showed deficient PDHc and KGDHc activities in P1, P2 and P3 with LIPT2 deficiency and an individual with *LIPT1* mutations. [(a)] adapted from [(7)]

Enzyme activities (pmol/min/mg protein) in fibroblasts

	LIPT2-P1	**Control**	**LIPT1-P** [(a)]	**Control** [(a)]
KGDHc	1400	9700	900	7000
PDHc	276	945	90	1117
Citrate synthase	49400	44500	27000	48000
Isocitrate dehydrogenase	14900	14300	21000	23000

Enzyme activities (nmol/mg protein/min) in isolated fibroblast mitochondria from LIPT2-P2

	LIPT2-P2	**Normal Range**
PDHc	1.7	6.0-19.7
Citrate synthase	173	225-459

Enzyme activities (mU/U citrate synthase) in fibroblasts from LIPT2-P3

	LIPT2-P3	**Normal Range**
PDHc	3.6	9.7-36

Table S4: Polarographic studies in fibroblasts from affected individual LIPT2-P1.

The polarographic assay (nmol O_2/min/mg protein) in LIPT2-P1 fibroblasts showed reduced oxygen consumption using pyruvate as substrate.

	LIPT2-P1	**Control**
Pyruvate+malate	1.9	10.0±1.5
Pyruvate+malate+glutamate	3.2	9.3±1.5

Table S5: Prediction of pathogenic relevance of *LIPT2* mutations

Mutation	**c.89T>C**	**c.314T>G**	**c.377T>G**
Chromosomal location	chr11:74204660A>G	chr11:74204435A>C	chr11:74204372A>C
Protein	p.Leu30Pro	p.Leu105Arg	p.Leu126Arg
1000 Genomes (heterozygous)	1	0	0
ExAC	0	0	0
MutationTaster Score (max. 1)	0.999989713071375	0.999832214029496	0.686451197788724
MutationTaster Prediction	Disease causing	Disease causing	Polymorphism
Provean Score (cutoff<-2.5)	-2.06	-5.96	-3.42
Provean Prediction	Neutral	Deleterious	Deleterious
Polyphen-2 Score (max. 1)	0.897	1	0.985
Polyphen-2 Prediction	Possibly damaging	Probably damaging	Probably damaging