Mutations in SLC25A46, encoding a UGO1-like protein, cause an optic atrophy spectrum disorder

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Dominant optic atrophy (DOA)1,2 and axonal peripheral neuropathy (Charcot-Marie-Tooth type 2, or CMT2)3 are hereditary neurodegenerative disorders most commonly caused by mutations in the canonical mitochondrial fusion genes OPA1 and MFN2, respectively4. In yeast, homologs of OPA1 (Mgm1) and MFN2 (Fzo1)5,6 work in concert with Ugo1, for which no human equivalent has been identified thus far7. By whole-exome sequencing of patients with optic atrophy and CMT2, we identified four families with recessive mutations in SLC25A46. We demonstrate that SLC25A46, like Ugo1, is a modified carrier protein that has been recruited to the outer mitochondrial membrane and interacts with the inner membrane remodeling protein mitofilin (Fcj1). Loss of function in cultured cells and in zebrafish unexpectedly leads to increased mitochondrial connectivity, while severely affecting the development and maintenance of neurons in the fish. The discovery of SLC25A46 strengthens the genetic overlap between optic atrophy and CMT2 while exemplifying a new class of modified solute transporters linked to mitochondrial dynamics. Mutations in MFN2 account for more than 20% of inherited axonal degenerative peripheral neuropathies, CMT2A3,8. A subset of affected patients also develop optic nerve atrophy9. Meanwhile, mutations in OPA1 account for as much as 60−70% of DOA10 and sometimes cause additional neurological symptoms, referred to as optic atrophy ‘plus’ (refs. 10,11). MFN2 and OPA1 are involved in the tethering and fusion of the outer and inner mitochondrial membranes4. Both CMT2 and DOA are genetically heterogeneous disorders, with up to 60% of patients undiagnosed genetically10,12. Therefore, we recruited families with both optic atrophy and axonal peripheral neuropathy to identify additional genes involved in disease, on the basis of the hypothesis that causative genes would uncover new factors in common biological pathways. By applying whole-exome sequencing with established methods and cutoffs for variant filtering in diseases inherited in a mendelian fashion13, we found four families with recessive variants in the nuclear-encoded mitochondrial gene SLC25A46. After excluding other candidate genes by segregation analysis, we identified the compound-heterozygous mutations c.165_166insC (p.His56fs*94) and c.746G>A (p.Gly249Asp) in a UK family (UK), a homozygous mutation, c.1005A>T (p.Glu335Asp), in a Palestinian family (PL), and the compound-heterozygous variants c.882_885dupTTAC (p.Asn296fs*297) and c.998C>T (p.Pro333Leu) in a US family (US). We then used conventional Sanger sequencing methods to screen SLC25A46 in similar cases without a genetic diagnosis and identified an additional family from Sardinia, Italy (IT), with the homozygous mutation c.1018C>T (p.Arg340Cys) (Fig. 1, Supplementary Fig. 1 and Supplementary Table 1).

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All non-truncating changes were predicted to be deleterious (Supplementary Table 1). Affected individuals in these four families presented with similar phenotypic core features, including optic atrophy, axonal CMT and cerebellar atrophy (Supplementary Note). Magnetic resonance spectroscopy (MRS) data in two of the affected individuals showed decreased N-acetylaspartate (NAA) and increased lactate levels in the central nervous system (Supplementary Fig. 2), which are typical of mitochondrial disorders and suggestive of a metabolic role for SLC25A46. However, analysis of a muscle biopsy from patient IT II-3 found no ragged red fibers or cytochrome c oxidase (COX)-negative fibers (data not shown).

SLC25A46 is a member of the mitochondrial solute carrier family14 (SLC25) and is predicted to function as a transporter across the inner mitochondrial membrane15. Using BLAST, we found that SLC25A46 was a reciprocal match to Ugo1 when querying between Homo sapiens, Caenorhabditis elegans, S. cerevisiae and Schizosaccharomyces pombe containing a mitochondrial carrier domain. This analysis identified SLC25A46 as the most similar to Ugo1. However, there was insufficient evidence to determine orthology (Online Methods and Supplementary Fig. 3), and SLC25A46 failed to complement ugo1 deletion in S. cerevisiae (data not shown).

During evolution, homologs of mitochondrial carriers, usually inner-membrane proteins15, have been modified and recruited to the mitochondrial outer membrane to perform specific functions, unrelated to metabolite transport. The list of these factors includes the mammalian mitochondrial carrier homologs MTCH1 (ref. 18) and interacts with mitofilin on the inner membrane. (a) High-resolution confocal images of COS-7 cells cotransfected to express SLC25A46-HA and mitofilin-Myc. TOM20 and mitofilin-Myc were used as markers of the outer and inner mitochondrial membranes, respectively. Scale bars, 1 µm. Right, linear profiles of the fluorescence intensity in arbitrary units (AU) along the 2-µm dotted lines in the corresponding merged image. (b) Mitochondria (M) isolated from HEK293T cells expressing SLC25A46-HA were submitted to brief sonication and centrifugation to fractionate soluble (S) and membrane-bound proteins. The pellet was subjected to alkaline extraction to separate soluble, membrane-extrinsic (CS) and membrane-intrinsic (P) proteins. Equivalent volumes of each fraction were analyzed by immunoblotting with antibodies against the soluble matrix protein mHSP70, the extrinsic membrane protein SDHA, the intrinsic membrane protein COX2 and HA. (c) Mitochondria and mitoplasts prepared by hypotonic swelling of mitochondria from HEK293T cells expressing SLC25A46-HA were treated with proteinase K where indicated. After treatment, samples were analyzed by immunoblotting with antibodies against the outer-membrane protein TOM20, the inner-membrane proteins Tim50 and COX2, and HA. (d) Co-sedimentation of SLC25A46-HA and mitofilin in a linear 7–20% sucrose gradient. Hemoglobin, a 67-kDa protein, and lactate dehydrogenase (LDH), a 130-kDa protein, were used to calibrate the gradient. M, mitochondria; Ex, total mitochondrial extract. Two exposures are shown for each protein. (e) Coimmunoprecipitation (co-IP) in HEK293T cells cotransfected to express mitofilin-Myc and SLC25A46-HA or SLC25A46-HA and Myc-tagged, mitochondria-targeted GFP (mito-GFP-Myc) as a negative control.
Immunocytochemistry studies in COS-7 cells demonstrated that hemagglutinin (HA)-tagged SLC25A46 colocalized more with the outer-membrane marker (TOM20) and less with the inner-membrane markers (Myc-tagged mitofilin and ANT2) (Fig. 2a and Supplementary Fig. 5a). We subsequently used mitochondria isolated from HEK293T cells expressing SLC25A46-HA to perform solubility and proteinase K protection assays, which demonstrated that SLC25A46 is an integral outer membrane protein (Fig. 2b,c). To gain insight into SLC25A46 function, we identified interacting partners by performing an unbiased HA immunoprecipitation assay combined with mass spectrometry analysis. Mitofilin was among the top hits in this assay (Supplementary Table 3). Consistently, sucrose gradient sedimentation analyses found mitofilin to co-sediment with a small fraction of SLC25A46-HA, indicating that these proteins may exist in the same high-molecular-mass complex (Fig. 2d). We further confirmed the interaction between SLC25A46 and mitofilin by coimmunoprecipitation assays using mitochondria isolated from cells transfected to express SLC25A46-HA (Supplementary Fig. 5b) or cotransfected to express SLC25A46-HA and mitofilin-Myc (Fig. 2e), followed by immunoblot detection. These results are congruent with those for Ugo1, which has been shown to co-fractionate with Fcj1 (ref. 21), the homolog of mammalian IMMT (mitofilin).

To determine whether SLC25A46 has a conserved role in mitochondrial dynamics, we compared the effects of SLC25A46 overexpression to overexpression of the ATP transporter ANT2 (also known as SLC25A5) and found that increased expression of SLC25A46 but not ANT2 led to mitochondrial fragmentation in cell lines (Fig. 3a,b). We next used small interfering RNA (siRNA) to knock down SLC25A46 (Supplementary Fig. 6) and found it to cause mitochondrial hyperfusion (Fig. 3c,d). To determine the degree of mitochondrial connectivity, we observed the instantaneous diffusion of matrix-targeted photoactivatable GFP, showing that this GFP diffused into larger areas in knockdown cells than in controls (Fig. 3e,f).

**Figure 3** SLC25A46 levels regulate mitochondrial morphology. (a) SLC25A46 overexpression causes fragmentation of the mitochondrial network in HeLa cells in comparison to overexpression of ANT2. (b) Percentage of transfected cells with fragmented mitochondria from three independent experiments. Error bars, s.d. *P = 0.00004, one-sided t test. ANT2, n = 22; SLC25A46, n = 21. (c) HeLa cells were treated with siRNA and stained with TOM20. Mitochondria are more hyperfilamentous when SLC25A46 is knocked down. (d) Quantification of mitochondrial morphology. The mean and s.d. were calculated from three independent experiments. Control siRNA, n = 219; SLC25A46 siRNA, n = 214. (e) The instantaneous diffusion of matrix-targeted, photoactivated GFP (mito PA-GFP) was used to assess mitochondrial connectivity. The region of interest indicated by the white circles in the preactivation image was targeted with the laser, and the corresponding signal in the post-activation image represents the extent of mitochondrial connectivity. (f) Quantification of the activated mitochondrial area in the post-activation image normalized to the region of interest (ROI) in the preactivated image. Error bars, s.d. *P = 0.0009, one-tailed t test. Control siRNA, n = 42; SLC25A46 siRNA, n = 38. (g) Representative electron micrographs of a mitochondrial cross-section with constriction sites (arrows) in COS-7 cells treated with SLC25A46 siRNA. (h) Percentage of cross-sections with visible constriction sites: 2 of 103 control sites versus 11 of 81 sites with knockdown. Error bars, s.d. *P = 0.0028, two-tailed Fisher’s exact test. Scale bars: 10 μm in a,c,e; 100 nm (top) and 200 nm (bottom) in g.
Assuming that mitochondrial hyperfusion is controlled by the balance between fusion and fission, this phenotype could be caused by either increased fusion or loss of fission. To test the rate of mitochondrial fusion, we performed photoactivation experiments where we measured the extinction of activated GFP over the course of 90 min and found no significant difference in the fusion rates of control and knockdown cells (Supplementary Fig. 7). This finding, in conjunction with the mitochondrial constriction sites observed in electron microscopy (Fig. 3g,h), which appear to be fission intermediates, suggests that delays in fission or elongation contribute more to the hyperfused morphology than an increase in the fusion rate.

We further examined fibroblasts from proband IT II-3, which displayed a hyperfilamentous and interconnected mitochondrial network in comparison to networks from both the unaffected sibling IT II-2 and a control, indicating the relevance of this phenomenon (Fig. 4a,b). Loss of SLC25A46 function was not associated with changes in total cellular ATP concentration, mitochondrial DNA content or membrane potential in either siRNA-treated cell lines or a knockdown zebrafish model (Supplementary Fig. 5b–d and data not shown). However, fibroblasts from patient IT II-3 showed a decreased oxygen consumption rate (OCR) and a glycolytic shift in metabolism (decreased OCR/ECAR (extracellular acidification rate) ratio) (Fig. 4c–e), consistent with the increased lactate peak found in MRS (Supplementary Fig. 2b). A decrease in ATP synthesis driven by complex I substrates was trending but not significant (data not shown). These metabolic changes are congruent with previous findings in patient-derived fibroblasts with OPA1 mutations.

Altogether, our data support the notion that, unlike Ugo1, SLC25A46 acts in a pro-fission manner. Ugo1 physically interacts with both Fzo1 (MFN2) and Mgm1 (OPA1) to coordinate the simultaneous fusion of the outer and inner mitochondrial membranes. Interestingly, mitochondrial fusion is not tightly coupled in mammalian cells, where the outer and inner membranes can fuse independently of each other. Consistent with these findings, SLC25A46 did not interact with MFN2 or OPA1 but formed a complex with mitofilin where the outer and inner membranes come together at constriction sites. Alternatively, SLC25A46 could mediate the translocation of an unknown pro-fission factor across the outer membrane, analogous to the translocation of tBID by MTPCH2 during apoptosis. Although the function of SLC25A46 in mitochondrial dynamics seems to deviate from the role of Ugo1 in yeast, the conservation of the interaction of both proteins with mitofilin suggests that SLC25A46 and Ugo1 might have similar roles at cristae junctions.
Figure 5 slc25a46 knockdown in zebrafish affects the growth and maintenance of neurons. (a) Dorsal view of Tg(islet2:EGFP) embryos at 72 h.p.f. RGC axons cross at the chiasm (CH) before innervating the optic tectum (OT). Scale bars, 100 µm. MO, morpholino; hRNA, RNA encoding the human SLC25A46 protein. (b) Quantification of the area of RGC-innervated tectum depicting means and s.d. P values were calculated from a one-tailed Student’s t test. Control MO, n = 3; 0.45 pmol of slc25a46 MO, n = 16; 0.3 pmol of slc25a46 MO + hRNA, n = 16; 0.3 pmol of slc25a46 MO + hRNA, n = 15. (c) Motor neuron axons in Tg(olig2::DsRed) embryos at 48 h.p.f. Truncated axons were commonly observed with knockdown (indicated by asterisks). Scale bars, 100 µm. (d) Quantification of the average common axon path length per fish with standard deviation between fish. Significance was determined with one-way ANOVA followed by Tukey’s post-hoc test (Supplementary Table 4); P values were calculated by one-tailed Student’s t test. Control MO, n = 4; 0.45 pmol of slc25a46 MO, n = 4. (e) Electron micrographs of zebrafish spinal cord (top, cell bodies; bottom, neuropil) at 48 h.p.f. Scale bars, 2 µm. (f) Quantification of the density of neuronal processes in the neuropil. Data represent means and s.d. P values were calculated by one-tailed Student’s t test. Control MO, n = 3; 0.45 pmol of slc25a46 MO, n = 3; 0.3 pmol of slc25a46 MO + hRNA, n = 3. (g) Motor neuron terminals labeled with membrane-bound YFP (memYFP) and postsynaptic acetylcholine receptors on muscle labeled with α-bungarotoxin (α-btx). Axonal blebbing (arrow) and dieback as indicated by receptors at the front of the axon (asterisk) were commonly observed in morphant motor neurons. Scale bars, 10 µm.

Genome-wide association studies have implicated SLC25A46 in atopic dermatitis, however, the transcript has been shown to be highly expressed in the neurons affected in patients, including in neurons from the spinal cord, cerebellum and optic chiasm. To examine the role of SLC25A46 in the vertebrate nervous system, we created a zebrafish knockdown model. Zebrafish Slc25a46 is 69% identical to the SLC25A46 protein encoded by the human ortholog of the zebrafish gene, and, in agreement with previous RNA in situ hybridization data, we found enrichment of slc25a46 transcripts in retinal ganglion cells (RGCs) and their corresponding neuronal projections in axons of the optic nerve and dendrites of the inner plexiform layer (Supplementary Fig. 8a-d). We then used a transient morpholino approach to knock down the transcript levels and demonstrated that knockdown was effective for up to 96 h post-fertilization (h.p.f.) (Supplementary Fig. 5c). When injected with slc25a46 morpholino, embryos (morphants) had fewer RGC axons that reached the tectum by 72 h.p.f. than embryos injected with control morpholino (Fig. 5a,b). We also saw decreased antibody staining of the inner plexiform layer, suggesting that RGC dendrites were also affected (Supplementary Fig. 8e,f). Around 40% of morphant embryos injected with 0.45 pmol of morpholino had a severe curly-tail morphology (Supplementary Fig. 8g,h); yet, even normal-appearing larvae displayed alterations in swimming, indicating dysfunction of neuronal circuits (Supplementary Fig. 8i).

We further investigated motor neurons and found significantly shorter axon tracts, many of which failed to innervate the rostral myotome at 48 h.p.f. (Fig. 5c,d and Supplementary Table 4). The spinal cord neuropil at the level of electron microscopy showed fewer neuronal processes in slc25a46 morphants, indicating either lack of development or degeneration of dendrites (Fig. 5e,f). By stochastically labeling motor neurons, we frequently observed axonal blebbing and dieback at morphant motor neuron terminals specifying degeneration, which is consistent with the pathology of CMT2 (Fig. 5g). These results suggest that Slc25a46 is important for both the growth and maintenance of neuronal processes.

We next investigated mitochondria in motor neurons in vivo. Consistent with previous findings, we observed that, in controls, the majority of the mitochondria were located within the lower half of motor neuron soma, clustered at the axon initial segment. In contrast, mitochondria in degenerating neurons exhibited a reversal in cell localization favoring the apical portion of the soma and also appeared to be aggregated (Fig. 6). At the level of electron microscopy, we identified numerous mitochondria that appeared to be in the process of fission within the cell bodies of morphants, which was never observed in controls (Fig. 6d,e). Incomplete fission of mitochondria could inhibit transport and distribution into neuronal processes.

In conclusion, we have identified a new mitochondrial disease gene associated with mitochondrial dynamics, optic atrophy and CMT2. Further investigation is needed to elucidate the role of SLC25A46 in mitochondrial dynamics, but, given the similar human phenotypes associated with MFN2, OPA1 and SLC25A46 mutations, these genes could be involved in common pathological mechanisms of neurodegeneration, thereby raising the possibility of future pathway-oriented treatments for these types of mitochondrial disorders.
Figure 6 Mitochondrial distribution and morphology in zebrafish motor neurons at 48 h.p.f. (a) Zebrafish motor neurons in vivo labeled by plasmids with expression of mitoCFP (human COX8A subunit fused to CFP; blue) and memYFP (black) driven by the hsp9 promoter. Scale bars, 10 μm. (b) Graph depicting the distributional density of mitoCFP pixels as a percentage either above or below the midline of the soma (defined as 0). In slc25a46-morphant motor neurons, mitochondria are shifted toward the top of the soma. The mitochondria in the morphants also appear to be more aggregated. Control MO, n = 8; slc25a46 MO, n = 8. P = 0.083, one-tailed Student’s t test. (c) Representative electron microscopy images showing mitochondrial distribution. Yellow, soma; blue, mitochondria. Scale bars, 1 μm. (d) Electron micrographs of mitochondria in spinal cord. slc25a46 morphants show accumulation of large mitochondrial aggregates that appear to be in the process of fission. Scale bars, 1 μm. (e) Serial sections of a clover-shaped mitochondrial aggregate, suggesting that these mitochondria are fused aggregates that appear to be in the process of fission. Scale bars, 1 μm. (f) Cross-sectional areas of mitochondria with distribution of sizes, showing that larger aggregates are present in slc25a46 morphants. Number of fish: control MO, n = 4; slc25a46 MO, n = 4; number of mitochondrial cross-sections: control MO, n = 138; slc25a46 MO, n = 201. The red line shows 3 s.d. of the average found for the controls; error bars, s.d. P = 0.0006, one-tailed Student’s t test comparing the total mitochondrial cross-sections per group.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
A.H.N., S.Z. and J.E.D. initiated the project, which was subsequently developed and led jointly by J.E.D., S.Z., T.H. and V.C. Experiments were conceived and carried out by A.J.A., A.R., C.Z., N.P., A.V.S., F.T., E.F., A.B., R.B.H., L.B.G., A.M. and R.S., with financial support from S.Z., T.H., A.A. and J.P. Zebrafish experiments were carried out by A.J.A., I.J.C. and S.G., with financial support from S.Z. and J.E.D. Patient recruitment, collection and analysis of human DNA, and genetic data analysis were carried out by M.A.G., F.S., I.A., S.M.D., I.C., C.L.M., R. Liguori, Z.M.A., K.L.S., X.W., L.A.K., Y.P., C.E.P., C.A.P., E.K.S., H.H.Z., O.A.A.-R. and Y.Y. Functional MRI-MRS data were generated by R. Lodi and Z.M.A. A.J.A. wrote the manuscript with critical input from S.Z. and J.E.D.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Whole-exome sequencing. Total genomic DNA was extracted from whole blood using standard methods. Library construction was performed on double-stranded DNA, sheared by sonication to an average size of 200 bp, in an automated fashion on an IntegenX Apollo324. After nine cycles of PCR amplification using the Clontech Advantage II kit, 1 µg of genomic library was recovered for exome enrichment using the NimbleGen EZ Exome V2 kit. Libraries were sequenced on an Illumina HiSeq 2500. The Genomes Management Application tool (GEM.app) was used for variant calling with the standard pipeline13.

Sanger sequencing. Sanger sequencing was performed using standard methods on the eight exons of the SLC25A46 gene (NM_138773.1). Primers were designed using Primer3. PCR products were amplified using 50 ng of DNA, with standard EconoTaq PCR reagents (Lucigen), on an ABI Veriti Thermal Cycler (Applied Biosystems). PCR products were precipitated, and sequencing PCR was performed using BigDye Terminator Ready Reaction Mix (Applied Biosystems).

Immunofluorescence. COS-7 or HeLa cells (originally obtained from the American Type Culture Collection; not tested for mycoplasma) were seeded to 75% confluence on glass coverslips and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were fixed with paraformaldehyde for 20 min, permeabilized with cold methanol for 5 min and stained with antibody to Myc (2276 and 2278) or HA (2367 and 3724) from Cell Signaling Technology. Antibody to TOM20 (sc-11415) from Santa Cruz Biotechnology was used to label the mitochondrial outer membrane. Corresponding Alexa Fluor–conjugated secondary antibodies (Life Technologies) were used for detection. Coverslips were mounted onto slides with DAPI mounting medium (Vectashield), and cells were imaged with a confocal microscope (Zeiss LSM710, 63×/1.4 NA oil objective with a pinhole of less than 1 airy unit). Images were processed with Fiji software (ImageJ).

Mitochondrial subfractionation and proteinase K protection assays. HEK293T cells (obtained from the NIH AIDS Research and Reference Reagent Program; not tested for mycoplasma) were grown in DMEM ( Gibco) supplemented with 10% FBS and incubated at 37 °C. Cells were transfected with construct expressing SLC25A46-HA, and mitochondria were isolated 24 h after transfection, as previously reported30. To test protein solubility, the purified mitochondria were ruptured by sonication. The membrane fraction was recovered by centrifugation at 20,000g for 15 min at 4 °C and was resuspended in 0.1 M Na2CO3, pH 11. Samples were incubated on ice for 30 min, and soluble extrinsic-membrane proteins and insoluble intrinsic-membrane proteins were separated by centrifugation at 20,000g for 15 min at 4 °C. Purified mitochondria were resuspended in buffer containing 10 mM Tris-HCl, pH 7.10 mM KCl, 0.15 mM MgSO4 and either 0.25 M sucrose or devoid of sucrose to allow for mitochondrial swelling and conversion to mitoplasts31. Samples were treated with proteinase K at a final concentration of 0.62 µg/ml and incubated on ice for 30 min. The reaction was inhibited with 2 mM phenylmethylsulfonyl fluoride (PMSF). Mitochondria and mitoplasts were recovered by centrifugation at 20,000g for 15 min at 4 °C and analyzed by immunoblotting.

Sucrose gradient analysis. Purified mitochondria (400 µg) were solubilized in 0.08 ml of extraction buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM MgSO4 and 0.5 mM PMSF) containing 1% digitonin, on ice for 15 min. Clarified extract was obtained by centrifugation at 20,000g for 15 min at 4 °C, mixed with standard proteins (hemoglobin and lactate dehydrogenase) and applied to a linear 7–20% sucrose gradient prepared in extraction buffer containing 0.1% digitonin. After centrifugation for 12 h at 74,000g in a Beckman 55Ti rotor, the gradient was fractionated in 12 equal fractions. Each fraction was assayed for hemoglobin by absorption at 409 nm and for lactate dehydrogenase activity by measuring NADH-dependent conversion of pyruvate to lactate. Subsequently, the proteins contained in each fraction were concentrated by trichloroacetic acid (TCA) precipitation and analyzed by immunoblotting.

Immunoprecipitation and mass spectrometry. HEK293T cells were transfected with construct expressing SLC25A46-HA using Turbofect reagent (Thermo Scientific). After 24 h, cells were lysed with a dounce homogenizer, and mitochondria were isolated with a mitochondria isolation kit for cultured cells (Pierce) according to the manufacturer’s protocol. Protein from mitochondria (800 µg) was incubated with 5 µg of mouse antibody to HA tag (Abcam, ab9110) at room temperature for 2 h. The protein-antibody mixture was then incubated with protein A/G magnetic beads (Thermo Scientific) at room temperature for 1 h on a rotator. Beads were collected using a magnetic stand and washed three times with the manufacturer’s IP Lysis/Wash buffer. Proteins were eluted with a low-pH elution buffer. Silver staining was performed using the Pierce Silver Stain kit (Thermo Scientific). Briefly, the SDS-PAGE gel was washed twice with ultrapure water for 5 min each wash and fixed with 30% ethanol:10% acetic acid solution. Samples were analyzed by incubation of the NuPAGE (Invitrogen) SDS-PAGE gel (4–20%) with silver staining solution for 5 min followed by incubation with the developing reagent for 3 min. The reaction was stopped by incubation with 5% acetic acid for 10 min. Bands were excised from the gel using a light box and submitted for mass spectrometry analysis at the Scripps Center for Metabolomics and Mass Spectrometry.

Coimmunoprecipitation. HEK293T cells were cotransfected with constructs expressing SLC25A46-HA and mitofilin-Myc or, as a negative control, with construct expressing SLC25A46 and pCMV/Myc/mitoGFP (Invitrogen). After 24 h, cells were lysed with a dounce homogenizer, and mitochondria were isolated with a mitochondria isolation kit for cultured cells (Pierce) according to the manufacturer’s protocol. Protein from mitochondria (800 µg) was incubated with 5 µg of mouse antibody to HA, rabbit antibody to Myc (Cell Signaling Technology), or control mouse or rabbit IgG at room temperature for 2 h. The protein-antibody mixture was then incubated with protein A/G magnetic beads (Thermo Scientific) at room temperature for 1 h on a rotator. Beads were collected using a magnetic stand and washed three times with the manufacturer’s IP Lysis/Wash buffer. Proteins were eluted with a low-pH elution buffer. Protein samples were analyzed by NuPAGE SDS-PAGE gel (4–20%) followed by immunoblotting using appropriate antibodies. Band signal was developed with either West Pico or West Femto chemiluminescent substrate (Thermo Scientific).

Overexpression and knockdown in mammalian cells. Cells were plated to 70% confluence on glass coverslips in a six-well plate. Plasmid transfections were carried out using Lipofectamine 2000 according to a standard protocol. Immunofluorescence was carried out as described earlier. Representative single-plane images were acquired with a Zeiss LSM710 confocal microscope, using a 63×/1.4 NA oil objective, and were processed with Fiji software (ImageJ) and assessed with blinding to sample identity for fragmentation. For knockdown, cells were plated to 50% confluence on glass coverslips in a six-well plate. Silencer Select (Ambion) SLC25A46 (s40579) and Negative Control No. 1 (4390843) siRNAs were used. The siRNAs (25 pmol per well) were transfected into cells with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. Effective knockdown of the SLC25A46 transcript was confirmed with RT-PCR and quantitative PCR using gene-specific primers (Supplementary Table 5). After 48 h, cells were washed in PBS and fixed in 4% paraformaldehyde, and immunofluorescence was carried out as described earlier. Images were acquired on a Zeiss LSM710 microscope, using a 63×/1.4 NA oil objective, and were processed with Fiji software (ImageJ). LUT-Grays. Representative images were assessed with blinding to sample identity.

Mitochondrial morphology analysis and energetic profiling of human fibroblasts. Fibroblasts were seeded in 36-mm dishes and grown in DMEM. Mitochondrial morphology was assessed by staining cells with 10 nM MitoTracker Red (Life Technologies) for 30 min at 37 °C. Cellular fluorescence images were taken with an inverted Nikon Eclipse Ti-U epifluorescence microscope equipped with a back-illuminated Photometrics Cascade CCD camera (Roper Scientific). Images were acquired using a 63×/1.4 NA oil objective. Data were acquired and analyzed using Metamorph software (Universal Imaging Corporation). OCR and ECAR were measured in adherent fibroblasts with an XFe24 Extracellular Flux Analyzer (Seahorse Bioscience). Each control and mutant fibroblast cell line was seeded in 5 wells of an XF 24-well cell culture microplate (Seahorse Bioscience) at a density of 30,000 cells/well in
250 µl of DMEM and incubated for 24 h at 37 °C under a 5% CO₂ atmosphere. The growth medium was replaced with 575 µl of prewarmed bicarbonate-free DMEM, pH 7.5, and cells were incubated at 37 °C for 1 h before starting the assay.

After baseline measurements of OCR and ECAR, OCR was measured after sequentially adding to each well 75 µl of oligomycin, 75 µl of FCCP, 75 µl of rotenone and 75 µl of antimycin A to reach working concentrations of 1 µM. OCR and ECAR values were normalized to protein content measured by Sulforhodamine (SRB) assay, following a standard protocol. For data analysis, the following parameters were evaluated: basal respiration, measurement before oligomycin injection subtracted by non-mitochondrial respiration (measurement after antimycin A injection); proton leak–linked respiration, measurement after oligomycin injection subtracted by non-mitochondrial respiration; ATP-linked respiration, basal respiration subtracted by proton leak–linked respiration; maximal respiration, measurement after FCCP injection subtracted by non-mitochondrial respiration; reserve capacity, maximal respiration subtracted by basal respiration; complex I–linked respiration, basal respiration subtracted by the measurement after rotenone injection; and maximal complex I–linked respiration, maximal respiration subtracted by the measurement after rotenone injection.

**Photoactivation assays.** The Mito PA-GFP assay was performed as previously described, using a Zeiss LSM710 microscope with a 63x/1.4 NA oil objective, heated stage and carbon dioxide chamber. After acquisition of the preactivated image, a circle of approximately 5 µm in diameter was selected using regions setup and the corresponding region was activated with the 488-nm laser. The post-activation image was immediately acquired using the 488-nm laser, and subsequent images were taken at 30-min intervals for a total of 90 min. Images were processed in Fiji software and displayed with LUT:fire. For the mitochondrial fusion assay, the mean pixel intensity was calculated for the region of interest and normalized to the post-activation image. For assessment of mitochondrial connectivity, the area of activated GFP was measured in the post-activation image and normalized to the area of the activated region.

**Zebrafish husbandry.** Experiments were carried out using wild-type Danio rerio strains (AB, TL, Tübingen) and the transgenic strains Tg(olig2::DsRed)33 and Tg(islet2b::EGFP)34. Adults were kept on a 14-h light/10-h dark cycle at 28 °C. Embryos were obtained from natural crosses after removing a divider at first light, and microinjections were performed into embryos at the one-cell stage. Embryos were reared in Petri dishes of system water in a 28 °C incubator with the same light/dark cycle. All experiments were conducted in accordance with University of Miami Institutional Animal Care and Use Committee guidelines. The sample size of embryos was not predetermined.

**Microinjections.** The slc25a46 morpholino (Gene Tools) was targeted to the exon 3–intron 3 junction. Effective blocking of splicing was confirmed with diagnostic primers (Supplementary Table 5) and was found to lead to the exclusion of exon 3, resulting in a frameshift and the introduction of a premature stop codon. The standard scrambled morpholino (Gene Tools) was used for the control. Stock solutions (1 mM) of the morpholinos were diluted in 1% Fast Green dye to a final concentration of 0.9 mM. Injection volumes used for the control. In the rescue experiments, mRNA encoding human SLC25A46 fused to s C-terminal HA tag was synthesized from the exon 3–intron 3 junction. Effective blocking of splicing was confirmed with diagnostic primers (Supplementary Table 5).

**In vivo imaging of the optic nerve.** RGCs were imaged at 72 h.p.f. after injection of slc25a46 and control morpholinos into Tg(islet2b::EGFP)34 embryos. At 24 h.p.f., N-phenylthiourea (Sigma) was added to the water to suppress pigment development. Live fish were anesthetized with tricaine methanesulfonate (Sigma), embedded in 1.5% agarose and imaged using a Leica confocal microscope with a 20x air lens. One-micrometer z stacks were processed with maximum intensity and thresholded for analysis. Calculations were performed by tracing GFP-positive areas. All possessing was carried out with Fiji software (ImageJ). LUT: green fire blue was used to generate the figure.

**Transmission electron microscopy and analysis.** COS-7 cells were cultured in 25-cm² flasks (Corning) and treated with siRNAs as described earlier. After 2 d, cells were fixed for 4 d in 2.5% glutaraldehyde in Millions phosphate buffer, post-fixed in 1% OsO₄ and uranyl acetate, dehydrated in an ethanol series and embedded in Spur. Zebrafish embryos were injected with 0.45 pmol of either control or slc25a46 morpholino. The more severely affected curved morphants were selected for electron microscopy. All embryos were fixed and processed for 48 h.p.f. as described earlier, with the exception that they were embedded in LR White resin (Fisher Scientific). Ultrathin, 60-nm sections were cut using a Leica microtome and stained with cold lead citrate for 6 min. Images were acquired with a Joel JEM-1400 transmission electron microscope with a Gatan camera. All images were processed and analyzed using Fiji software (ImageJ).

**Plasmids.** Hb9::Gal4-VP16 was generated from the pmT-hb9s-YFP construct kindly provided by the Nonet laboratory (Washington University). UAS-E1b::memYFP::mitoCFP was kindly provided by B. Schmid from the German Center for Neurodegenerative Diseases (DZNE). Constructs for SLC25A46 and mitoflin-Myc were obtained from Origene. The SLC25A46 sequence was subcloned into pcDNA3.1 (Invitrogen) with a sequence encoding either a C-terminal HA or Myc tag. For RNA synthesis, the sequence encoding SLC25A46-HA was also subcloned into pCS2+.

**Phylogenetic analysis.** The concise list of members of the human mitochondrial solute carrier family (SLC25) was obtained from the Bioparadigms web site, and all proteins with a mitochondrial carrier domain in C. elegans, S. cerevisiae and S. pombe were retrieved from the European Bioinformatics Institute web site. We used multiple-sequence alignment by ClustalW to draw unrooted neighbor-joining trees. We then created a maximum-likelihood tree using PhyML by Mobyle, with 1,000 bootstraps. The consensus tree was visualized and drawn using ITOL.

**ATP and mitochondrial DNA analysis.** ATP was measured according to the manufacturer’s protocol using the ATP Determination kit (Molecular Probes). COS-7 cells were transfected with siRNAs as described earlier and homogenized in ATP assay buffer 48 h after transfection, or five whole zebrafish embryos were homogenized in ATP assay buffer at 4 h.p.f. Homogenates were centrifuged at 12,000g for 5 min to pellet tissue debris. Supernatant (10 µl) was added to a 96-well plate with each well containing 90 µl of the luciferase reaction mix from the ATP Determination kit. Luminescence was immediately read on a luminometer. ATP values were calculated by comparison to a standard curve generated from a series of ATP concentrations. Values were then normalized by the total amount of protein present in each sample. Samples were run in triplicate. Mitochondrial DNA amounts were quantified by quantitative PCR as described.

**In vivo imaging of motor neurons.** Motor neuron outgrowth was assayed at 48 h.p.f. using Tg(olig2::DsRed)33 fish. Live fish were anesthetized with tricaine methanesulfonate, embedded in 1.5% agarose and imaged using a Leica confocal microscope with a 20x air lens; images were processed with Fiji software (ImageJ). For analysis, 1-µm z stacks were imaged between myotome segments 6 and 13 (ref. 35), and the lengths of the caudal anterior primary axons were measured from the base of the spinal cord to the end of the rostral myotome using the Simple Neurite Tracer in Fiji software (ImageJ). LUT:edges was used to generate the figure. To stochastically label motor neurons and mitochondria, 300 pl of a cocktail containing 25 ng/µl each HB9: Gal4-VP16, UAS-E1b::memYFP::mitoCFP36 and transposase RNA was injected before the morpholinos. Embryos were presorted for YFP expression at 48 h.p.f. before imaging. Acetylcholine receptors were labeled in embryos at 48 h.p.f. by injection of α-bungarotoxin conjugate (Molecular Probes) into the yolk of anesthetized fish 1 h before imaging with a Leica confocal microscope, using a 63x water lens. Motor neuron cell bodies and mitochondria were processed with LUT:edges/memYFP and 16 colors/mitoCFP. For mitochondrial distribution analysis, 1-µm z stacks were projected with maximum intensity and auto-thresholded. The midline was measured as the halfway point between the top of the soma and the axon hillock. The area of pixels corresponding to mitochondria was calculated for the regions above and below the midline, with the resulting values then converted to a percentage using Fiji software (ImageJ).
RNA in situ hybridization. For RNA in situ hybridization, wild-type embryos were incubated in N-phenylthiourea to suppress pigment development. The slc25a46 probe sequence was PCR amplified from zebrafish cDNA (Supplementary Table 5), and DIG RNA Labeling Mix (Roche) was used to synthesize the antisense probe. Probe hybridization and signal development were carried out using a standard protocol39, followed by post-fixation in 4% paraformaldehyde and dehydration in 30% sucrose. Embryos were embedded in tissue freezing medium (Triangle Biomedical Sciences), sliced with a cryostat into 30-µm sections and imaged with an Olympus BX60 compound light microscope.

Retinal ganglion cell staining. Morpholino-injected larvae were fixed at 96 h.p.f. in 4% paraformaldehyde overnight and then decapitated. Zn-5 antibody (ZFIN) was used at a 1:200 dilution followed by incubation with a 1:200 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody; staining was finally developed with 3,3′-diaminobenzidine (DAB) (Vector Laboratories). After DAB development, fish were depigmented with hydrogen peroxide and potassium hydroxide, post-fixed and dehydrated in ethanol. Samples were embedded in freezing medium, sliced into 30-µm sections and imaged with an Olympus BX60 compound light microscope. Eight-bit grayscale numbers were calculated from TIF files of sections that contained the optic nerve using Fiji image analysis, such that 0 = white and 256 = black, and was converted to percentages, whereby 0 = white and 100 = black. Five evenly spaced pixels from the inner plexiform layer were assessed to find the average intensity per eye.

Analysis of swimming. High-speed videos were taken using a Fastcam 1024PCI (Photron USA) with a Fujinon lens mounted in a customized behavioral chamber. The parameters to assess swimming kinematics were as follows: shutter speed of 1/1,000 s using an LED array for backlight illumination (Advance Illumination, Backlight LED Illuminator), 512 x 512 resolution and a frame rate of 500 frames/s. Three vibration-evoked behaviors, elicited with an S40 stimulator using a DAC timer, were recorded from a 35-mm dish containing up to five larvae. Videos were acquired from multiple runs containing more than 30 larvae in total. Videos were analyzed using Flote40 to quantify changes in axis curvature over time. Three representative overlapping swim traces from different fish were chosen from over eight analyzable swim responses.

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