Characterization of Novel Neurodegenerative Genes Implicated in Mitochondrial Dynamics

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A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

CHARACTERIZATION OF NOVEL NEURODEGENERATIVE GENES
IMPLIED IN MITOCHONDRIAL DYNAMICS

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Mitochondria are dynamic organelles undergoing constant fusion, fission, and migration within cells. The mobile nature of the mitochondria is essential for nerve health, as mutations in two of the major mitochondrial fusion genes, \textit{MFN2}\textsuperscript{1} and \textit{OPA1}\textsuperscript{2,3}, cause axonal peripheral neuropathy (Charcot-Marie-Tooth Type 2, CMT2), and dominant optic atrophy (DOA) respectively.

Through collaborative exome sequencing and data sharing, we identified four families with recessive mutations in the nuclear encoded mitochondrial gene, \textit{SLC25A46} (Chapter 2). The patients in these families present a clinical spectrum of features ranging from optic atrophy, spasticity, peripheral neuropathy, and ataxia, to lethal infantile neurodegeneration. \textit{SLC25A46} is one of 53 members of the mitochondrial solute carrier family (SLC25)\textsuperscript{11}, which typically transport metabolites across the inner mitochondrial membrane. Interestingly, \textit{SLC25A46} is similar to Ugo1, an essential component of the mitochondrial fusion mechanism in yeast. However, unlike Ugo1, \textit{SLC25A46} seems to play a greater role in mitochondrial fission in both cells and zebrafish models (Chapter 3). \textit{SLC25A46} strengthens the genetic overlap between optic atrophy and peripheral neuropathy, and is a novel mitochondrial dynamic factor.
While mitochondrial dysfunctional plays a prominent role in nerve degeneration, abnormal protein aggregation is also another common feature. Here we describe a novel frame-shift mutation in *NEFH* associated with CMT2 in two families of dominant inheritance (Chapter 4). The frameshift mutations leads to the stop-loss and extended translation of 40 amino acids that would otherwise encode the 3'-UTR. Overexpression of this frameshift mutation in cultured cells results in prominent protein aggregation that is absent when wildtype NEFH is overexpressed. *In vivo* expression of the mutant protein in developing zebrafish larvae negatively affects the development of motor neurons in comparison to wildtype NEFH overexpression.

In conclusion, we have identified a novel mitochondrial gene associated with dynamics, characterized a novel aggregation mechanism in neurofilaments, and developed models to study neurodegenerative diseases genes in zebrafish.
DEDICATION

This work is dedicated to my grandmother, Mary Abrams, who had Parkinson’s disease and passed away during the course of my candidacy, to my mother, Catherine Abrams, and to the rest of my family who encouraged my success. In particular, I would like to thank my wife, Emily, and 1 year old son, Jacob, who supported me. I could not have done it without you all.
ACKNOWLEDGEMENTS

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Hufnagel for joining forces. Dr. Toni Barrientos and Dr. Flavia Fontanesi for helping us through the last hurdle. I would also like to thank all of my committee members; Dr. Juan Young, Dr. Mustafa Tekin, and Dr. Grace Zhai, for their time and commitment, and to my external examiner, Marni Falk who traveled all this way to be here for my defense.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ANT</td>
<td>adenosine nucleotide transporter</td>
</tr>
<tr>
<td>ARSAC</td>
<td>autosomal recessive spastic ataxia of Charlevoix-Sanguenay</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CH</td>
<td>optic chiasm</td>
</tr>
<tr>
<td>CJ</td>
<td>cristae junctions</td>
</tr>
<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth neuropathy</td>
</tr>
<tr>
<td>CMT1</td>
<td>CMT type 1 demyelinating</td>
</tr>
<tr>
<td>CMT2</td>
<td>CMT type 2 axonal</td>
</tr>
<tr>
<td>DOA</td>
<td>dominant optic atrophy</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>ECAR</td>
<td>extra-cellular acidification rate</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization</td>
</tr>
<tr>
<td>HSMN</td>
<td>hereditary sensory and motor neuropathy</td>
</tr>
<tr>
<td>HSP</td>
<td>hereditary spastic paraplegia</td>
</tr>
</tbody>
</table>
IMM  inner mitochondrial membrane
IMS  inner mitochondrial membrane space
INL  inner nuclear layer
IPL  inner plexiform layer
MGC  3-methylglutaconic acid
MINOS mitochondrial inner membrane organizing system
MRI  magnetic resonance imaging
MRS  magnetic resonance spectroscopy
mtDNA mitochondrial DNA
NAA  n-acetyl-aspartate
OCR  oxygen consumption rate
OMIM Online Mendelian Inheritance of Man
OMM  outer mitochondrial membrane
ON  optic nerve
PCR  polymerase chain reaction
PD  Parkinson’s disease
RGC  retinal ganglion cell
RNA  ribose nucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction to Mitochondrial Dynamics and Neurological Diseases

Introduction

Mitochondria within a cell undergo constant fusion and fission, changing shape, size, number, and location. Mitochondrial morphology differs between tissue type and developmental stages, ranging from granules, sausages, to threadlike networks. The dynamic nature of the mitochondria has been observed under the microscope for over 100 years\(^1\), but the concept of mitochondrial dynamics has only recently been accepted as a mechanistic pathway due to advances in genetics\(^2\). Mitochondrial dynamics play a crucial role in many different neurological diseases and remain an attractive therapeutic target\(^3\). This chapter will review the major genes involved in mitochondrial dynamics, the history of their discoveries, the human diseases caused by their dysfunction, and the current hypothesized pathological mechanisms.

History of Mitochondrial Dynamics

Mitochondria are the powerhouse of the cell providing most of the cell’s energy in the form of ATP through oxidative phosphorylation. The metabolic pathways leading to the production of ATP are only one facet of mitochondrial function. Mitochondria within a cell are highly dynamic, and the disruption of their movement is associated with cellular dysfunction\(^4\). In 1914 Lewis and Lewis stated, “Any one type of mitochondria such as a granule, rod or thread may at times change into any other type or may fuse with another mitochondrion, or it
may divide into one or several mitochondria……..Can we infer from these observations anything concerning the real nature of the mitochondria?" The molecular mechanisms governing this motion was not accessible until advances in genetics. The field of mitochondrial dynamics as we know it today kick started after the discovery and characterization of several genes implicated in mitochondrial fusion, which were identified through forward genetic screens in drosophila, yeast, and through identifying disease genes in humans. The dynamic nature of the mitochondria is conserved in all eukaryotes and the homologous genes across species are still being discovered.

Fuzzy Onions

The first mitochondrial fusion protein to be identified was fuzzy onions (Fzo) found through a forward genetic screen in Drosophila. Fzo mutant male flies are sterile because the mitochondria in their sperm do not fuse properly. During spermatogenesis, the mitochondria aggregate and fuse to form two giant mitochondria that wrap around each other to form the Nebenkern, a super mitochondria that provides the energy needed for swimming. This normally onion like structure is said to resemble a fuzzy onions in mutant fly sperm at the level of electron microscopy.

Fzo is conserved in yeast where it is required for mitochondrial fusion and mitochondrial DNA (mtDNA) maintenance during mating. The homologs of Fzo in mammals are the mitofusins; MFN1 and MFN2 which are essential for mitochondrial fusion and cause embryonic lethality when knocked out in mice.
Mutations in *MFN2* were found to be the primary cause of Charcot-Marie-Tooth neuropathy type 2 (CMT2), a disorder leading to the degeneration of long peripheral neurons\(^9\). There are actually two mitofusin homologs in flies; *Fzo* and *Marf*\(^10\). Because *Fzo* is primarily expressed in testes, *Marf* is considered the functional equivalent of the mammalian mitofusins. *Fzo/mitofusin* are transmembrane GTPases in the outer mitochondrial membrane, which help to tether adjacent mitochondria during the fusion of the outer membrane\(^11\).

**Optic Atrophy**

In the genetics field, the nuclear encoded mitochondrial gene *OPA1* was linked to autosomal dominant optic atrophy and said to most closely resemble the dynamin related large GTPase Mgm1 in yeast\(^12\). Mgm1 which had been shown to be essential for mtDNA maintenance in yeast, was later implicated in the fusion of the inner mitochondrial membrane\(^13,14\). OPA1 is now the accepted functional homolog of Mgm1 in mammals\(^14,15\), and both proteins also play an important role in cristae remodeling independent of fusion\(^16\).

**Ugo (Fusion)**

Mitochondrial fusion is balanced by mitochondrial division or fission, which in yeast is carried out by a dynamin-like GTPase, Dnm1\(^17\). The loss of mtDNA and respiratory deficiency in yeast FZO mutants can be rescued by blocking fission\(^14,18\). Based upon this principle Sesaki and Jensen developed a screen to
identify other genes that cause loss of mtDNA, which could be rescued by blocking fission. This lead to the discovery of Ugo1 and Ugo2 (Pcp1/Rbd1)\textsuperscript{18} named so because Ugo is Japanese for fusion. Mitochondria are double membrane bound organelles, so fusion requires the coordinated merging of these four lipid bilayers. In yeast Fzo1, Ugo1, and Mgm1 were found to exist in a protein complex mediating the coordinated fusion of the outer and inner membranes\textsuperscript{19}, however the fusion mechanism in mammals is not as well understood because the outer and inner membranes can fuse independently of each other\textsuperscript{20}.

Ugo1 is a modified mitochondrial carrier protein and is most similar to SLC25A46. We found that mutations in SLC25A46 cause a phenotypic spectrum encompassing both optic atrophy and peripheral neuropathy, however the protein seems to play a greater role in mitochondrial fission\textsuperscript{21}. Ugo2 which is a Rhomboid-like metalloprotease implicated in Mgm1 processing goes by the other aliases PCP1 or RBD1. Homologs of this family in mammals include the presenilin-associated rhomboid protease (PARL)\textsuperscript{22}, Paraplegin (SPG7)\textsuperscript{23}, OMA1 and YME1L. OMA1 and YME1L are believed to directly cleave OPA1\textsuperscript{24}, while SPG7 is implicated in mitochondrial ribosome assembly, degradation of unfolded mitochondrial proteins, and the formation of the mitochondrial transition pore\textsuperscript{25,26}. 
These are among the first genes implicated in mitochondrial dynamics, however there are likely many other proteins involved. An RNAi screen in *C. elegans* found that approximately 80% of mitochondrial genes knocked down resulted in mitochondrial fragmentation or elongation\(^\text{27}\), which implicates that many mitochondrial proteins are either directly or secondarily involved in mitochondrial dynamics\(^\text{28}\). Even the lipids within the mitochondrial membranes themselves and the enzymes that process them could play a role in mitochondrial dynamics through physical effects on membrane fluidity\(^\text{29}\). The most widely accepted mitochondrial fusion/fission genes as well as their homologs, functions, domain structure, and associated human diseases are presented in table 1.1

<table>
<thead>
<tr>
<th>Table 1.1 Archetypal mitochondrial fusion and fission genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Function</strong></td>
</tr>
<tr>
<td>OMM Fusion</td>
</tr>
<tr>
<td>IMM Fusion</td>
</tr>
<tr>
<td>IMM remodeling</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Fission</td>
</tr>
<tr>
<td>IMM remodeling</td>
</tr>
</tbody>
</table>

**Presumed function:** Outer mitochondrial membrane (OMM); inner mitochondrial membrane (IMM). Protein families, accepted homologs in *S.cerevisae* (Yeast); *D.melanogaster* (Fruit fly); *H.sapiens* (Human). Human diseases caused by mutations in the genes. Alternative aliases are in (parenthesis); alternative homologs separated by / slash.
Canonical Mechanisms of Mitochondrial Dynamics

Mitochondrial Fusion

Mitochondria are double membrane bound organelles, so mitochondrial fusion requires the sequential merging of four membranes; the two outer mitochondrial membranes (OMM) followed by the two inner mitochondrial membranes (IMM) (figure 1.1). In mammals the mitofusins, MFN1 and MFN2, have overlapping roles in tethering the outer membranes before the first fusion event\textsuperscript{8,11,30}, while OPA1 is essential for the subsequent fusion of the inner membranes\textsuperscript{20}. In yeast, Ugo1 sits in the outer membrane and forms complexes between the outer (Fzo1) and inner membrane fusion proteins (Mgm1\textsuperscript{19}). In this way Ugo1 is thought to function as a fusogenic adaptor that coordinates the merging of the four lipid membranes\textsuperscript{31}. Mitochondrial fusion is important for mitochondrial morphology and mitochondrial DNA (mtDNA) maintenance. Cells that are null for the canonical fusion proteins have fragmented mitochondria that lose the mtDNA nucleoids. Such pathologies can be rescued by blocking fission\textsuperscript{6,18,32}.

Mitochondrial Fission

Mitochondrial fission is an antagonistic process to fusion\textsuperscript{17}. Loss of mitochondrial fission leads to increased mitochondrial connectivity\textsuperscript{33}. Mitochondrial fission is mediated through membrane constriction via DRP1\textsuperscript{34}. DRP1 is located in the cytosol and is recruited to the OMM\textsuperscript{35} through several proposed adaptor proteins such as FIS1\textsuperscript{36} (Figure 1.1). Mitochondrial fission
plays an important role in mitochondrial morphology and distribution, as well as in cell division, and in the segregation and maintenance of mtDNA\textsuperscript{37}.

**Figure 1.1 Canonical mechanisms of mitochondrial fusion and fission**

**Steps of mitochondrial fusion:** 1. Adjacent mitochondria come into close proximity 2. OMM tethering through mitofusins (MFN1/2) 3. OMM fusion, mixing of IMS and IMM tethering through OPA1 4. IMM fusion and merging of matrix. **Steps of mitochondrial fission:** 1. Single mitochondria 2. DRP1 recruitment from the cytosol to the OMM through a fission receptors including FIS1 3. DRP1 mediated membrane constriction 4. Membrane scission. **Key:** Outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), inner membrane space (IMS), and matrix, and key for proteins involved in fusion/fission.

**Mitochondrial-ER tethering**

Mitochondria are closely associated with the endoplasmic reticulum (ER) and physical contacts between the two organelles are important for mitochondrial lipid biosynthesis and calcium exchange\textsuperscript{38}. MFN2 has also been proposed to function...
in the dynamics between the mitochondria and the ER. MFN2 localizes to the outer mitochondrial membrane and a small fraction is also found in the ER\textsuperscript{38}. Based on this evidence MFN2 has been hypothesized to directly tether the mitochondria to the ER in a similar manner to mitochondria-mitochondria tethering prior to fusion. However, recent data shows that MFN2 ablation actually increases mitochondrial ER contacts contradicting this popular notion\textsuperscript{39}.

1.2 MFN2’s proposed roles in mitochondrial-ER tethering.
MFN2 localizes to the OMM as well as to the ER and the protein is hypothesized to directly tether to two organelles. Figure Adapted from A. Strickland 2015

Cristae Remodeling
Mitochondrial fusion and fission are the most obvious dynamics in live cells by conventional microscopy, however the inner mitochondrial membrane is also highly dynamic and is only visible through fixed sections of electron microscopy. The inner mitochondrial membrane is a complex structure composed of invaginations, termed cristae, which contact the outer membrane at junction sites. The inner membrane contains the respiratory chain complexes, ATP synthase, and metabolite transporters\textsuperscript{40}. The cristae folds increase the surface
area of the mitochondrial inner membrane, which is the main site of ATP production. The diameter of the cristae junctions adjacent to the outer membrane, respond dynamically to changes in nutrient availability and to the energetic state of the mitochondria. OPA1 helps to form the cristae junctions independently of its role in inner membrane fusion, and junction dynamics are mediated by the oligomerization and cleavage of OPA1. OPA1 is cleaved by the AAA+ proteases OMA1 and YME1L.

1.3 OPA1 and Mitofilin in cristae remodeling.

The inner mitochondrial membrane (IMM) is composed of cristae that respond dynamically to the energetic state of the cell. In high glucose conditions, cultured cells primarily undergo glycolysis and mitochondria are not utilized as much for oxidative phosphorylation. In high nutrient conditions, OPA1 exists as a monomer and the cristae junctions (CJ) are wide. In low glucose, mitochondria are forced to respire, and OPA1 forms oligomers that helps to maintain tight cristae junctions. OPA1 null cells do not have well-formed cristae and are more susceptible to cytochrome c release, upon exposure to apoptotic stimuli. Mitofilin is another protein directly
implicated in the formation of the cristae junctions. Mitofilin knockdown causes almost complete loss of the cristae junctions.

Cristae dynamics play an important role in apoptosis as the widening of the junctions occurs during cytochrome C release\textsuperscript{16} and OPA1 null cells are more susceptible to apoptotic stimuli\textsuperscript{44}. OPA1 directly interacts with IMMT(mitofilin)\textsuperscript{45} and members of the mitochondrial inner membrane organizing system MINOS\textsuperscript{40}. Mitofilin plays a direct role in the formation of the cristae, but not in fusion fission dynamics\textsuperscript{46}. Likewise, knockdown of mitofilin causes almost complete loss of the cristae junctions, loss of mtDNA, and increases the susceptibility of cells to apoptosis\textsuperscript{46}.

**Mitochondrial Dynamics in Human Disease**

Alterations in mitochondrial dynamics are a hallmark feature in a number of neurodegenerative disease including amyotrophic lateral sclerosis (ALS), Parkinson’s and Alzheimer’s diseases\textsuperscript{47}. Mitochondrial dynamics are intricately involved in many pathways including mitochondrial biogenesis, mitophagy, respiratory complex assembly, and sensitivity to oxidative stress\textsuperscript{48}. One of the critical and lingering questions in the field, is why defects in mitochondrial dynamics seem to selectively affect the nervous system. For this reason, functional studies into the causative genes of rare neurological disorders such as Charcot-Marie-Tooth (CMT) neuropathy types 2A, Dominant optic atrophy (DOA) have provided an important foundation from which to understand the role
mitochondrial dynamics in the human diseases\textsuperscript{4}. A summary of other diseases and genes involved in mitochondrial dynamics is outlined in Table 1.2.

<table>
<thead>
<tr>
<th>GENE</th>
<th>Disease</th>
<th>inheritance</th>
<th>Location</th>
<th>Family</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFN2</td>
<td>CMT axonal</td>
<td>AD</td>
<td>OMM</td>
<td>GTPase</td>
<td>Fusion</td>
</tr>
<tr>
<td>GDAP1</td>
<td>CMT intermediate</td>
<td>AR or AD</td>
<td>OMM</td>
<td>GST</td>
<td>Fission</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glutathione S-transferase</td>
<td></td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy</td>
<td>AD</td>
<td>IMM</td>
<td>GTPase</td>
<td>Fusion and cristae remodeling</td>
</tr>
<tr>
<td>OPA3</td>
<td>Optic atrophy</td>
<td>AR</td>
<td>OMM</td>
<td>?</td>
<td>Fission and susceptibility to apoptosis</td>
</tr>
<tr>
<td>SLC25A46</td>
<td>Optic atrophy</td>
<td>AR</td>
<td>OMM</td>
<td>Modified mitochondrial carrier protein</td>
<td>Fission</td>
</tr>
<tr>
<td>SPG7</td>
<td>HSP</td>
<td>AR</td>
<td>IMM</td>
<td>AAA+ metalloprotease</td>
<td>Degradation of unfolded proteins</td>
</tr>
<tr>
<td>(Paraplegin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SACS</td>
<td>Ataxia</td>
<td>AR</td>
<td>Cytosol with mitochondrial enrichment</td>
<td>?</td>
<td>Chaperone</td>
</tr>
<tr>
<td>PINK1</td>
<td>Parkinson’s</td>
<td>AR</td>
<td>OMM</td>
<td>PTEN putative Kinase</td>
<td>Mitophagy</td>
</tr>
<tr>
<td>PARK2</td>
<td>Parkinson’s</td>
<td>AR</td>
<td>Cytosol</td>
<td>E3 Ubiquitin Ligase</td>
<td>Mitophagy</td>
</tr>
</tbody>
</table>

Table 1.2 Human diseases caused by mutations in genes associated with mitochondrial dynamics. Genes; diseases; modes of inheritance, autosomal dominant (AD), autosomal recessive (AR); subcellular location, outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM); protein family; implied function.

Charcot-Marie Tooth Neuropathy (CMT)

CMT is the most commonly inherited disorder of the peripheral nervous system with an incidence of 1 in 2,500\textsuperscript{49}. The neuropathy affects the longest motor and sensory nerves causing axonal degeneration, but sparing the cell bodies. As the disease progresses, it weakens the lower limbs causing muscle wasting, accompanied by sensory loss, decreased reflexes, and foot deformities. CMT is
categorized as demyelinating (CMT1) or axonal (CMT2) based upon measurements of nerve conduction velocities\textsuperscript{53}.

The axonal form of CMT is primarily caused by dominant mutations in \textit{MFN2}\textsuperscript{97}, which is among the first mammalian proteins implicated in mitochondrial fusion\textsuperscript{7}. The exact reason that mutations in MFN2, a ubiquitously expressed protein, cause the selective degeneration of long axons is not completely understood and is hindered by the lack of access to patient tissues and the discordant phenotypes in animal models. \textit{MFN2} mutations can likely function through both dominant gain-of-function and loss-of-function mechanisms\textsuperscript{51}. Mfn2 is essential for embryonic development in mice\textsuperscript{8}. A conditional homozygous knockout mouse showed cerebellar degeneration and died several weeks after birth\textsuperscript{52}, while two separate knock-in models are post-natal lethal in the homozygous state and die shortly after birth\textsuperscript{53}. Interestingly, a loss-of-function zebrafish model showed a neuromuscular phenotype and reduced life span in the homozygous state\textsuperscript{54}.

While some of these animal models do recapitulate neuronal degeneration, pathology is only apparent in the homozygous state and results in metabolic defects and shortened lifespan. Meanwhile, two transgenic mouse models have recapitulated motor neuron phenotypes with longer life spans\textsuperscript{55,56}. While MFN2 mutations do not typically result in severe metabolic defects or directly affect human lifespan, some patients with \textit{MFN2} mutations have additional clinical features such cognitive impairment, white matter alterations,
hearing loss, and optic atrophy\textsuperscript{57} suggesting central nervous system involvement that is not always specific to long axons\textsuperscript{51}.

None the less, alterations in mitochondrial transport within axons is the leading hypothesis as to why defects in \textit{MFN2} lead to the selective degeneration of long axons, which has been demonstrated in various models\textsuperscript{56–58}. Other proposed mechanism of \textit{MFN2} include mtDNA depletion\textsuperscript{61}, and loss of mitochondrial ER contacts affecting calcium homeostasis\textsuperscript{38}.

A recessive intermediate form of CMT, which affects both the axon and the myelinating Schwann cells is caused by mutations in \textit{GDAP1}\textsuperscript{54} which has been characterized as a mitochondrial fission protein\textsuperscript{55}. Interestingly though \textit{GDAP1} also functions in glutathione metabolism, which has led to speculation as to whether its role in fission is secondary to an imbalance of oxidation and reduction within the mitochondria\textsuperscript{64}.

\textit{Optic Atrophy}

Autosomal dominant optic atrophy (DOA) is primarily caused by mutations in the nuclear encoded mitochondrial gene, \textit{OPA1}\textsuperscript{65}. The prevalence of DOA has been estimated around 1 in 50,000\textsuperscript{66,67} and mutations in \textit{OPA1} account for 60\% of cases\textsuperscript{25}. Heterozygous mouse models for Opa1 show age progressive visual defects and reduced synaptic connectivity of retinal ganglion cells\textsuperscript{68,69}, while homozygous mutations cause lethality. It remains to be elucidated as to why defects in \textit{OPA1} lead specifically to retinal ganglion cell dysfunction\textsuperscript{70}. Although optic atrophy primarily effects the retinal ganglion cells, patient’s with \textit{OPA1}
mutations have also been reported to have neurological symptoms including peripheral neuropathy, spasticity, and even Parkinosnianism\textsuperscript{57,71,72}.

The clinical spectrum of features associated with optic atrophy have been described as ‘plus’ syndromes\textsuperscript{57,73}. Costeff syndrome caused by recessive mutations in \textit{OPA3} is a syndromic optic atrophy with variability in clinical features such as ataxia, spasticity, and elevated urinary excretion of 3-methylglutaconic acid (MGC)\textsuperscript{74}. Although the precise function of the OPA3 protein is unknown, it is integral to the outer mitochondrial membrane and has been shown to play a role in mitochondrial fragmentation and susceptibility to apoptotic stimuli\textsuperscript{44}. Similarly, recessive mutations in \textit{SLC25A46}, cause optic atrophy with additional features including spasticity, cerebellar ataxia, peripheral neuropathy, and elevated MGC\textsuperscript{21}.

Optic atrophy is also associated with other diseases caused by mutations in nuclear encoded mitochondrial genes, such as Friedreich’s ataxia caused by mutations \textit{in FXN}\textsuperscript{75}, Charcot-Marie-Tooth type 2A (CMT2A) caused by mutations in \textit{MFN2}\textsuperscript{76}, and hereditary spastic paraplegia (HSP) caused by mutations in \textit{SPG7} \textsuperscript{77,78}. Most of the proteins involved in optic atrophy phenotypes are mitochondrial, indicating that the retinal ganglion cells are particularly susceptible to mitochondrial dysfunction\textsuperscript{79}.

\textit{Hereditary Spastic Paraplegias (HSP)}

Hereditary spastic paraplegias (HSP) are a group of neurodegenerative disorders that primarily affect the corticospinal tracts with an incidence of 0.5 to 12 per
100,000 people\textsuperscript{80}. Over 48 genetic loci have been identified with the SPG nomenclature; SPG7 and SPG5 being the most common\textsuperscript{78}. SPG7 encodes the paraplegin protein which is a mitochondrial membrane associated AAA+ ATPase metalloprotease implicated in the degradation of misfolded proteins, ribosome maturation\textsuperscript{81}, and most recently the formation of the mitochondrial permeability transition pore\textsuperscript{26}. The mitochondrial permeability transition pore is a large channel that forms during apoptosis, or programmed cell death. It functions by connecting the inner and outer mitochondrial membranes and allowing the diffusion of cytochrome C from the inner membrane and into the cytosol where it induces the cascade of events leading to apoptosis\textsuperscript{47}.

In yeast Mgm1 cleavage is mediated by the rhomboid AAA+ protease, Pcp1\textsuperscript{82}. Based on its homology to Pcp1, Paraplegin was originally thought to cleave OPA1\textsuperscript{43}, but that function is now attributed to the YME1L and OMA1 homologs\textsuperscript{24}. Interestingly though, some patients with SPG7 mutations have been shown to have multiple mtDNA deletions and reduced complex I, III, and IV activity\textsuperscript{23}, which is similar to what has been observed in OPA1 patients\textsuperscript{71,83}. A mouse model of SPG7 recapitulates the axonal degeneration and shows mitochondrial abnormalities associated with impaired axonal transport\textsuperscript{84}.

\textit{Cerebellar Ataxia}

Autosomal recessive spastic ataxia of Charlevoix-Sanguenay (ARSACS) is an early onset neurological disease characterized by spasticity and cerebellar ataxia, and is caused by mutations in the SACS gene\textsuperscript{85}. Functional studies in
patient fibroblasts and mouse models demonstrate that the loss-of-function of the SACS protein leads to increased mitochondrial connectivity and affects the distribution of mitochondria in Purkinje cell neurons\textsuperscript{86,87}. Purkinje cells have elaborate dendritic arbors that integrate the large amount of information processed in the cerebellum. The improper fission of the mitochondria in the SACS model does affect the mitochondrial distribution and transport into the dendritic arbors of Purkinje cells, but it is unclear if the SACS proteins plays a direct role in mitochondrial fission or if the hyperfused phenotype is secondary to its presumed chaperone function\textsuperscript{88}.

\textit{Parkinson’s Disease (PD)}

PD is one of the most common neurodegenerative disease affecting up to 2 in 100 people over the age of 65\textsuperscript{89}. Although the genetics and environmental factors contributing to PD are complex, several genes have been identified in early-onset Parkinsonism including PINK1 and Parkin\textsuperscript{10,89,90}. These proteins are implicated in the selective degradation of dysfunctional mitochondria, a process known as mitophagy\textsuperscript{91}. The hypothesized mechanism is that when PINK1, a mitochondrial associated kinase, builds up on the outer mitochondrial membrane it recruits Parkin. Parkin is an E2 ubiquitin ligase which targets other proteins for degradation such as MFN1 and MFN2\textsuperscript{90,91}. This could lead to loss of fusion and or increased fission which is thought to initiate the process of mitophagy\textsuperscript{10}. 
**Summary**

As described above, many neurodegenerative diseases converge on common aspects of mitochondrial dysfunction associated with dynamics. It is an attractive hypothesis to think that the canonical mechanisms of mitochondrial fusion and fission are so ubiquitously involved in such a wide range of disorders. In yeast, it is well established that restoring the balance between fusion and fission can rescue the metabolic defect in mitochondrial fusion deficient cells\(^{17}\). Furthermore, current research is demonstrating the therapeutic potential of overexpressing OPA1\(^{92}\) in mitochondrial or the inhibition of DRP\(^{1,93}\) in neurological disease models. Future research using higher throughput approaches, will hopefully help us to further delineate which fusion and fission factors can be modulated to ameliorate neurological phenotypes in various disease models.

**Mitochondrial Dynamics in Neurons**

*Introduction*

Overall, the genetic evidence and animal models corroborate the importance of mitochondrial dynamics for nervous system function. Neurons are energetically demanding tissues, thus relying heavily on oxidative phosphorylation. Yet in these diseases heart and skeletal muscle, which also rely on oxidative phosphorylation are not typically affected.

In order to appreciate why the neurons may be differentially affected by alterations in mitochondrial dynamics, it should be noted that neurons are a
highly polarized cell. The axon and dendrites, are often far away from the soma and form elaborate processes. It has long been noted that mitochondria are not randomly distributed within axons, but cluster at regions of high energy demand such as the axon initial segment\textsuperscript{94}, Nodes of Ranvier\textsuperscript{60}, and the synapse\textsuperscript{95} (Figure 1.4). Mitochondria support nerve function by producing the energy required for cytoskeletal development, axonal transport, producing and recycling synaptic vesicles, maintaining membrane potential, and sequestering the calcium released after an action potential\textsuperscript{96}.

The axonal transport of mitochondria

A major dogma in the field of mitochondrial dynamics is that the proper distribution and transport of mitochondria in neurons is essential for their function. MFN2's role in the degeneration of long peripheral axons in CMT2A supports this notion. CMT affects the longest peripheral axons which can reach up to a meter long. Despite little direct evidence, it is widely assumed that the bulk of mitochondrial biogenesis and degradation or mitophagy occurs within the soma\textsuperscript{96}. The reason being that most of the mitochondria's proteins are produced from nuclear DNA and the degradation machinery is also found in the soma\textsuperscript{96}. Assuming this is the case, mitochondria in long axons would need to be transported down the axon to the pre-synaptic axon terminal, in order to replace pools of aged mitochondria.
1.4 Mitochondrial distribution in a neuron. Neurons are highly polarized cells with dendrites, soma, axon and synapse. Mitochondria cluster at distinct regions within a neuron including the axon initial segment or hillock, nodes of Ranvier between the myelinating Schwann cells, and at the synapse.

Only 13% of mitochondria in cultured neurons are, with two thirds moving away from the nucleus, while one third moves toward the nucleus at $1.02 \pm 0.02$ um/s and $1.41 \pm 0.02$ um/s respectively. Given a 1 meter long axon it would take more than a week for a mitochondrion to be transported between the soma and the terminals assuming it did not stop along the way. Various genetic models of MFN2 exhibit decreased axonal transport of mitochondria. Mitochondrial fusion and fission are interconnected with axonal transport. For example mitochondrial fusion requires the merging of mitochondria which must presumably be transported close enough together. Additionally mitochondrial fission is required in order to have mitochondria small enough to be transported down the axon. Disruption of the fission protein drp1 in drosophila leads to mitochondrial depletion at the neuromuscular junction.
The notion of a disrupted axonal transport mechanism in CMT is an attractive hypothesis that could explain the length dependence of the disease. In addition, mutations in the genes encoding the kinesin motors (KIF) also cause length dependent axonal degeneration. For example, mutations $KIF1C$ cause hereditary spastic paraplegia$^{99}$, while $KIF1B$ has been implicated in CMT2$^{100}$. The KIF proteins are involved in the transport of diverse cargos including mitochondria, neurofilaments, vesicles, organelles, RNA granules, receptors, trophic factors, and injury signals$^{101}$.

Axonal mitochondria are transported along microtubules via kinesin, dynein, and myosin motor proteins through interactions with various adaptor proteins$^{96}$. MFN2 has been shown to interact with Miro/Milton adaptor complex which interacts with kinesin motors to mediate the transport of mitochondria along microtubules$^{59}$, suggesting a direct involvement in axonal transport (Figure 1.5).

While MFN2’s role in axonal transport is a widely accepted as the pathological mechanism in peripheral neuropathy, there is direct evidence indicating that mitochondria can be both generated$^{102}$ and degraded$^{103}$ within the axon itself. Considering that mitochondrial fusion and fission are so interconnected with mitochondrial biogenesis and mitophagy, it is unclear to what extent mitochondrial transport actually plays the dominant role in degeneration. For example, one study found that depleting pyramidal neuron dendrites of mitochondria increased branching$^{104}$. There is clearly a lack of understanding of the life cycle of mitochondria in neurons and the correlative versus causative
relationship of mitochondrial transport in degenerating neurons. Therefore, the field could benefit from better tools and model systems.

1.5 MFN2’s proposed role in axonal transport.

MFN2 interacts with the MIRO/Milton (TRAK) adaptor proteins, which bind to the kinesin (KIF) motor proteins which mediate fast axonal transport.

Additionally, altered axonal transport is not believed to be the causative mechanism of OPA1 dysfunction. Misko et al. reports that while loss of MFN1 or MFN2 alters the axonal transport of mitochondria, OPA1 knockdown does not\textsuperscript{59}. OPA1 has secondary roles in cristae remodeling, adaptation to metabolic demand, mtDNA maintenance, calcium uptake, and apoptosis all of which could contribute to the pathology of DOA\textsuperscript{105}. The broadened optic atrophy phenotypic spectrum, includes spasticity, cerebellar atrophy, and white matter alterations, indicating that OPA1 dysfunction is not entirely specific to retinal ganglion cells\textsuperscript{57,73}. Likewise the \textit{MFN2} phenotypic spectrum is also widening to include more systemic phenotypes including cognitive impairment, white matter alterations, sensorineural hearing loss, and optic atrophy\textsuperscript{51}. A conditional Mfn2 knockout
mouse model develops cerebellar degeneration\textsuperscript{52}. The phenotypic overlap between $MFN2$ and $OPA1$ could reflect a mechanistic overlap between the pathological mechanisms of the two proteins.

**Hypothesis**

I hypothesize that more of the genes involved in rare hereditary disorders will further converge on common pathways and mechanism involved in mitochondrial dynamics. Our lab through collaborative exome sequencing, discovered recessive mutations in a previously uncharacterized gene, $SLC25A46$, associated with optic atrophy, spasticity, and peripheral neuropathy. We hypothesized that knocking down this gene in zebrafish would cause a neurological phenotype outlined in Chapter 3. Based on sequence comparison, we also hypothesized that this protein encoded an atypical mitochondrial carrier protein resembling Ugo1, an essential mitochondrial fusion gene in yeast. The results of this study are discussed in Chapter 2. Finally, we identified a novel stop-loss mutation in $NEFH$ causing CMT2, which we hypothesized could cause aggregation and gain-of-function toxicity in a zebrafish (Chapter 4).
Chapter 2: Characterization of SLC25A46 as a UGO1-like Protein Associated with Neurological Disease

The data presented in this chapter was previously published in Nature Genetics (Doi:10.1038). Genetics studies are large collaborative projects including many co-authors who have made contributions through either clinical evaluations, clinical diagnostics, data sharing, and through direct functional contributions. When we started this project, we had identified only a single family with mutations in SLC25A46, and by the time we were ready to publish, this number had increased to a total of four families widening the phenotypic spectrum of the disease. The major clinical contributions were made by groups led by Taosheng Huang, Andrea Németh, and Valerio Carelli. Experimental contributions in terms of biochemistry including characterizing the outer membrane localization of SLC25A46 and its interaction mitofilin, were made by Adriana Rebelo, Flavia Fontanesi, and Antonio Barrientos. All cell experiments and analysis were carried out by myself with assistance by Adriana Rebelo and Alleene Strickland, with electron microscopy by Neville Patel. Metabolic and imaging analysis in the patient fibroblasts was performed by Claudia Zanna and Alessandra Maresca.

Background

Mitochondria are dynamic organelles undergoing constant fusion and fission. MFN1 and MFN2 are the major proteins thought to be responsible for the fusion of the mitochondrial outer membranes, while OPA1 is responsible for inner
membrane fusion\textsuperscript{106}. Dominant optic atrophy (DOA)\textsuperscript{15,65} and axonal peripheral neuropathy (Charcot-Marie-Tooth Type 2 or CMT2)\textsuperscript{9} are hereditary neurodegenerative disorders most commonly caused by mutations in the canonical mitochondrial fusion genes \textit{OPA1} and \textit{MFN2}, respectively\textsuperscript{106}. In yeast, homologs of \textit{OPA1}(Mgm1) and \textit{MFN2}(Fzo1) work in concert with a third protein, \textit{Ugo1}\textsuperscript{18,19}. \textit{Ugo1} has been shown to function as an adaptor protein by physically interacting with Mgm1 and Fzo1 to coordinate the sequential merging of the four lipid bilayers during the fusion\textsuperscript{19}. \textit{Ugo1} also interacts the cristae remodeling protein \textit{Fcj1} whose human equivalent in mitofilin\textsuperscript{46,107}. \textit{Ugo1} is modified mitochondrial solute transporter with unidentified homologs in humans\textsuperscript{36}.

\textbf{Summary}

By whole exome sequencing patients with optic atrophy and CMT2, we identified four families with recessive mutations in the previously uncharacterized gene, \textit{SLC25A46}. We demonstrate that \textit{SLC25A46}, like \textit{Ugo1}, is a modified carrier protein that has been recruited to the outer mitochondrial membrane and interacts with the inner membrane remodeling protein, mitofilin(\textit{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 \textit{SLC25A46} strengthens the genetic overlap between optic atrophy and CMT2, while exemplifying a novel class of modified solute transporters linked to mitochondrial dynamics.
Clinical Descriptions

Table 2.1 Clinical features of SLC25A46 patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Optic atrophy</th>
<th>Axonal neuropathy</th>
<th>Cerebellar ataxia</th>
<th>Genetic mutations</th>
<th>Protein change</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK II-2</td>
<td>F</td>
<td>8 years</td>
<td>43 years</td>
<td></td>
<td>c.165_166insC</td>
<td>p.His56fs*94</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.746G&gt;A</td>
<td>p.Gly249Asp</td>
</tr>
<tr>
<td>UK II-1</td>
<td>F</td>
<td>5 years</td>
<td>~40 years</td>
<td></td>
<td>c.165_166insC</td>
<td>p.His56fs*94</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.746G&gt;A</td>
<td>p.Gly249Asp</td>
</tr>
<tr>
<td>IT II-3</td>
<td>M</td>
<td>2 years</td>
<td>18 years</td>
<td>28 years</td>
<td>c.1018C&gt;T</td>
<td>p.Arg340Cys</td>
</tr>
<tr>
<td>PL II-8</td>
<td>F</td>
<td>&gt;2 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL II-7</td>
<td>M</td>
<td>&gt;2 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL II-5</td>
<td>F</td>
<td>11.5 years</td>
<td>5 years</td>
<td>5.2 years</td>
<td>c.1005A&gt;T</td>
<td>p.Glu335Asp</td>
</tr>
<tr>
<td>US II-3</td>
<td>F</td>
<td>&lt;1 year</td>
<td></td>
<td>&lt;1 year</td>
<td>c.882_885dupTTAC</td>
<td>p.Asn296fs*297</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.998C&gt;T</td>
<td>p.Pro333Leu</td>
</tr>
</tbody>
</table>

Table 2.1 Summary of clinical descriptions of patients with recessive SLC25A46 mutations. Years represent ages of clinical diagnosis.

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 joint 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/reinnervation 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, he 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 that progressively worsening over time, and were 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.

Materials and Methods

Whole exome sequencing
Whole genomic DNA was extracted from whole blood by standard methods.
Library construction was performed on dsDNA, sheared by sonication to an
average size of 200 bp, in an automated fashion on an IntegenX Apollo324. After
9 cycles of PCR amplification using the Clontech Advantage II kit, 1ug of
genomic library was recovered for exome enrichment using the NimbleGen EZ
Exome V2 kit. Libraries were sequenced on an Illumina HiSeq2500. The
Genomes Management Application tool (GEM.app) was used for variant calling
using the standard pipeline.¹⁰⁸
**Sanger sequencing**

Sanger sequencing was performed by standard methods on the 8 exons of the *SLC25A46* gene (NM_138773.1). Primers were designed using Primer3. PCR products were amplified using 50ng DNA, with standard PCR reagents Econotaq (Lucigen, WI), on an ABI Veriti Thermocycler (Applied Biosystems, Austin, TX). PCR products were precipitated and sequencing PCR performed using BigDye Terminator Ready Reaction Mix (ABI Biosystems).

**Immunofluorescence**

COS-7 or HeLa cells were seeded to 75% confluence onto glass coverslips and transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells were fixed with paraformaldehyde for 20 min, permeabilized with cold methanol for 5 min and stained with anti-myc (#2276 and #2278) or anti-HA antibodies (2367 and 3724) from Cell Signaling. Tom20 (SC-11415) from Santa Cruz was used to label mitochondrial outer membrane. Corresponding Alexa-fluor-conjugated secondary antibodies (Life Technologies) were used for detection. Coverslips were mounted onto slides with DAPI mounting media (Vectashield), and imaged with a confocal microscope, Zeiss LSM710, 63X/1.4 oil objective with a pinhole of less than one Airy unit and processed with Fiji (Image J).
**Mitochondrial subfractionation and proteinase K protection assay**

HEK293T cells were grown in DMEM (Gibco) with 10% fetal bovine serum and incubated at 37°C. Cells were transfected with SLC25A46-HA and mitochondria were isolated 24 hours post transfection as previously reported\textsuperscript{109}. To test proteins solubility, purified mitochondria were ruptured by sonication. The membrane fraction was recovered by centrifugation at 20,000 x g for 15 minutes at 4°C and was resuspended in 0.1M Na\textsubscript{2}CO\textsubscript{3} pH 11. The sample was incubated on ice for 30 minutes and both soluble extrinsic membrane proteins and insoluble intrinsic membrane proteins were separated by centrifugation at 20,000 x g for 15 minutes at 4°C. Purified mitochondria were resuspended in 10 mM TrisHCl pH 7, 10mM KCl, 0.15mM MgSO\textsubscript{4} buffer either containing 0.25 M sucrose or devoid of sucrose to allow mitochondrial swelling and conversion to mitoplasts\textsuperscript{110}. Samples were treated with proteinase K at final concentration of 0.62 µg/ml and incubated on ice for 30 min. The reaction was inhibited with 2mM phenylmethylsulfonyl fluoride (PMSF). Mitochondria and mitoplasts were recovered by centrifugation at 20,000 x g for 15 min at 4°C and analyzed by Western blotting.

**Sucrose gradient analysis**

Four hundred µg of purified mitochondria were solubilized in 0.08 mL of extraction buffer (20mM TrisHCl pH 7.4, 100 mM KCl, 1 mM MgSO\textsubscript{4}, 0.5 mM PMSF) containing 1% digitonin, on ice for 15 minutes. The clarified extract was obtained by centrifugation at 20,000 x g for 15 minutes 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. Following centrifugation for 12 hours at 28,000 r.p.m. 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 lactate dehydrogenase activity by measuring NADH-dependent conversion of pyruvate to lactate. Subsequently, proteins contained in each fraction were concentrated by TCA precipitation and analyzed by immunoblotting.

**Immunoprecipitation and mass spectrometry**

HEK293T cells were transfected with SLC25A46-HA using Turbofect reagent (Thermo Scientific). After 24 hr cells were lysed with a dounce homogenizer and mitochondria were isolated with the mitochondria isolation kit for cultured cells (Pierce) following manufacture’s protocol. 800 ug of protein from mitochondria were incubated with 5 ug of anti-HA tag mouse (Cell signaling ab9110) at room temperature for 2 h. Then, the proteins/antibody mixture was incubated with protein A/G magnetic beads (Thermo Scientific) at room temperature for 1 hr on a rotator. Beads were collected using a magnetic stand and washed 3X with 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, SDS-PAGE gel was washed 2X with ultra-pure water for 5 min and fixed with 30% ethanol: 10% acetic acid solution. Samples were analyzed by NuPAGE® (Invitrogen) SDS-PAGE Gel (4-20%) incubated with silver staining solution for 5 min followed by the developing reagent for 3 min. Reaction was
stopped with 5% acetic acid for 10 min. Bands were excised from the gel using a light box and submitted for mass spectrometry analysis at Scripps Center for Metabolomics and Mass Spectrometry.

Co-immunoprecipitation

HEK293T cells were double transfected with SLC25A46-HA and mitofilin-myc or as a negative control SLC25A46 and pCMV/myc/mitoGFP (Invitrogen). After 24 hr cells were lysed with a dounce homogenizer and mitochondria were isolated with the mitochondria isolation kit for cultured cells (Pierce) following manufacture’s protocol. 800 ug of protein from mitochondria were incubated with 5 μg of either anti-HA mouse, anti-myc rabbit (Cell signaling), or control mouse and rabbit IgG at room temperature for 2 h. Then, the proteins/antibody mixture was incubated with protein A/G magnetic beads (Thermo Scientific) at room temperature for 1 hr on a rotator. Beads were collected using a magnetic stand and washed 3X with manufacturer’s IP Lysis/wash buffer. Proteins were eluted with a low PH elution buffer. Protein samples were analyzed by NuPAGE® (Invitrogen) SDS-PAGE Gel (4-20%) followed by western blot using appropriate antibodies. Band signal was developed with either chemiluminescent substrates West Pico or West Femto (Thermo).

Overexpression and knockdown in mammalian cells

Cells were plated to 70% confluence on glass coverslips in a 6 well plate. Plasmid transfections were carried out using Lipofectamine 2000 (Invitrogen)
Mitochondrial morphology analysis and energetic profiling of human fibroblasts

Fibroblasts were seeded onto 36 mm diameter 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 acquired with an inverted Nikon Eclipse Ti-U epifluorescence microscope equipped with a back-illuminated Photometrics Cascade CCD camera (Roper Scientific). Images were collected using a 63X/1.4 oil objective. Data were acquired and analyzed using the Metamorph software (Universal Imaging Corp.). Oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR)
were measured in adherent fibroblasts with a XFe24 Extracellular Flux Analyzer (Seahorse Bioscience). Each control and mutant fibroblast cell lines were seeded in 5 wells of a 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 hours at 37 °C in 5% CO2 atmosphere. After replacing the growth medium with 575 μL of bicarbonate-free DMEM, pH 7.3, pre-warmed, cells were incubated at 37 °C for 1 hour before starting the assay.

After baseline measurements of OCR and ECAR, OCR was measured after sequentially adding to each well 75 μL of oligomycin (O) and 75 μl of carbonyl cyanide 4-(trifluoromethoxy) phenylhy- drzone (FCCP), 75 ul of rotenone (R) and 75ul of Antimycin A (AA) to reach working concentrations of 1μM. OCR and ECAR values were normalized on protein content measured by Sulforhodamine (SRB) assay, following standard protocol. For data analysis the following parameters were evaluated: BASAL RESPIRATION: measurement prior oligomycin injection minus non-mitochondrial respiration (measurement after antimycin A injection); PROTON LEAK-LINKED RESPIRATION: measurement after oligomycin injection minus non-mitochondrial respiration; ATP-LINKED RESPIRATION: basal respiration minus proton leak; MAXIMAL RESPIRATION: measurement after FCCP minus non-mitochondrial respiration; SPARE CAPACITY: maximal respiration minus basal respiration; CI-LINKED RESPIRATION: basal - measurement after rotenone; MAXIMAL CI-LINKED RESPIRATION: maximal respiration minus measurement after rotenone.
**Photoactivation assay**

The Mito-PA-GFP assay was performed as previously described\(^{112}\), using a Zeiss LSM710 with a 63X/1.4 oil objective, heated stage, and carbon dioxide chamber. After acquisition of the preactivated image, a circle of approximately 5 µm was selected using the regions setup and was activated with the 405 nm laser. The postactivation image was immediately acquired using the 488 nm laser and subsequent images were taken at 30 minute increments for a total of 90 minutes. Images were processed in Fiji and displayed with LUT:fire. For the mitochondrial fusion assay, the mean pixel intensity was calculated for the region of interest (ROI) and normalized to the postactivation image. For the assessment of the mitochondrial connectivity, the area of the activated GFP was measured in the post activation image and normalized to the area of the activated region.

**Transmission electron microscopy and analysis**

COS-7 cells were cultured in 25 cm\(^2\) flasks (Corning) and treated with siRNAs as previously described. After 2 days, cells were fixed for four days in 2.5% glutaraldehyde in Millonig's phosphate buffer\(^{113}\), post-fixed in 1% OsO\(_4\) and uranyl acetate followed by dehydration in an ethanol series and embedded in Spur's. Ultrathin, 60 nm sections were made using a Leica microtome and were stained with cold lead citrate for 6 minutes\(^{113}\). Images were acquired with a Joel JEM-1400 transmission electron microscope with Gatan camera. All images were processed and analyzed using Fiji (image J).
**Phylogenetic analysis**

The concise list of the human the mitochondrial carrier (SLC25) family was obtained from the Bioparadigms web site, while all mitochondrial carrier domain containing proteins of *C.elegans*, *S. cerevisiae*, and *S. pombe* were retrieved from the European Bioinformatic institute. 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 1000 bootstraps. The consensus tree was visualized and drawn using iTol.

**ATP and mtDNA analysis**

ATP was measured according to manufactures protocol using the ATP Determination Kit (Molecular Probes). COS-7 cells were transfected with siRNAs as previously described and homogenized in ATP assay buffer at 48 hours post transfection, or 5 whole zebrafish embryos were homogenized in ATP assay buffer at 24 hours post fertilization. Homogenates were spun at 12,000 g for 5 min to pellet tissues debris. 10 ul supernatant were added to a 97 well dish containing 90 ul of the luciferase reaction mix from the ATP Determination Kit (Molecular Probes). 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. mtDNA levels were quantified by quantitative PCR as described. 

\[^{114}\]
Results

By applying whole exome sequencing with established methods and cut-offs for variant filtering in Mendelian inherited diseases\textsuperscript{108}, we found four families with recessive variants in the nuclear-encoded mitochondrial gene \textit{SLC25A46}. After excluding other candidate genes by segregation analysis, we identified the compound heterozygous mutations \textit{c.165_166insC}, \textit{p.His56fs*94} and \textit{c.746G>A}, \textit{p.Gly249Asp} in a British family (UK), a homozygous mutation \textit{c.1005A>T}, \textit{p.Glu335Asp} in a Palestinian family (PL), and the compound heterozygous variants \textit{c.882_885dupTTAC}, \textit{p.As296fs*297} and \textit{c.998C>T}, \textit{p.Pro333Leu} in an American family (US). We then used conventional Sanger sequencing methods to screen \textit{SLC25A46} in similar cases without a genetic diagnosis, and identified an additional family from Sardinia, Italy (IT), with the homozygous mutation \textit{c.1018C>T}, \textit{p.Arg340Cys} (Fig. 2.1).

All non-truncating changes were predicted to be deleterious. Patients in these four families presented with similar phenotypic core features including optic atrophy, axonal CMT, as well as cerebellar atrophy with varying severity. Magnetic resonance spectroscopy (MRS) data in two of the patients found decreased N-Acetyl-Aspartate (NAA) and increased lactate in the central nervous system (data not shown) 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).
Figure 2.1 Pedigrees and clinical features of optic atrophy plus syndromes with variants in SLC25A46. (a) Filled symbol, affected individual; symbol with a slash, deceased individual; triangle, miscarriage; M, mutant allele; +, wild-type allele; asterisk, individual for whom the whole exome was sequenced; arrow, proband. (b) Schematic diagram of the SLC25A46 gene, NM_138773, located on chromosome 5 (cDNA: 1,257 bp, 418 amino acids), with the positions of associated mutations indicated by arrows. The conserved mitochondrial carrier domain is indicated by a blue rectangle. (c) Image of the optic disc from patient UK II-2 showing primary temporal optic nerve pallor when compared to an unaffected individual. (d) Photograph of the legs of patient IT II-3 showing muscle wasting stereotypic of CMT2. (e) Magnetic resonance imaging (MRI) showing bilateral hyperintensity of white matter (arrows) in the cerebellum, on a FLAIR T2-weighted image of patient IT II-3.

SLC25A46 is a member of the mitochondrial solute carrier family\textsuperscript{115} (SLC25) and is predicted to function as a transporter across the inner mitochondrial membrane\textsuperscript{116}. Querying for a similar carrier protein in yeast, we found a reciprocal BLAST (http://blast.ncbi.nlm.nih.gov/) match between SLC25A46 and the putative Ugo1 ortholog of Schizosaccharomyces japonicus, indicating significant homology. Ugo1 is a modified mitochondrial solute carrier in
the outer mitochondrial membrane (OMM)\textsuperscript{117,118} that operates as a mitochondrial fusion factor in \textit{Saccharomyces cerevisiae}\textsuperscript{18} with no homologs identified in metazoans\textsuperscript{36}. To unbiasedly determine the most similar protein to Ugo1 in metazoans, we created a bootstrapped, maximum-likelihood phylogenetic tree with all mitochondrial carrier domain-containing proteins in \textit{Homo sapiens}, \textit{Caenorhabditis elegans}, \textit{Saccharomyces cerevisiae}, and \textit{Schizosaccharomyces pombe}. This revealed SLC25A46 as the most similar to Ugo1 (Fig 2.2 c,d). However, there is insufficient evidence to determine orthology and SLC25A46 fails to complement the \textit{ugo1} deletion in yeast (data not shown).

Figure 2.2 Phylogentic trees of mitochondrial carrier domain containing proteins in various species. (a) Phylogenetic tree of all 35 mitochondrial carrier domain containing proteins in \textit{S. cerivisae}. Demonstrating the sequence divergence of Ugo1 portrayed by longer branch length. (b) Phylogenetic tree of all 53 mitochondrial carrier domain containing proteins in \textit{H.sapiens} demonstrating the divergence of SLC25A46 and the mitochondrial carrier homologs
(MTCH1 and MTCH2). (c) Neighbor joining tree showing the relative similarity between all carrier proteins in *H. sapiens*, *C. elegans*, *S. cerevisae*, and *S. pombe*, drawn with 1,000 bootstraps. Red arrow indicates the node at which the SLC25A46 and Ugo1 orthologs bifurcate. (b) Collapsed tree depicting the node with calculated branch support values out of 1000 and respective branch lengths (blue). SLC25A46 is orthologous to Y40B1B.8 in *C. elegans*, while Ugo1 is orthologous to Ugo1p in *S. pombe*; however the branch support value between Ugo1 and SLC25A46 is under 700, and is therefore insufficient evidence for orthology between these proteins.

During evolution, homologs of mitochondrial carriers, usually inner membrane proteins\textsuperscript{116}, have been modified and recruited to the OMM to perform specific functions, unrelated to metabolite transport. The list includes the mammalian mitochondrial carrier homologs MTCH1\textsuperscript{119} and MTCH2\textsuperscript{120,121}, critical players in apoptosis, and yeast Ugo1\textsuperscript{18}. Because human SLC25A46 is also a highly derived carrier protein (Fig 2.2 a), we investigated its submitochondrial localization.

Immunocytochemistry studies in COS-7 cells demonstrate that HA-tagged SLC25A46 co-localizes more with the OMM marker (TOM20) and less with the inner membrane markers (myc-tagged mitofilin or ANT2) (Figure 2.3 a). 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 (Figure 2.3 b,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 (data not shown). Consistently, sucrose gradient sedimentation analyses found mitofilin to co-sediment with a small fraction of SLC25A46-HA, indicating that they may exist in the same high molecular mass complex (Figure 2.3 d). The interaction
between SLC25A46 and mitofilin was further confirmed by co-immunoprecipitation assays using mitochondria isolated from cells transfected with SLC25A46-HA (data not shown) or co-transfected with SLC25A46-HA and mitofilin-myc (Figure 2.3 e), followed by western blot detection. These results are congruent to Ugo1, which has been shown to co-fractionate with Fcj1\textsuperscript{122}, the homolog of mammalian IMMT(mitofilin).

Figure 2.3 SLC25A46 localizes to the outer mitochondrial membrane and interacts with mitofilin on the inner membrane. (a) High resolution confocal images of COS-7 cells co-transfected with SLC25A46-HA and Mitofilin-myc. TOM20 and Mitofilin-myc were used as markers of the outer and inner mitochondrial membranes respectively. Scale bar=1 μm. Linear profiles of the fluorescence intensity in arbitrary units along 2 μm dashed lines in merged images. (b) Mitochondria (M) isolated from SLC25A46-HA transfected HEK293T cells 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 mitoflin 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) Co-immunoprecipitation in HEK293T cells co-transfected with mitofilin-myc and SLC25A46-HA or SLC25A46-HA and myc-tagged mitochondria-targeted GFP as negative control.

To determine if SLC25A46 plays a conserved role in mitochondrial dynamics, we compared the effects of protein overexpression to the ATP transporter ANT2 (also known as SLC25A5) and found that SLC25A46, unlike ANT2, leads to mitochondrial fragmentation in cell lines (Figure 2.4 a,b). Next we used siRNA to knockdown SLC25A46 and found it to cause mitochondrial hyperfusion (Figure 2.4 c,d). To determine the degree of mitochondrial connectivity, we observed the instantaneous diffusion of matrix targeted photoactivatable GFP, revealing that the GFP diffuses into larger areas of the knockdown cells versus controls (Figure 2.4 e,f).

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 minutes and found no significant difference in the fusion rates between control and knockdown cells (Figure 2.5). This in conjunction with the mitochondrial constriction sites in electron microscopy
(Figure 2.4 g,h), which appear to be fission intermediates\textsuperscript{123} suggests that delays in fission or elongation contribute more to the hyperfused morphology than an increase in fusion rate.

![Image](image1.png)

**Figure 2.4 SLC25A46 levels regulate mitochondrial morphology.** (a) SLC25A46 overexpression causes fragmentation of the mitochondrial network in HeLa cells in comparison to ANT2. (b) Percentage of transfected cells with fragmented mitochondria from three independent experiments. Error bars indicate standard deviation (s.d.). One-sided T-test was used to determine significance. $P$ value = 0.00004. ANT2 n=22; SLC25A46 n=21. (c) HeLa cells treated with siRNAs and stained with Tom20. Mitochondria are more hyperfilamentous when the SLC25A46 is knocked down. (d) Quantification of mitochondrial morphology. 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 was used to assess mitochondrial connectivity. The region of interest (ROI) white circle in the pre-activation image was targeted with the laser, while the corresponding signal in the post-activation image represents the extent of the mitochondrial connectivity. (f) Quantification of the activated mitochondrial area in the post-activation image normalized to the ROI of the pre-activated image. Error bars represent s.d. and $P$ value was calculated by one tailed T-test $P = 0.0009$. Control siRNA n=42; SLC25A46 siRNA n=38. (g) Representative electron micrographs of a mitochondrial cross-section with constriction sites (arrows) in SLC25A46 siRNA treated COS-7 cells. (h) Percentage of cross-sections with visible constriction sites; 2 out of 103 control versus 11 out 81
in the knockdown. Error bars represents s.d. and significance was determined by a 2-tailed Fisher’s exact test, $P$ value 0.0028. Scale bars (a,c,e) = 10 µm; (g) = 100 nm top, 200 nm bottom.

Figure 2.5 Mitochondrial fusion assay by the extinction of mitochondrial-targeted photoactivated GFP (Mito PA-GFP). Mito PA-GFP was activated in an ROI (white circle in the preactivated image), and images were acquired up to 90 min after activation. There was no significant difference between the extinction rates of control and knockdown cells by two-tailed $t$ test, suggesting that there is neither an increase nor decrease in the mitochondrial fusion rate. Scale bars, 10 µm.
Figure 2.6 Knockdown in cell lines and zebrafish does not affect total ATP or mtDNA amount (a) RT-PCR confirmation of SLC25A46 knockdown in COS-7 cells 72 hpt. (b) q-PCR confirmation of SLC25A46 knockdown in HeLa cells. (c) Total cellular ATP measured in COS-7 cells 72 hpt. (d) Total ATP measured in zebrafish 24 hpf. (e) Total mtDNA amount in HeLa cells 72 hpt.

We further examined fibroblasts from proband IT II-3, which display a hyper-filamentous and interconnected network in comparison to both, the unaffected sibling IT II-2 and control, indicating the relevance of this phenomenon (Figure 2.7 a,b). Loss-of-function of SLC25A46 is not associated with changes in total cellular ATP, mitochondrial DNA content, or membrane potential in either siRNA-treated cell lines, or a knockdown zebrafish model.
(Figure 2.6 a-e). However, patient IT II-3 fibroblasts show decreased oxygen consumption rate (OCR) and a glycolytic shift of metabolism (decreased OCR/ECAR ratio) (Figure 2.7 c-e) consistent with the increased lactate peak found in MRS (data not shown). A decrease in ATP synthesis driven by complex I substrates, was trending, but not significant (data not shown). These metabolic changes are congruent to previous findings in patient fibroblasts with OPA1 mutations.83

Figure 2.7 Patient fibroblasts have hyper-filamentous mitochondria and are respiratory deficient.

(a) Patient fibroblasts from IT II-3, homozygous for SLC25A46R340C, have a hyper-filamentous and interconnected mitochondrial network in comparison to unaffected heterozygous sibling IT II-2 and control. Scale bars = 25 µm. (b) The distribution of mitochondrial morphology into three categories by blind test in human fibroblasts: P=0.001, calculated with chi square test for control, IT II-2, and IT II-3. Control n=200; IT II-2 n=200; IT II-3 n = 200. (c) Oxygen consumption rate (OCR) traces in control and mutant fibroblasts, expressed as pMolesO2/min, after the injection of oligomycin (O), FCCP (F), rotenone (R) and antimycin A (AA). The homozygous IT II-3 fibroblasts show reduced OCR compared to controls and heterozygous IT II-2 fibroblasts. OCR values are normalized to protein content. Error bars indicate the standard error of mean (s.e.m.) of four independent experiments. (d) BASAL, PROTON LEAK-linked , ATP-linked, MAXIMAL, Complex
I-linked, MAXIMAL Complex I-linked respiration and SPARE CAPACITY were calculated from OCR traces and reported in the graph as mean ± SEM. Dunnett’s test of control vs mutant fibroblasts was performed, * indicates p<0.01. (e) Analysis of OCR/ECAR in basal conditions. Extra-cellular acidification rate (ECAR), is an indicator of lactic acid production. The reduced OCR/ECAR ratio observed in the homozygous IT II-3 fibroblasts suggests the occurrence of a glycolitic shift. Bars indicate the confidence intervals (Conf.Int.). Analysis was performed from 5 replicate wells for each fibroblasts line. Dunnett’s test of control vs mutant fibroblasts was performed, * indicates P = 0.002.

Discussion

Altogether, we demonstrate that mutations in SLC25A46 cause a spectrum of neurological feature which overlap with optic atrophy and CMT. SLC25A46 is a modified mitochondrial transporter, most similar to Ugo1. The discovery of SLC25A46 has a number of implications in the field. SLC25A46 has been categorized by the Online Mendelian Inheritance in Man (OMIM) database as hereditary sensory motor neuropathy (HSMN) type 6B, while MFN2 is HSMN type 6A. This further broadens the phenotypic spectrum associated with CMT2. For example CMT2A or HSMN type 6A caused by mutations in MFN2 is not typically thought of as a metabolic disease, however animal models which are lethal in the homozygous state indicate that there is a dosage dependent metabolic dysfunction associated with Mfn2. The SLC25A46 patient in the US family presented with an early onset and lethal neurodegenerative disorder. It is likely that because SLC25A46 follows an autosomal recessive inheritance pattern, that patients present with a much broader phenotypic spectrum depending on the functionality of various hypo-morphic mutations.

In terms of the orthology between SLC25A46 and Ugo1, both proteins evolved from the homologous mitochondrial carrier family of inner membrane transporters, and have translocated the outer mitochondrial membrane. Our data
supports 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. We found that SLC25A46 does not interact with MFN2 or OPA1, but forms a complex with mitofilin that is independent of MFN2 (data not shown). Interestingly, mitochondrial fusion is not tightly coupled in mammalian cells, where the outer and inner membranes can fuse independently of each other. Therefore, it is possible that SLC25A46 still functions as an adaptor protein, but the loss-of-function is more apparent on the fission mechanism where the outer and inner membranes come together at the 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 MTCH2 during apoptosis.

Ugo1 has been experimentally shown to have three transmembrane domains and forms homodimers. In yeast Ugo1 does interact with both Fzo1 and Mgm1, however it seems to do so independently suggesting that these interactions are dynamic and that Ugo1 can play dual roles in remodeling either inner membrane and outer membrane proteins. Another possible function of Ugo1 and SLC25A46 is in the formation of a mitochondrial fusion pore, which could support the transfer of protons and other metabolites between tethered or partially fused mitochondria.

Although the function of SLC25A46 in mitochondrial dynamics seem to deviate from the role of Ugo1 in yeast, the conservation of the interaction of both
proteins with mitofilin suggest that SLC25A46 and Ugo1 might play similar roles at the cristae junctions.

In conclusion, we have identified a novel 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, which opens the possibility of future pathway-oriented treatments for these types of mitochondrial disorders.
Chapter 3. Developing Assays to Evaluate Neurological Phenotypes in Zebrafish

Some of the data presented in this chapter was previously published in Nature Genetics (Doi:10.1038)\textsuperscript{21}. I worked directly to design and test morpholinos as well as to characterize phenotypes in zebrafish larvae. Microinjections and some of the data analysis was done with the assistance of undergraduate students, John Campeanu and Saskia Groenewald. Electron microscopy sectioning and imaging was carried out by Neville Patel with assistance and supplies provided by Jeffrey Prince. Zebrafish maintenance and husbandry was performed by Ricardo Cepeda.

Background

Zebrafish as a genetic model

When choosing how to characterize novel genes and variants discovered through exome sequencing, zebrafish are an attractive model for a number of reasons including low cost of husbandry, large clutch size, higher similarity to humans in comparison non-vertebrate models, and ease of genetic manipulations\textsuperscript{126–128}. Zebrafish are an established functional model in neurogenetic disorders including optic atrophy\textsuperscript{70,129}, HSP\textsuperscript{130} and CMT\textsuperscript{254,131–133}. Finally, given the transparency of embryos and transgenic lines available that label mitochondria\textsuperscript{134,135}, it makes it the ideal model to study the role of mitochondrial dynamics in neurodegenerative disorders.
The methods of genetic manipulations used in these studies include, morpholino antisense oligonucleotides, RNA injection, and plasmid transgenesis. Morpholinos are a single stranded antisense oligonucleotide that resemble RNA with the exception that the sugar backbone moieties are composed of 6 rather than 5 carbon rings, which increases their stability\textsuperscript{126}. Morpholinos have been the gold standard in the zebrafish community for at least 15 years\textsuperscript{126,127}. The oligonucleotides can be injected into 1-cell stage embryos as a transient method of gene knockdown and gradually wear off by 3-6 days post fertilization (dpf)\textsuperscript{126}. This window does limit analysis to developmental phenotypes, although by 5 dpf the larval nervous system is entirely functional\textsuperscript{136}.

**Summary**

Through collaborative exome sequencing we identified recessive mutations in \textit{SLC25A46} in two sisters which presented with juvenile onset optic atrophy and late onset spasticity and peripheral neuropathy. No functional data was available for the gene except that it had been implicated in atopic dermatitis through genome wide association studies\textsuperscript{137}. Interestingly though, the transcript was shown to be highly expressed in the mammalian central nervous system including the optic chiasm, cerebellum, and spinal cord\textsuperscript{116}. We found through in situ hybridization that the gene was ubiquitously expressed in zebrafish with enrichment in retinal ganglion cells. Based on this evidence we hypothesized that knocking out slc25a46 in zebrafish larvae would create a nervous system phenotype affecting swimming circuits, motor neuron outgrowth, and vision.
Indeed we found that loss-of-function did cause a congruent phenotype. We observed morphological defects in a subset of embryos, alterations in vibration evoked swimming, truncations in motor neurons, dystrophic appearance of cell bodies in the spinal cord, delayed outgrowth of retinal ganglion cells, and abnormal mitochondrial morphology. Based on these experiments, we have now developed and collected the tools needed to assess the function of other novel genes in the vertebrate nervous system.

**Materials and Methods**

*Zebrafish husbandry*

Experiments were carried out using *Danio rerio* wild type strains: AB, TL, Tubingen and the transgenic stains, Tg(*Olig2:DsRed*)	extsuperscript{138} and Tg(*Islet2b:GFP*)	extsuperscript{139}. Adults were kept on a 14 hour light/10 hour dark cycle at 28°C. Embryos were obtained from natural crosses after removing a divider at first light and microinjections were performed into one-cell stage embryos. 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 the University of Miami Institutional Animal Care and Use Committee guidelines.

*Microinjections*

The *slc25a46* morpholino (Gene Tools) was targeted to the exon 3/intron 3 junction. Effective splice blocking was confirmed with diagnostic primers and found to lead to the exclusion of exon 3 resulting in a frame-shift and premature
stop. The standard scrambled morpholino (Gene Tools) was used for the control. 1mM stock solutions of morpholinos were diluted in 1% fast-green dye to a final concentration of 0.9 mM. Injection volumes of 333 pL or 500 pL were calibrated for a final injected amount of 0.3 picomoles and 0.45 picomoles (pmol) respectively. For the rescue experiments, human SLC25A46-HA mRNA was synthesized from the PCS2+ plasmid using mMessage Machine SP6 Kit (Ambion) and 400pg were injected.

**Analysis of swimming**

High-speed videos were taken using a Fastcam 1024PCI (Photron USA Inc., San Diego, CA, USA) with a Fujinon lens mounted in a customized behavioral chamber. Parameters were as follows: shutter speed of 1/1000 using an LED array for backlit illumination (Advance Illumination; Rochester VT; Backlight LED Illuminator), 512 x 512 resolution, a frame rate of 500 f/s to assess swimming kinematics. Three vibration-evoked behaviors, elicited with S40 stimulator using DAC timer, were recorded from a 35 mm dish containing up to 5 larvae. Videos were acquired from multiple runs containing more than 30 total larvae. Videos were analyzed using Flote\textsuperscript{140} to quantitate changes in axis curvature over time. Three representative overlapping swim traces from different fish were chosen from over 8 analyzable swim responses.
In vivo imaging of motor neurons

Motor neuron outgrowth was assayed at 48 hpf using Tg(Olig2:Dsred)\textsuperscript{138} fish. Live fish were anesthetized with tricaine methanesulfonate (Sigma), imbedded in 1.5% agarose, and imaged using a Leica confocal microscope 20X/air lens and processed with Fiji (Image J). For analysis 1 μm Z-stacks were imaged between myotome segments 6 and 13\textsuperscript{141} and the lengths of the caudal anterior primary (CaP) axons were measured from the base of the spinal cord to the end of the rostral myotome using the Simple Neurite Tracer in Fiji (Image J). LUT: edges was used to make the figure. To stochastically label motor neurons and mitochondria, 300pL of a cocktail containing 25 ng/ul of each: HB9:Gal4-VP16, UAS-E1b:mYFP:mitoCFP \textsuperscript{134}, and transposase RNA was injected prior to the morpholinos. Embryos were presorted for YFP expression at 48 hpf before imaging. Acetylcholine receptors were labeled in 48 hpf embryos by injecting, α-bungarotoxin conjugate (Molecular Probes) into the yolk of anesthetized fish an hour before imaging with Leica confocal microscope, 63x/water lens. Motor neuron cell bodies and mitochondria were processed with LUT:edges/memYFP and 16 colors/mitoCPF. For mitochondrial distribution analysis, 1 μm Z-stacks were maximum intensity projected and auto-thresholded. The midline was measured as the half-way point between the top of the soma and the axon hillock. The area of mitochondrial pixels was calculated for the region above and below the midline, which was then converted to a percentage using Fiji (image J).
**RNA in situ hybridization**

Wild type embryos were incubated in n-phenylthiourea (Sigma) to suppress pigment development. The *slc25a46* antisense probe was amplified using the following primers 5'-GCCTGGTTCTTTACCTGCTG-3' and 5'-AAACCCAAATCGGTGTTGTC-3' and the DIG RNA Labeling Mix (Roche) was used to synthesize the probe. Probe hybridization and development were carried out using standard protocol\textsuperscript{142}, followed by post fixation in 4% PFA and dehydration in 30% sucrose. Embryos were embedded in tissue freezing media (Triangle Biomedical Sciences), sliced with a cryostat into 30μm sections, and imaged with an Olympus BX60 compound light microscope.

**Retinal ganglion cell staining**

Morpholino injected larvae were fixed at 96 hpf in 4% PFA overnight and then decapitated. Zn-5 antibody (ZFIN) was used at 1:200 followed by incubation with 1:200 HRP-conjugated secondary and finally developed with 3,3'-Diaminobenzidine DAB (Vector). After DAB development fish were depigmented with H2O2 and KOH, post fixed and dehydrated in ethanol. Samples were embedded in freeing media, sliced into 30μm sections, and imaged with an Olympus BX60 compound light microscope. 8-bit grey scale numbers were calculated from TIF files of sections contained the optic nerve using Fiji image analysis such that 0=white and 256=black and was converted to percentage whereby 0=white 100=black. 5 evenly spaced pixels from the inner plexiform layer were measured to find the average intensity per eye. Fluorescent images
were generated in a similar manner, Zn-5 antibody (ZFIN) was used at 1:200 followed by incubation with 1:200 Alexaflor-568 secondary, 1:1000 DAPI, and 1:1000 Peanut agglutinin (PNA) 488. Samples were dehydrated in 30% sucrose, embedded in freeing media, sliced into 30μm sections, and imaged with Leica confocal microscope 63X/Oil lens.

In vivo imaging of optic nerve

Retinal ganglion cells were imaged at 72 hpf after injected slc25a46 and control morpholinos into Tg(Islet2b:eGFP)139 embyros. At 24 hpf, 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 20X/air lens. 1 μm Z-stacks were processed with maximum intensity and thresholded for analysis. Calculations were made by tracing GFP positive areas. All possessing was done with Fiji (Image J). LUT: Green Fire Blue was used to make the figure.

Transmission electron microscopy and analysis

Zebrafish embryos were injected with 0.45 pmoles of either control or slc25a46 morpholino. The more severely affected curly morphants were selected for EM. All embryos were fixed and processed at 48 hpf and were fixed for four days in 2.5% glutaraldehyde in Millonig's phosphate buffer113, post-fixed in 1% O₅O₄ and uranyl acetate followed by dehydration in an ethanol series and embedded in LR white resin (Fisher Scientific). Ultrathin, 60 nm sections were made using a Leica
microtome and were stained with cold lead citrate for 6 minutes.\textsuperscript{113} Images were acquired with a Joel JEM-1400 transmission electron microscope with Gatan camera. All images were processed and analyzed using Fiji (image J).

**Plasmids**

HB9:Gal4-VP16 was generated from the pmT hb9s YFP kindly provided by the Nonet Lab at Wash U. UAS-E1b:mYFP,mitoCFP was kindly provided by Bettina Schmid\textsuperscript{134} from the German Center for Neurodegenerative Diseases (DZNE). SLC25A46 and mitofilin-myc were obtained from (Origene). SLC25A46 was subcloned into pcDNA3.1 (Invitrogen) with either C-terminal HA or myc. For RNA synthesis SLC25A46-HA was also subcloned into PCS2+.

**Results**

*Morpholino effectively knocks down slc25a46*

The two major strategies applied for morpholino based gene knock down include translation blocking and splicing interference at the mRNA level. We chose to use the splice blocking method because the effectiveness of the morpholino can be tested through reverse transcription PCR and Sanger sequencing. The Gene Tools website provides tools to design exon-intron boundary targets. We chose to target the exon3-intron3 boundary of slc25a46 and designed diagnostic primers from exon 1 to exon 6 (**Figure 3.1 a**). Through reverse transcription PCR we identified the expected 566 bp as well as a 508 bp amplicon, which was confirmed to be the result of exon 3 skipping by Sanger sequencing (**Figure 3.1**)
b). This results in a frameshift and premature stop codon truncating the protein. This morpholino was shown to be effective up to 4 dpf (data not shown).

_Slc25a46 knockdown causes a morphological and early swimming phenotype_

After confirming the effectiveness of the gene knockdown, we characterized gross morphological phenotypes in _slc25a46_ morpholino injected (morphants) versus scrambled control morpholino. The most obvious phenotype was the curly tail, which is has been observed in other disease models such as _pnpla6_ associated with HSP$^{130}$, and _lrsam1_ associated with CMT$^{132}$. We tried two different dosages of morpholino; 0.3 and 0.45 pico moles (pmol). At the higher dosage we observed that just under 40% of morphants had the curly tail morphology, while at the lower 0.3 pmol dosage, we observed no obvious morphological defects at 2 days post fertilization (dpf) (Figure 3.1 c,d). By 96 hpf larvae display robust upright swimming behaviors. At this time point we compared vibration evoked swimming in morphant and control morpholino injected fish, only assessing normal looking fish which were able to swim. While control fish display a robust initial bend followed by beat and glide swimming, morphants are inefficient swimmers. They have a reduced amplitude of body curvature and do not achieve enough forward momentum to glide, hence they continue to beat their tails after the evoked stimuli (Figure 3.1 e).
Figure 3.1 slc25a46 morpholino targeting, confirmation, and behavioral phenotype.

(a) Schematic depicting zebrafish slc25a46 and the relative position of the exon3-intron3 splice site targeted morpholino. (b) Reverse transcription PCR demonstrating the presence of a smaller 508 bp amplicon which was confirmed to be the product of exon 3 exclusion resulting in frameshift and premature stop codon. (c) Representative images of the gross morphology at 48 hpf. Scale bar = 1 mm. (d) Quantification of morphology: the curly phenotype was predominantly observed at the 0.45 pmol dosage. 0.45 pmol control MO n = 220; 0.45 pmol slc25a46 MO n = 223; 0.3 pmol; slc25a46 MO n = 181. (e) Vibration induced swim traces from three different larvae illustrating the angle of body curvature at 96 hpf.

Knockdown effects the growth of motor neurons:

We next wanted to characterize the growth and morphology of the motor neurons. In zebrafish, primary motor neurons begin to elongate between 18 and 36 hours post fertilization (hpf)\textsuperscript{141}, and embryos are capable of evoked swimming as early as 36 hpf\textsuperscript{136}.
The transgenic line, Tg(olig2:DsRed)$^{138}$, labels oligodendrocyte precursors cells as well as motor neurons. The caudal anterior primary (CaP) axons are the first motor neurons to exit the spinal and by 48 hours post fertilization have fully innervated the ventral muscle from where they turn dorsally to grow along the rostral myotome$^{141}$. We injected embryos from Tg(olig2:DsRed) crosses in order to visualize motor neuron development in vivo. We assessed motor neuron outgrowth at 36, 48, and 72 hpf. At 36 hpf we found significantly more stalled axons in morphant larvae suggesting delayed outgrowth (Figure 2.2 a,b), however we found that only a few hours could lead to drastic differences in length measures so we chose to further analyze the 48 hpf time point.

By 48 hpf we observed truncated axons at both the high and low dose injected fish. Furthermore, we were able to rescue the motor neuron truncation by co-injecting the morpholino with RNA encoding the human transcript (Figure 2.2 c,d). By 72 hpf a large number of secondary motor neurons fasciculate along the common path and the morpholino begins to wear off making it more difficult to assess the motor neuron phenotype at this time point.

In order to answer whether the motor neuron phenotype is due to lack of development or degeneration, we stochastically labeled axons by plasmid injection. We found blebing and captured movies of destabilized axons at 48 hpf (Figure 3.3), indicating that the motor neuron phenotype is a combination of developmental delay and degeneration.
Figure 3.2 Motor neuron morphology assessed in vivo at 36 and 48 hours post fertilization (hpf) using transgenic Tg(Olig2:DsRed) zebrafish (a) Tg(Olig2:DsRed) zebrafish at 36 hpf. Morphants show delayed axon outgrowth. (b) Quantification of axons lengths in individual control morpholino (CoMo) and morphant fish. (c) Representative images at 48 hpf, including high 0.45 pmol and low 0.3 pmol morpholino doses and rescue by injection of human RNA. Scale bar = 100 µm (d) Quantification of axons lengths.

Figure 3.3 Stochastic labeling of motor neurons by injection of Hb9:memYFP and labeling of postsynaptic receptors on muscle. At 48 hpf morphants nerve terminals have axonal blebing and what appear to be retraction bulbs. Acetylcholine receptors on muscle, labeled with alpha-bungarotoxin (α-btx), are in front of axon suggesting retraction. Scale bars = 20 µm.
Knockdown affects optic nerve development

Zebrafish slc25a46 is 69% identical to the human ortholog, and in agreement with previous RNA in situ hybridization data, we found enrichment in retinal ganglion cells (RGCs) and their corresponding neuronal projections in the axons of the optic nerve (ON) and in the dendrites of the inner plexiform (IPL) at 72 hpf (Figure 3.4). In zebrafish, retinal ganglion cells begin to differentiate at 32 hpf. At 36 hpf only between 1-6 axons of the optic nerve will have exited the eye, and visual information is not relayed until 120 hpf fertilization. All cell types within the zebrafish retina continue to proliferate even in adult fish, so in a sense, the development of the optic nerve is never completed. Morpholinos have a transient effect in blocking gene expression, so all of our analysis is limited to a 120 hour window. Based on this these time constraints, we analyze retinal ganglion cell processes at 72 and 96 hpf. Before directly assessing the optic nerve we noticed a decrease in pigment in the eye at 24 hpf and by 96 hpf as a small subset of severely affected embryos showed coloboma, or incomplete eye closure (Figure 3.5 a). This coloboma phenotype was observed in a zebrafish model of abcb6, a golgi and ER transporter associated with ocular colobomas in humans, but was not an entirely penetrant phenotype in the slc25a46 morphants.

In order to visualize the retinal ganglion cells (RGC) and their corresponding projections in the inner plexiform layer (IPL), optic nerve (ON), and optic tectum (OT), we used the Zn5-antibody in fluorescent and diaminobenzidine (DAB) stained sections (Figure 3.5 b). At the level of
fluoresence, we saw what appears to be the de-fasciculation of the optic nerve and the exit point at a different plane of section, while the DAB stain shows decreased staining intensity in both the optic nerve and inner plexiform layer, suggesting decreased RGC processes (Figure 3.5 b).

Figure 3.4 RNA *in situ* hybridization showing enrichment of slc25a46 in optic tracts of 72 hpf zebrafish. (a) sense control (b) slc25a46 probe notating optic nerve (ON) (c) Optic tectum. (d) Inner plexiform layer (IPL) retinal ganglion cells (RGC), as well as Inner nuclear layer (INL). Scale bars = 50 µm.

As a more direct means of confirmation we obtained the transgenic line Tg(*Islet2:eGfp*)\textsuperscript{139}, generously donated by Fabienne Poulain. Here we found that the innervation of the optic tectum is reduced in morphant embryos and can be rescued by coinjecting with the human transcript (Figure 3.3 d). Overall these results, indicate that the morphant embryos have decreased outgrowth of RGC processes, however a stable line is needed for further analysis of a potential degenerative phenotype. In a stable line, the visual system could also be indirectly assessed using phototaxis and optokinetic responses\textsuperscript{150}. 
Knockdown effects the morphology and distribution of mitochondria in vivo

The transparency of zebrafish larvae makes them an ideal vertebrate model in order to visualize mitochondria in real-time. To date a number of stable transgenic lines that label mitochondria have been generated including ones with mitochondrial targeted cyan fluorescent protein (mitoCFP)\textsuperscript{134} or red fluorescent protein (mitoDsRed)\textsuperscript{135}. We did not have access to a stable line at the time we started these experiments and instead injected the mitoCFP/memYFP construct expressed under the HB9 motor neuron promoter. This provides stochastic labeling of motor neurons and can be co-injected with the morpholino (Figure 3.6.
a). In doing so we found that the mitochondria normally clustered at the axon hillock, lose this polarity in motor neurons when slc25a46 is knocked down (Figure 3.6 a,b). This effect was also observed at the level of transmission electron microscopy (TEM) (Figure 3.6 c), however it is unclear if the change in mitochondrial polarity is secondary to the dystrophic appearance of the motor neuron. TEM also revealed unusual mitochondrial aggregates (Figure 3.6 d,e) and a significant increase in mitochondrial cross sectional sizes (Figure 3.6 f). These findings are congruent to the results found in siRNA treated cell lines and patient fibroblasts discussed in chapter 2. Future experiments should focus on directly measuring mitochondrial transport in order to assess whether or not it is altered given the increased connectivity and change in cell polarity of the mitochondria. These experiments could be performed using the mitoDsRed135 line obtained from Alvaro Sagasti’s lab.
Figure 3.6 Visualization of mitochondria in zebrafish motor neurons. (a) Zebrafish motor neurons in vivo labeled with plasmids driving mitoCFP (blue) and memYFP (black) under the Hb9 promoter. Scale bar = 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. Control MO n = 8; slc25a46 MO n = 8. P value = 0.083 by one-tailed Student’s T-test. The mitochondria in the morphants also appear to be more aggregated which is more apparent in electron microscopy images. (c) Representative electron microscopy images showing mitochondrial distribution. Soma (yellow) and mitochondria (blue). Scale bar = 1 μm. (d) Electron micrographs of mitochondria in spinal cord. Scale bar = 1 μm. Slc25a46 morphants show accumulation of large mitochondrial aggregates that appear to be in the process of fission. (e) Serial section of a clover shaped mitochondrial aggregate, suggesting that these mitochondria are fused at a narrow junction point, only visible in the right plane of section. (f) Cross-sectional areas of mitochondria with distribution of sizes show 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. Red line=3 times the s.d. of the average are found in controls. P value = 0.0006 by one-tailed Student’s T-test when comparing total mitochondrial cross-sections per group.

Discussion

Overall I have outlined various methods to assess optic nerve and motor neuron phenotypes in zebrafish, as well as methods to visualize mitochondria in
vivo. Morpholinos have come under recent scrutiny by the zebrafish and genetics communities, due to the discordant phenotypes observed between morphants and stable mutants\textsuperscript{151}. Therefore future work should aim to develop stable zebrafish lines using other methods of genetic modification. The assays outlined here can still be applied to other methods of genetic manipulation such as plasmid and RNA overexpression discussed in chapter 4, or through CRISPR\textsuperscript{128} based methods of genome editing.

Zebrafish have the potential to be a powerful and informative model in order to tease apart the causative versus correlative role of altered mitochondrial transport in various diseases. Many novel fluorescent biomarker proteins are being developed to measure parameters such as mitochondrial age\textsuperscript{152} and ATP\textsuperscript{153}, which could be implemented into zebrafish to elucidate our understanding of mitochondria within neurons \textit{in vivo}.
Chapter 4: Expression of an Amyloidgenic Neurofilament Mutation in vivo

The story of the neurofilament NEFH aggregation is the work of Adriana Rebelo. My contribution was testing the functionality of the NEFH mutation in zebrafish larvae. The following data is under revision at the American Journal of Human Genetics, with myself as second author.

Background

Neurons are highly polarized cells with axons and dendrites composed of microtubules, microfilaments, and neurofilaments. Neurofilaments (NF) are 10-nm intermediate filaments that provide structural support, a means of axonal trafficking, and the scaffolding needed for the cells organelles\textsuperscript{154}. They are primarily composed of three subunits classified by their molecular weight; neurofilament light (NEFL), medium (NEFM), and heavy (NEFH) chain\textsuperscript{155}. Abnormal NF structure is a hallmark feature of motor neuron disorders including amyotrophic lateral sclerosis (ALS)\textsuperscript{156}, and can cause global effects on neuronal function by disrupting axonal transport processes\textsuperscript{64}. The aggregation of NFs in motor neuron diseases can occur through either primary or secondary mechanisms. For example the massive accumulations of NFs that have been observed in post mortem spinal cords of ALS patients with SOD1 mutations\textsuperscript{156} are likely secondary, because SOD1 (Superoxide Dismutase 1) has no known direct relationship to the neurofilaments. In contrast, Charcot-Marie-Tooth neuropathy (CMT2), a related motor neuron disease, is directly caused by
mutations in NEFL\textsuperscript{157}. Different NEFL have been found to affect the assembly of the intermediate filament networks pointing to a direct effect on NF aggregation in CMT2\textsuperscript{158}. Consequently, we report for the first time mutations in NEFH causing CMT2, and have further characterized a novel mechanism of aggregation due to the translation of the 3'UTR of NEFH. Variants in NEFH have been associated with an increased susceptibility to ALS, however NEFH does not represent a causative high-penetrance gene for the disease\textsuperscript{159,160}. Our finding suggests a direct effect of NF aggregation in motor neuron diseases, and represents a novel amylogenic mutational motif in the NF genes.

**Summary**

We identified families with Charcot-Marie-Tooth disease type 2 (CMT2) carrying frameshift mutations in the neurofilament heavy gene (NEFH) leading to stop-loss and extended translation of 40 amino acids in the 3'UTR. *In silico* aggregation prediction analysis combined with experimental results demonstrated that the terminal 20 amino acid residues in the alternative protein is amyloidogenic and critical for the formation of aggregates. Expression of the amyloidogenic mutant NEFH in cells causes prominent protein aggregates and in zebrafish disrupts motor neurons. Interestingly though the levels of the mutant NEFH protein are significantly reduced in the fish, suggesting that the mutant protein is rapidly degraded *in vivo.*
Materials and Methods

Families

The families were identified as part of our ongoing genetic studies in CMT. The families were ascertained in Austria and Great Britain. Participants were recruited, enrolled, and sampled according to the institutional review board protocols of the University of London and Vienna. A complete description of the study was provided to the subjects, and written informed consent was obtained. Whole blood was collected from all participants by venipuncture. Affection status was determined by consensus of physicians and clinical staff experienced in clinical CMT research, and was based on medical records and in-person evaluation.

Sanger and exome sequencing

We performed exome sequencing in 269 index patients with autosomal dominant CMT. The SureSelect Human All Exon 50MB kit (Agilent, Santa Clara, CA, USA) was used for in-solution enrichment and the HiSeq2500 instrument (Illumina San Diego, CA, USA) was used to produce 100bp paired end sequence reads. BWA (24), Picard (http://picard.sourceforge.net), and GATK (25) software were used to align sequence reads and call variants. This data were imported into GEM.app (16) for further analysis. Variants were filtered for variants that segregated in an autosomal dominant fashion and met the “strict” criteria, which requires that variants are rare (NHLBI ESP6500 MAF<0.05%), present in less than three
families within GEM.app (~4,300 exomes), conserved (GERP score > 2 or PhastCons score > 0.6), and have sufficient quality scores (Genotype Quality > 75). NEFH variant calls identified were validated using conventional Sanger sequencing.

**Immunocytochemistry**

Neuro-2a cells were grown in complete DMEM media (Gibco) to 75% confluence and transfected with Lipofectamine 2000 (Invitrogen) following manufacturer’s protocol. After 24hr, cells were fixed with paraformaldehyde for 20 min, permeabilized with cold methanol for 5 min and stained with anti-myc antibody (Cell Signaling) and anti-tubulin (Invitrogen). Cells were mounted onto microscope slides and imaged with a confocal microscope, Zeiss LSM710, using a 60X objective lens. Aggregation was quantified by visual counting the ratio of cells with aggregates over the total number of GFP fluorescent cells in at least 100 cells from 3 independent experiments. Plasmid injected 48 hpf zebrafish larvae were fixed in 4% PFA overnight. Larvae were dehydrated in 30% sucrose, embedded in freeing media, sliced into 30μm sections, and incubated with 1/200 anti-GFP primary overnight followed by 1/200 Alexaflor-488 secondary with 1/1000 DAPI for 1 hr. After mounting slides with coverslip, samples were imaged with Leica confocal microscope 63X/Oil lens.
Zebrafish Husbandry

Experiments were carried out using *Danio rerio* AB/TL/Tubingen wildtype or Tg(*Olig2:DsRed*)\textsuperscript{138}. Adults were kept on a 14 hour light/10 hour dark cycle at 28°C. Embryos were obtained from natural crosses after removing a divider at first light. All experiments were conducted in accordance with the University of Miami Institutional Animal Care and Use Committee guidelines.

Microinjections

mRNAs were synthesized from GFP-WT-NEFH and GFP-FS-NEFH cloned into pcDNA3.1/NT-GFP-Topo (Invitrogen), using mMessage mMACHINE T7 Ultra (Ambion) and 400pg of each RNA were microinjected into one cell stage embryos. Embryos were reared in petri dishes in a 28°C incubator with the same light dark cycle. Motor neuron outgrowth was assayed at 48 hpf. Live fish were anesthetized with tricaine methanesulfonate (Sigma), placed against a shelf of 1.5% agarose, and imaged using a Leica confocal microscope. 1 μm Z-stacks were taken between segments 6 and 15 and the lengths of the first 4 caudal anterior primary axons were measured using the Simple Neurite Tracer in Fiji. Plasmids were injected in a 300pL cocktail containing 25 ng/ul of each: HB9:Gal4-VP16\textsuperscript{21}, UAS-E1b:DsRed:GFP-WT/FS-NEFH, and transposase RNA, or a 300pL cocktail containing 25 ng/ul of each HB9:memCherry, HB9:GFP-WT/FS-NEFH and transposase RNA. Embryos were presorted for RFP expression at 48 hpf before imaging as previously described.
**Plasmid constructs**

The gene encoding the human NEFH was synthesized by Genscript and the GFP-WT-NEFH construct was generated by subcloning into pcDNA3.1/NT-GFP-Topo (Invitrogen). Site-directed mutagenesis was used to generate the patient’s frameshift mutation, c.3010_3011delCA, which is referred to as GFP-FS-NEFH. GFP-FS-NEFH and GFP-WT-NEFH where then subcloned into pENTR/D-TOPO (Invitrogen) and recombined into UAS-E1b:DsRed:R1-R2\textsuperscript{161} via LR recombinase gateway reaction (Invitrogen) Hb9:Gal4-VP16, Hb9:memCherry, Hb9:GFP-WT-NEFH, and Hb9:GFP-FS-NEFH were generated as previously described\textsuperscript{21}, from Hb9:memYFP donated by the Nonet Lab at Wash U.

**Results**

We performed whole exome sequencing on three affected patients belonging to different generations of family UK1 reported with autosomal-dominantly inherited CMT2 (Figure 4.1 a). Exome data was analyzed by a strict filtering approach for segregation of non-synonymous heterozygous variants using the Genomes Management Application (GEM.app) software\textsuperscript{22}. A frameshift mutation in NEFH was identified as a top candidate for the disease. The variant co-segregated with the disease across three generations in this family. This variant, c.3010_3011delCA, chr22: 29,886,637, results in a stop-loss mutation leading to continued translation of an alternative open reading frame and extension of 40 amino acids of the C-terminal tail, Asp1004fs*56 (Figure 4.1 d). We then
screened an additional 322 families with CMT where whole exome sequencing
had been performed. We identified an additional CMT2 family (AT1) with four
affected patients carrying a heterozygous frameshift mutation in *NEFH*
(c.3021_3024insAGCC, chr22: 29,886,645) that interestingly also results in a
stop-loss mutation leading into an identical open reading frame extension of 40
amino acids of the C-terminal tail, Pro1008fs*54 (Figure 4.1 b and d).

![Family Tree Diagrams]

**NEFH**

![Gene Structure Diagram]

- ▼ Coding KSP deletions in ALS
- ▲ Coding KSP insertion in ALS
Because neurofilaments have a considerable tendency to aggregate in neurodegenerative diseases, we decided to investigate the intrinsic aggregation propensity of the extension of amino acids present in the NEFH mutants. Constructs encoding GFP-tagged wild type (GFP-WT-NEFH) and frameshift mutation from UK1 family, Asp1004fs*56 (GFP-FS-NEFH) proteins were transfected into neuro-2a cells. GFP-FS-NEFH encodes the extended of 40 extra amino acid derived from translation of the NEFH-3'UTR reading-frame 3. In contrast to GFP-WT-NEFH that was evenly distributed in the cytoplasm, confocal microscopy revealed prominent abnormal perinuclear aggregation of GFP-FS-NEFH in the cytoplasm of neuro-2a analyzed 24hrs after transfection (Figure 4.2 a). Quantification of the aggregates shows that over 75% of cells transfected with GFP-FS-NEFH contained aggregates compared to less than 1% in GFP-WT-NEFH cells (Figure 4.2 b). In cells transfected with the mutant NEFH we also observed abnormal cellular morphology with round-shaped cells lacking neuronal projections (Figure 4.2 a). Cells transfected with the wild-type NEFH retained
their typical neuro-2a cell morphology with ‘neuronal’ projections extending from the cell body. By contrast, the percentage of GFP-FS-NEFH-expressing cells containing neuronal projections was significantly reduced (Figure 4.2 c). Using a combination of online prediction tools and experimental testing of truncated constructs, it was determined that the last 22 of the 40 amino acid extensions necessary and sufficient for the aggregation propensity of NEFH (data not shown). These 22 residues (SSRIRVTQFSLFLSLCKKKLLR) are referred to as the cryptic amyloidogenic element (CAE).

![Figure 4.2](image)

**Figure 4.2** Neuro-2a cells transfected with wildtype GFP-WT-NEFH and the stop-loss mutant GFP-FS-NEFH. (a) GFP-FS-NEFH, the stop loss mutation results in prominent aggregates and rounding of cells compared to wild type GFP-WT-NEFH. Red astrix denotes the position of stop codon either before or after the 3’ untranslated region (UTR) and cryptic amyloidogenic element (CAE). (b) Quantification of percentage of positively transfected cells with aggregates. (c) Quantification of the percentage of transfected cells with projections.

In order to assess the effects of the NEFH frameshift mutation *in vivo* we injected RNA into one-cell stage zebrafish embryos. Equal amounts of RNA encoding either GFP-WT-NEFH or GFP-FS-NEFH were injected into transgenic Tg(Olig2:DsRed) embryos at a dosage at which there was no apparent effect on body morphology (Figure 4.3 a), but a measurable difference in motor neuron
outgrowth. We assessed the common path of the caudal anterior primary motor neurons at 48 hours post fertilization (hpf). We found that the GFP-FS-NEFH RNA injected embryos have significantly decreased axon lengths compared to both GFP-WT-NEFH and uninjected larvae, while there is no significant difference between the motor neurons lengths of the uninjected and embryos injected with GFP-WT-NEFH RNA (Figure 4.3 b,c). This supports the pathogenicity of NEFH frame-shift mutations and indicates that the addition of the 3'-UTR CAE can function through a toxic gain-of-function mechanism.

Contrary to the cell data, we did not observe visible protein aggregation from the GFP-FS-NEFH expression, but instead saw a considerable decrease in protein levels. We tried three different methods of overexpression, none of which produced visible protein aggregates. First we injected either GFP-WT-NEFH or GFP-FS-NEFH mRNA as previously described, which should drive early, high, and ubiquitous expression of the GFP-tagged proteins. While we did observe GFP fluorescence in GFP-WT-NEFH injected embryos as early as 8 hpf and 24 hpf, we found only the slightest signal that was barely visible in the GFP-FS-NEFH injected embryos (Figure 4.3 d,e). In congruence, the level of full length GFP-FS-NEFH was hardly detectable by western blot in comparison GFP-WT-NEFH (Figure 4.3 f). As a control, both RNA transcripts are detectable in the same cohort of injections by RT-PCR indicating that the RNA is still present (Figure 4.3 e).
Figure 4.3 NEFH overexpression in zebrafish larvae by RNA injection.

(a) Zebrafish embryos injected with RNA encoding either GFP-WT-NEFH or GFP-FS-NEFH do not show major morphological defects at 48 hpf. (b) Motor neurons labeled in the transgenic line, Tg(Olig2:DsRed). Embryos injected with GFP-WT-NEFH show normal motor neuron development, while of zebrafish injected with GFP-FS-NEFH show examples of stunted axons. Asterix indicates where the axon fails to innervate the rostral myotome. Scale bar 100 µm (c) Quantification of the average axon length shows a decrease in GFP-FS-NEFH injected fishes compared to both GFP-WT-NEFH injected and uninjected larvae. Axon length is not significantly different between GFP-WT-NEFH injected fishes and uninjected control fish. The average axon length per fish was calculated from the first four myotomes. Data was compiled from three independent experiments and significance was determined by a one way ANOVA with Bonferroni post test. P-values are *0.024 and **0.014. (d) Confocal images of GFP-WT/FS-NEFH RNA injected embryos at 24 hpf. GFP fluorescence is significantly reduced in GFP-FS-NEFH injected larvae. Scale bar 100 µm (e) Quantification of the average GFP intensity in tail as a 16-bit gray value (0-255). (f) Quantification
of full length GFP tagged WT and FS NEFH proteins. Levels of GFP-FS-NEFH are significantly reduced even when embryos were incubated with the autophagy inhibitor chloroquine (Chq). (e) RT-PCR confirms the presence of the injected GFP-WT/FS-NEFH mRNAs indicating that the reduced protein levels are not due to mRNA degradation.

As a secondary means of validating these results we cloned GFP-WT-NEFH and GFP-FS-NEFH into the transgenic gateway construct, UAS-E1b:DsRed:(R1-R2)\(^{161}\). This bidirectional UAS construct allows for the simultaneous expression of the GFP tagged NEFH proteins along with DsRed, under the transcriptional control of the Gal4 element. We co-injected these constructs with the motor neuron driver plasmid, HB9:Gal4-VP16\(^{21}\) and transposase RNA which integrates the plasmid elements flanked by the TOL2 sites (Figure 4.4 a). This system stochastically drives the expression of the transgenes in motor neurons in which we assume that most of the red cells also express the NEFH proteins. Although the GFP signal from the NEFH fusion proteins were not easily detectable \textit{in vivo}, the presence of the DsRed indicates that they should be expressed (Figure 4.4 b). We found that motor neurons expressing the mutant GFP-FS-NEFH were significantly shorter than those expressing wildtype GFP-WT-NEFH, which is congruent to the dominant gain-of-function effect measured with the injection of the mRNA (Figure 4.3 b). Interestingly, we never observed protein aggregates from GFP-FS-NEFH expression \textit{in vivo} or in fixed spinal cord sections in which the GFP was amplified with a \(\alpha\)-GFP antibody. (Figure 4.4 a). We did notice that the UAS-E1b:DsRed:GFP-WT/FS-NEFH constructs did not produce particularly high levels of the transgenes, so we also chose to put the GFP-tagged NEFH transcripts directly under the HB9 promoter, which we co-injected with
HB9:memCherry (Figure 4.5 a). Using this system we were able to clearly visualize the GFP-WT-NEFH fusion protein in vivo, however in congruence to our RNA injection data the GFP-FS-NEFH protein was barely detectable (Figure 4.5 b).

Figure 4.4 Motor neuron specific overexpression of NEFH transcripts with DsRed reporter.
(a) The bi-direction UAS reporter construct drives GFP-FS-NEFH or GFP-WT-NEFH with DsRed in motor neurons when coinjected with the Hb9:Gal4 driver construct. The bidirectional system
ensures that most red cells will also express the NEFH proteins. (b) In vivo imaging of NEFH expressing motor neurons. Motor neurons expressing the mutant GFP-FS-NEFH transcript are significantly shorter compared to wildtype GFP-WT-NEFH. P=0.03 by 2-tailed T-test. (c) UAS:DsRed:GFP-FS-NEFH does not cause the formation of aggregates in vivo (not shown) or in spinal cord sections amplified with anti-GFP antibody (shown).

Figure 4.5 Motor neuron specific overexpression of NEFH transcripts under direct control of Hb9.

(a) Schematic showing independent expression of GFP-NEFH and memCherry directly under the Hb9 promoter. This method drives high expression of both transgenes, but does not guarantee that all motor neurons will express both transgenes. (b) Using this method we did observe the presence of GFP in vivo in GFP-WT-NEFH, but not in GFP-FS-NEFH injected larvae at 48 hpf. Higher magnification images of GFP-FS-NEFH motor neurons does show the presence of some
GFP along with dystrophic appearance of cell bodies, but the GFP levels are significantly reduced similar to what was observed through mRNA injection.

Discussion

Neurofilament aggregation is a well characterized feature in neurodegeneration that occurs in amyotrophic lateral sclerosis\textsuperscript{156}, Alzheimer’s disease, CMT2, and giant axonal neuronpathy\textsuperscript{162}. Our results are unique in that they presents a novel mechanism of neurofilament self-aggregation in CMT2, which could help us to understand the causative versus correlative relationship between neurofilament aggregation and neurodegeneration. We found that the GFP-FS-NEFH protein can function by a dominant gain-of-function mechanism, however the pathology of the protein aggregation is more complex in an \textit{in vivo} system and it is likely that the mutant protein is being degraded and or not being stably assembled. The kinetics of AB, a protein aggregate associated with Alzheimers, is slowed with aging suggesting that these types of pathogenic protein aggregates are normally cleared in young and healthy individuals\textsuperscript{163}. Considering that not much is known about the mechanism of neurofilament degradation\textsuperscript{164}, a stable animal model that can be used to further dissect the mechanism of the FS-NEFH protein turnover would be a valuable asset to the field.
Chapter 5: Future Perspectives

SLC25A46 as a Modified Carrier Protein

Mitochondria are composed of two lipid membranes. While the outer membrane is permeable to solutes, the inner membrane has specialized carriers that allow for the selective transport of nutrients and metabolites. These channels are formed by proteins of the SLC25A family\textsuperscript{115}, however all eukaryotes seem to contain highly derived members of this family including MTCH1, MTCH2 and Ugo1 of yeast. These modified carriers have lower conservation of the carrier domain repeats and have been shown to localize to the outer mitochondrial membrane\textsuperscript{119,18}. Many of these derived carriers have been difficult to study because they cannot be expressed in bacteria and reconstituted into liposomes\textsuperscript{165}. Furthermore, it is assumed that that these proteins do not function as solute transporters because they lack the conserved tripartite carrier domain structure\textsuperscript{121} and because the outer membrane is permeable to solutes up to 5 kDa\textsuperscript{118}. My work reclassifies SLC25A46 as a modified carrier, so future experiments should be guided by what is known of the other derived family members for clues as to its function.

Ugo1 has been proposed to function as an adaptor protein in that it directly interacts with the fusion machinery on the outer (Fzo1) and inner (Mgm1)\textsuperscript{19} mitochondrial membranes of yeast as well as with the inner membrane cristae organizer (Fcj1)\textsuperscript{166}. The interaction between SLC25A46 and mitofilin (Fcj1 homolog) suggests that it could function at mitochondrial contact sites which have important roles in fusion/fission dynamics, metabolite transport, as well as
protein and lipid translocation. Future studies discussed below should aim to further elucidate the function of SLC25A46.

Functional Domains of SLC25A46

Typical mitochondrial carrier proteins of the SLC25A family have three carrier repeats containing electron transfer motifs (ETM) defined by the conserved amino acid residues; PX[D/E]XX[R/K]. SLC25A46 has only one well conserved carrier domain with semi-conserved ETM repeats. Interestingly, most of the patient mutations fall into the ETM residues of the conserved carrier domain suggesting that they are highly important for the function of the protein. In Ugo1, changing the charge of some of these ETM residues affects the ability of Ugo1 to dimerize and prevents mitochondrial fusion. We speculate that the ETM residues in the conserved carrier domain of SLC25A46 also mediate homodimerization. We have preliminary evidence suggesting that SLC25A46 dimerizes, which could be definitively confirmed by reciprocal pull down assays of SLC25A46-MYC and SLC25A46-HA transfected cells, and by running blue native gels of SLC25A46-HA transfected cells. Full length SLC25A46 runs at 46 KD and we expect the dimer to run at 92 KD under native conditions.

It should further be tested which domains of SLC25A46 directly interact with mitofilin, and if SLC25A46 can directly influence cristae morphology. This would support its role as a physical adaptor protein like Ugo1.
Mitochondrial Fusion Pore Hypothesis

In the heart, mitochondria are small and fragmented and there is essentially no detectable fusion and fission events\textsuperscript{168}. Interestingly though, the mitochondria show electrical coupling and have well aligned cristae junctions between adjacent membranes suggesting trans-mitochondrial organization of the junctions\textsuperscript{169,170}. The tethering of the mitochondria and alignment of the cristae is independent of the mitofusins suggesting that other proteins are facilitating this arrangement.

It has been speculated that the alignment of the junctions between adjacent mitochondria can facilitate the diffusion of inner membrane space solutes between adjacent mitochondria. In support of this theory, mitochondria in cultured cells display synchronous depolarizing and hyperpolarizing events suggesting the presence of pore that allows for the diffusion of protons\textsuperscript{125}. This indicates that there is a channel that can exist between incompletely fused mitochondria. Such a channel would allow for greater electrical coupling and signaling across the mitochondrial network, independent of full membrane fusion events. Transient fusion in which matrix is exchanged, but morphology is unchanged has been described as kiss-and-Run fusion\textsuperscript{171}, which could be mediated through the fusion pore.

Given that both Ugo1 and SLC25A46 share homology to mitochondrial proton transporters\textsuperscript{115,118}, and interact with mitofilin (Fcj1)\textsuperscript{21,40}, an organizer of the cristae junctions, it is possible they have dual roles in facilitating the exchange of protons between adjacent mitochondria as well as in organizing the cristae
alignments. If SLC25A46 is a component of this pore, it is possible that in its absence the mitochondria still undergo fusion but that the subsequent fission is inhibited due to the altered kinetics of the lipid mixing.

5.1 Mitochondrial fusion pore hypothesis.
In this hypothetical figure, SLC25A46 through interactions with mitofilin helps to organize cristae junction alignments and facilities the diffusion of protons between the inner membrane spaces independent of mitochondrial fusion. It is also possible that SLC25A46 could facilitate matrix exchange depending on the configuration of the outer-inner membrane contacts.

To determine if SLC25A46 could be forming this type of transient pore, we could directly measure mitochondrial membrane potential oscillations, or pH flashes in Slc25a46 KO cells. This can be achieved by overexpressing Mito-SypHer and measuring the frequency and extent of synchronous pH flashes in Slc25a46 KO MEFs as compared to the matrix connectivity by photoactivatable
GFP$^{125}$. Using the latter assay, we have already determined that SLC25A46 knockdown cells have increased matrix connectivity, which could provide a compound variable when assessing the extent of pH flashes. As a control we may need to modulate other fusion and fission factors in Slc25a46 KO MEFs to control for these variables.

*Therapeutic Implication of Mitochondrial Dynamics*

A number of studies have demonstrated that modulating the balance between mitochondrial fusion and fission has benefits in many different disease models. Knocking down Drp1 with siRNA or with the small molecules mdiviA and mdiviB rescues mitochondrial function in *in vitro* and *in vivo* models of glutamate toxicity and oxygen-glucose deprivation$^{172}$. Furthermore, overexpression of the dominant-negative DRP1 K38A in mouse models of Parkinson’s disease$^{3}$ and glaucoma$^{93}$ have shown therapeutic potential.

The overexpression of MFN1 has been proposed as a therapeutic mechanism for CMT2 patients with mutations in *MFN2*. In mammals MFN1 and MFN2 are 81% similar to each other and can complement each other *in vitro*$^{30,59}$. Based on this evidence and the lower expression of MFN1 in the nervous system, it has been proposed that increasing the endogenous expression MFN1 in CMT2A patient could have therapeutic benefits. Currently, small molecules are being tested for their ability to increase endogenous MFN1 in neurons and the concept of the MFN1 rescue is being tested in a dominant negative MFN2
OPA1 has dual roles in both mitochondrial inner membrane fusion and in cristae remodeling. The transgenic overexpression of Opa1 has been shown to improve mitochondrial respiration and phenotypes in two mouse models of mitochondrial dysfunction suggesting that the therapeutic benefits are relevant across multiple subtypes of mitochondrial disease.

Mice that are deficient in Mff, a protein involved in mitochondrial fission and die due to heart failure, can also be rescued by concomitantly deleting Mfn1. However, while Mfn1 deletion rescues the heart in Mff KO mice, it fails to rescue defects in the cerebellum. This indicates that while there is tissue specificity when it comes to balancing fusion and fission dynamics in vivo.

SLC25A46 could be a potential therapeutic target given its interaction with mitofilin and homology to Ugo1. Mitofilin is essential for the formation of the cristae junctions independent of mitochondrial fusion/fission, and has been shown to interact with various disease genes including Pink1, MFN2, and SLC25A46. The transgenic overexpression of mitofilin has been shown to have therapeutic benefits in a mouse model of cardiac disease, but its function is largely unexplored in the central nervous system. Future findings related to SLC25A46 could help identify more specific therapeutic targets as a strategy to boost mitochondrial fitness with implications in both the heart and nervous system.
References


