Supplementary note

Clinical summary for family UK

UK proband II-2 was found to have optic atrophy at 8 years of age after a routine optometry exam. Visual acuities were 20/30 right eye (OD) and left eye (OS). She had gradual progressive central visual loss and by 19 years of age visual acuities were 20/80 OD, 20/120 OS correcting to 20/60 OD and 20/80 OS with -0.5 refraction. Anterior segments, intraocular pressures and retinas were normal, but symmetrical predominantly temporal bilateral optic atrophy was recorded. Visual field testing showed patchy scotomata within the central 30 degrees, but no field constrictions, nor blind spot enlargements. Her sister was diagnosed with optic atrophy at 5 years of age; she was examined because of her sister's diagnosis. Her visual acuities at that time were 20/40 OD and 20/80 OS (left amblyopia). Examination of their parents revealed normal fundi and normal vision apart from the mother who had right squint and dense amblyopia with visual acuities of <20/200 OD, and 20/30 OS. Both parents were in good health. The proband II-2 was registered as sight impaired in her 30's and had progressive slow visual deterioration.

The proband and sibling were fit and well without any systemic disorders until their 40's. II-2 presented at the age of 43 with symptoms of altered sensation and stiffness in her legs. On examination she had increased tone in both legs with brisk reflexes and extensor plantars associated with a reduction in light touch sensation and join position sense.

Both Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) of the brain and spinal cord were normal. Nerve conduction studies of the legs revealed unrecordable sensory nerve action potentials, markedly depressed compound muscle action potentials and slowed motor nerve conduction velocities, all consistent with an axonal motor and sensory polyneuropathy. CSF examination did not reveal any abnormalities. Muscle biopsy showed regular muscle fibres with no evidence of inflammation or necrosis, no ragged red fibres, normal oxidative enzyme activity, no upregulation of succinic dehydrogenase (SDH; mitochondrial enzyme), all fibers expressed cytochrome oxidase; there was no evidence of denervation/renervation and no evidence of major mitochondrial rearrangements. Genetic testing for mutations in OPA1 (sequencing and Multiplex Ligation-dependent Probe Amplification-MLPA), MFN2, mitochondrial genome mutations m.3243A>G and m.1351G>A, PolG sequencing and targeted mutation screening of PEO1 were all negative.

Clinical summary for family IT

IT proband II-3 is a 51 year-old man from the Italian island Sardinia, born to non-consanguineous parents, who presented poor vision bilaterally since he was 2 years old. Vision deteriorated over the years and at 14 years he started having difficulties in walking progressively worsening over time, associated with distal muscular atrophy. At about the same time he also started having speech difficulties. At 18 years of age he received the diagnosis of HMSN type VI, and the neurologic exam showed bilateral optic atrophy and slight bilateral deafness, cerebellar signs including nystagmus and intentional tremor with dysmetria, ataxic and stepping gait, marked hypotrophy of

antero-lateral muscles at lower limbs, pes cavus, hypopallestesia at lower limbs, brisk tendon reflexes except for Achilles reflex, which was absent. EMG showed axonal sensorimotor polyneuropathy. Brain MRI at 28 years of age was remarkable for diffuse brain and cerebellar atrophy with hyperintensity T2-weighted cerebellar white matter changes, and marked chiasm atrophy. At this time the patient also received a diagnosis of Leber Hereditary Optic Neuropathy (LHON) in combination with Charcot-Marie Tooth (CMT).

We observed this patient when he was 44, and our neurologic exam was similar to the previous demonstrating severe visual loss and optic atrophy, marked worsening of gait ataxia and steppage (standing and walking required support), marked pes cavus, hypo-apallestesia at lower limbs, with severe hypotrophic legs (see Figure 1d), and absent deep tendon reflexes. Creatine kinase was slightly elevated (225 U/L; normal <170), and both basal and post-exercise lactic acid levels were at the upper end of the normal range (basal=20 mg/dl, post-exercise=23.6 mg/dl; normal 5.8-22 mg/dl). A brain CT scan disclosed small calcifications in the basal ganglia bilaterally. Muscle CT showed severe muscle atrophy with fibrotic and fat substitution of antero-lateral and posterior muscles of the legs. EMG confirmed a severe axonal sensorimotor polyneuropathy. Muscle biopsy was described as essentially normal except for slight neurogenic signs, in particular there were no RRF, nor COX negative fibers at histoenzymatic stains. Audiometry had selective loss for high tones. Brain MR-spectroscopy showed increased lactic acid with neurodegenerative changes in the cerebellum and optic radiations. Cardiac examination and EKG were normal. Molecular

investigation excluded the occurrence of the canonical LHON mtDNA mutations, as well as the complete mtDNA and OPA1 sequences were also normal.

The proband's sister II-1 has an autoimmune disorder including thyroiditis and painful fibromyalgia with muscle fatigability. Her neurologic exam was normal as well as the EMG. The family history was positive for autoimmune disorders and deafness, but the clinical syndrome of the proband was unique to him.

Clinical summary for family PL

Patients II-8, II-7, and II-5 are siblings of a consanguineous marriage (first cousins) with normal vision and development through the first 1-2 years of life. They developed progressive developmental delay and vision loss with optic nerve pallor and normal fundus, loss of gross and fine motor skills, hypertonia, hyperreflexia, and ataxia, and were wheelchair-bound by late childhood. Another sibling died in early childhood with similar symptoms per report, though not evaluated at our hospital.

Brain MRI of patient II-5 was notable for prominent extra-axial spaces at 22 months of age. By 5.2 years, bilateral cerebellar encephalomalacia developed with increased gliosis signal and vermal sparing. At 11.5 years of age, brainstem and cerebral volume loss were found along with progressive bilateral optic nerve atrophy. On MR spectroscopy, a lactate peak was noted in the cerebellum. Electromyography (EMG) performed on II-5 at age 5 revealed absent motor responses with maximal stimulation of the left tibial and bilateral peroneal nerves. However, motor responses in the right median nerve were normal. Sensory studies were normal for right median and right sural nerves, though conduction velocity of the right median was mildly slow. Sural

nerve and muscle biopsy at age 8 demonstrated abnormal nerve tissue, autonomic denervation with onion-bulb morphology and rare foci of axonal demyelination and degeneration. Electron microscopy of muscle biopsy showed normal myofibrils and sarcoplasmic reticulum, abnormal peripheral nerve axons, and increased mitochondria with normal morphology (data not shown). Elevations in urine 3-methylglutaconic acid (3-MG) were detected in II-5 (13.2 mg/g creatinine; age 11.5 years), II-7 (27.8 mg/g creatinine; age 6.0 years), and II-8 (56.1 mg/g creatinine; age 13 months).

Clinical summary for family US

The index patient was born to a 26 year old gravidity 3, parity 2 mother and 34 year old father at 39 weeks by spontaneous vaginal delivery after an uncomplicated pregnancy. The family history was noncontributory and without evidence of consanguinity. Growth parameters at birth were normal. Her delivery was complicated by meconium aspiration. APGAR scores were 5 and 6 at 1 and 5 minutes, respectively. She had significant hypotonia and contractures at birth. On exam, the infant had bitemporal narrowing, mildly overriding sutures, crimped posterior helices, persistent helical root extending to the antihelix, upturned nose with bulbous tip, tented upper lip, narrow palate, flat midface, inverted nipples, tapered fingers with extra flexion creases, hypoplastic thenar and hypothenar eminences with palms measuring 4.2cm and midfinger length of 3cm, and bilateral knee contractures. Ophthalmologic evaluation was significant for severely small, pale optic discs with a wide area of depigmented retina consistent with optic hypoplasia and secondary foveal hypoplasia. She required intubation due to increased apneic spells and worsening acidosis. She failed extubation on multiple attempts and

eventually required a tracheostomy. An EEG showed multifocal discharges, but without electrographic seizures. Initial MRI imaging revealed moderate cerebellar atrophy with mild atrophy of the brainstem and mild volume loss involving the pons and adjacent cerebellar peduncles. Subsequent MRI at three months of age showed significant progression with severe atrophy of the bilateral cerebellar hemispheres and brainstem as well as diffuse volume loss without evidence of hemorrhage or restricted diffusion. Electromyography with nerve conduction studies showed generalized neuropathy. A muscle biopsy demonstrated myopathy with small fibers and relative atrophy of type I fibers. The patient expired at 15 weeks of life.

Normal laboratory testing included carbohydrate deficient transferrin, very long chain fatty acid analysis, 7-dehydrocholesterol, serum copper level, TSEN54 sequencing, POLG1/2 sequencing and LAMA2 sequencing. Creatine kinase was initially elevated, but subsequently normalized. A SNP microarray revealed a de novo 2.9Mb-deletion from 6q22.33q23.2, which was considered nonpathogenic. Based on other reports of patients with a similar deletion and the genes within this area, the 6q deletion did not sufficiently explain the spectrum of findings in our patient.

Supplementary Tables

Supplementary Table 1. Pathogenic predications for SLC25A46 mutations

Variant	rsID	EVS	PhastCons	Polyphen2	GERP	CAAD	MutationAssessor	MutationTaster	SIFT
c.165_166insC p.His56fsX94									
c.746G>A p.Gly249Asp	rs200725073	A=1/G=13001	0.999	0.826	5.96	15.15	L	D	T
c.882_885dupTTAC p.Asn296fsX297									
c.998C>T p.Pro333Leu		C=13004	0.882	1.0	4.82	22.1	H	D	D
c.1005A>T p.Glu335Asp		A= 13004	0.999	0.993	1.98	20.2	N	D	T
c.1018C>T p.Arg340Cys		C = 13004	1.000	0.999	4.82	18.44	H	D	D

Exome Variant Server (EVS): Frequency of observed alleles in NHLBI Exome Project data set. PhastCons: Probability that each nucleotide belongs to a conserved element, based on hidden Markov-based method. PolyPhen2: benign (0 to 0.446), possibly damaging (0.447 to 0.908), probably damaging (0.909-1). GERP(Genome Evolution Rate Profiling): measurements from -12.3 to 6.17, with higher scores indicating higher conservation. CADD(Combined Annotation Dependent Depletion): C-score of greater or equal to 10 indicates that these are predicted to be the 10% most deleterious substitutions that you can do to the human genome, a score of greater or equal to 20 indicates the 1% most deleterious; MutationAssesor: N=Neutral, L=Low Impact, M=Medium Impact, H=High Impact; MutationTaster and SIFT: T=Tolerated, D=Damaging.

Supplementary Table 2a. Reciprocal BLASTP Matches

Query	Top Hit	Identity score	Query coverage	Expected value
SLC25A46 (NP_620128.1) Human to *S. japonicus* (taxid:4897)	Ugo1	24%	59%	5e-5
Ugo1 (XP_002172607.1) *S. japonicus* to Human	SLC25A46	24%	55%	9e-4

Supplementary Table 2b. BLASTP Query Ugo1p *S. cerevisiae* (NP_010758.3)

Searched Set	Top Hit	Length	Identity score	Query coverage	Expected value
S. cerevisiae	Ugo1p	502	100%	100%	0
***S. pombe* (taxid:4896)**	Ugo1p	421	24%	77%	1e-25
***S. japonicus* (taxid:4897)**	Ugo1p	433	27%	70%	1e-27
Human	SLC25A46*	418	22%	47%	2.9

Supplementary Table 2c. BLASTP Query SLC25A46 Human (NP_620128.1)

Search Set	Top Hit	Length	Identity score	Query Coverage	Expected value
Mouse (*M. musculus)*	Slc25a46	418	89%	100%	0
Zebrafish (*D. rerio)*	slc25a46	405	69%	99%	0
Fruit fly *(D. melanogaster)*	CG8931	421	40%	74%	2e-79
Nematode (*C. elegans)*	Y40B1B.8	500	34%	74%	3e-57
Yeast (S. cerevisiae)	Ugo1p*	502	22%	56%	1.5

BLAST (Basic Logarithmic Alignment Search Tool) is a database designed to predict orthology based on multiple sequence comparison[1]. Query: the input sequence. Search set: species queried. Top Hit: most likely ortholog based on lowest expected value or by highest query coverage (*). Length: amino acid length of Top Hit. Identity score: percent identical amino acids between query and hit. Query coverage: percent of the total query sequence that aligns to the hit. Expected value: statistical measure that takes into account query size and the number of alignments to determine the probability that any hit happens by chance, values closer to 0 are more significant.

Supplementary Table 3. Coimmunoprecipitation Mass spectrometry results

ID	Gene	Aliases	Description	Unique peptides identified
gi 31542947	HSPD1	Chaperonin HSP60	60 kDa heat shock protein, mitochondrial	6
gi 28336	ACTB	beta'-actin	mutant beta-actin (beta'-actin)	5
gi 51476841	IMMT	mitofilin	hypothetical protein, partial [Homo sapiens]	3
gi 6650826	ALB	Serum albumin	Pro2044	2
gi 182401	SLC25A46	TB1	Mitochondrial solute carrier member 46	2
gi 292059	MTHSP75	mortalin	Heat shock 70kDa protein 9	1
gi 37460	PRSS3	Protease Serine, 3	Protease Serine 3	1
gi 914044	TXN	Surface-associated sulphydryl protein	SASP Thioredoxin homolog	1
gi 386758	GRP78	GPR78 precusor	G protein-couple receptor 78	1
gi 6912286	CASP14	Caspase 14 precursor	Apoptosis Related Cysteine Protease	1
gi 51476370	CCDC180	KIAA1529 C9orf174	Coiled-coil domain containing 180	1

Supplementary Table 4. ANOVA for motor neuron lengths (referred to in **Fig. 5d**)

Tukey's Multiple Comparison Test	Mean Diff.	q	P < 0.05	Summary
Control MO vs 0.45 pmol *slc25a48* MO	66.77	14.76	Yes	0.0001
Control MO vs 0.3 pmol *slc25a48* MO	50.57	11.52	Yes	0.0001
Control MO vs 0.3 pmol *slc25a48* MO + hRNA	14.30	3.227	No	ns
0.45 pmol *slc25a48* MO vs 0.3 pmol *slc25a48* MO	-16.19	3.579	No	ns
0.45 pmol *slc25a48* MO vs 0.3 pmol *slc25a48* MO + hRNA	-52.47	11.49	Yes	0.0001
0.3 pmol *slc25a48* MO vs 0.3 pmol *slc25a48* MO + hRNA	-36.28	8.187	Yes	0.0001

ANOVA used to determine significance of motor neuron lengths in various treatments. Embryos injected with either 0.45 or 0.3 pico moles (pmol) of morpholino have significantly shorter axons. There is no significant difference between the dosages. Embryos co-injected with 0.3 pmol and human RNA (hRNA) “rescued’, have significantly longer axons than those only injected with either 0.3 or 0.45 pmol. There is no significant difference between control and “rescued” embryos.

****

Supplementary Table 5. Oligonucleotide sequences

Species	Description	Sequence
Human	Diagnostic primers for SLC25A46 siRNA knockdown	Forward 5'-TGGTGGCAATGCCTTTTTAT-3' Reverse 5'-TCAAGGAAAGAAGCGGAAGA-3'
zebrafish	Morpholino targeting the exon 3 intron 3 junction of slc25a46	Antisense 5'-CAGTGTCTTATTCTGCATACCTGAC -3'
zebrafish	Diagnostic primers for slc25a46 morpholino knockdown	Forward 5'-GCCACTGGGTGACGACTC-3' Reverse 5'-GAAGCGGAAGAAGTCGTTTG-3'
Zebrafish	RNA *in situ* hybridization primers used to generate probe	Forward 5'-GCCTGGTTCTTTACCTGCTG-3' Reverse 5'-AAACCCAAATCGGTGTTGTC-3'