Identification of Novel Phospholipid Related Functions of Mitofusin 2 in Cell Models of Charcot-Marie-Tooth Disease 2A

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IDENTIFICATION OF NOVEL PHOSPHOLIPID RELATED FUNCTIONS OF MITOFUSIN 2 IN CELL MODELS OF CHARCOT-MARIE-TOOTH DISEASE 2A

By

Donald S. McCorquodale III

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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IDENTIFICATION OF NOVEL PHOSPHOLIPID RELATED FUNCTIONS OF
MITOFUSIN 2 IN CELL MODELS OF CHARCOT-MARIE-TOOTH DISEASE 2A

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The mitofusin 1 and 2 (MFN and MFN2) proteins reside in the outer mitochondrial membrane and have been shown to regulate mitochondrial network architecture by mediating tethering and fusion of mitochondria. Mitochondria normally form a tubular and branched reticular network dynamically regulated by a balance of fusion and fission events. Absence of either \(Mfn1\) or \(Mfn2\) results in a fragmented mitochondrial network. Züchner et al. previously described mutations in the gene \(mitofusin 2\) (\(MFN2\)) as the cause of the major autosomal-dominant, axonal form of Charcot-Marie-Tooth neuropathy (CMT2A). CMT type 2 (CMT2) is characterized by chronic axonal degeneration of peripheral nerves leading to the loss of functional nerve fibers. Mutations in MFN2 are the most common cause of CMT2, and in Chapter 2 we report the results from a genetic screen of MFN2 in a CMT2 patient cohort.

The original finding that mutations in \(MFN2\) cause CMT2A led to investigations focused on deficiencies of mitochondrial fusion and transport, specifically in the context of long axonal processes affected in CMT. While some experimental work supports disrupted mitochondrial transport in the etiology of CMT2A, other studies on CMT2A
patient fibroblasts and cell models suggest abnormal mitochondrial fusion and dynamics
do not underlie the etiology of this. In the first half of Chapter 3, we present some of our
initial investigations prior to de Brito and Scorrano’s report published in 2008 regarding
a novel role for Mfn2 in tethering the endoplasmic reticulum (ER) to mitochondria. In
Mfn2 null mouse embryonic fibroblasts (MEFs) regions of contact between mitochondria
and the endoplasmic reticulum (ER) are significantly reduced. These regions of contact
are thought to form specialized subdomains of the ER, called mitochondrial associated
membranes (MAM). Besides observing a fragmented ER network in Mfn2 knockout
(KO) mouse embryonic (MEF) cells, de Brito and Scorrano presented several lines of
evidence which suggest that the underlying pathogenic mechanism in CMT2A stems
from disrupted ER-mitochondria. As this observation had not been replicated in the
literature, we describe our attempts to replicate these finding in the last half of Chapter 3.

The MAM represents a sub-domain of the ER in close association with the
mitochondrial outer membrane. The movement of phosphatidylserine (PS) from the
MAM domains of the ER to mitochondria and its subsequent decarboxylation to
phosphatidylethanolamine (PE) by the enzyme PS decarboxylase (Pisd) has been well
characterized and is known to depend on the existence of an outer mitochondrial
membrane protein. As PE has curvature inducing and fusogenic biophysical
characteristics, a deficiency in PE would be an attractive mechanism contributing to the
morphological and fusion defects observed in Mfn2 null cell models. We hypothesized
that loss of Mfn2 would lead to specific decreases in mitochondrial and cellular levels of
PE. Chapter 4 describes experiments designed to test this hypothesis. We observed
significantly lower levels of PE in Mfn2 null cells, yet observe similar changes in Mfn1
null cells. Likewise, other lipid species such as ether linked PE (ePE) are decreased. To investigate how CMT2A mutations in MFN2 influence cellular phospholipid profiles, we then profiled cellular phospholipids of CMT2A patients and control lymphoblasts. We hypothesized that mutations in MFN2 would result in decreased levels of PE. In Chapter 5, we report the results of a phospholipid screen which reveal changes in ePE in CMT2A patient lymphoblasts, without the drastic decreases in PE previously observed in Mfn2 null lines.

In conclusion, our data indicates an important role for both mitofusins in the mitochondrial synthesis of PE. In the context of CMT2A mutations, ePE levels are specifically reduced. Future studies may reveal how deficiencies in ePE might have important functional consequences in the pathogenesis of CMT2A.
DEDICATION

This work is dedicated to my family. My mother and father taught me the value of hard work and self driven learning not through pressure or force, but through example. My wife Pallas, whose understanding and commitment during these years has been essential to my success. Lastly, I’d like to acknowledge my sons Iver and Raef, who have enriched my life beyond measure. Everyone seems to wonder how I “did it” with a wife and kids. Instead, I wonder how Ph.D. students do it without them.
ACKNOWLEDGEMENTS

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Additionally, I would like to thank the members of my thesis committee who have helped shape and model this work. There support and ideas along with their practical understanding of how thesis progress helped make this process as painless as possible.

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I would also like to thank David Chan, Ph.D., for the kind gift of the Mfn2 and Mfn1 null mouse lines. These cell lines proved to be an essential part of my thesis. Likewise, I would also like to thank Mary Roth of the Kansas State University Lipidomics Core for her technical assistance in setting up the phospholipidomic screens.
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CHAPTER 1. INTRODUCTION

Charcot Marie Tooth disease and \textit{MFN2}.

Charcot Marie Tooth disease (CMT) is one of the most common inherited disorders in humans, with a prevalence estimated at 1 in 1,214 to 2,500 individuals (Braathen and others 2011; Skre 1974). The clinical hallmarks of CMT are distal weakness beginning in the lower legs which progresses to the hands and arms with accompanying sensory loss, decreased reflexes and foot and hand deformities. Charcot-Marie-Tooth disease can be clinically classified into two groups. CMT type 1 (CMT1) is associated with reduced nerve conduction velocities stemming from demyelination of the peripheral nerve. CMT type 2 (CMT2) is associated with chronic axonal degeneration and regeneration of peripheral nerves leading to the loss of functional nerve fibers. The resulting nerve conduction velocities are normal but decreased in amplitude. The identification of a number of genes causing CMT has led to genetic subclassification of CMT1 and 2. A CMT type 2A (CMT2A) locus was assigned to chromosome to 1p35-36 in 1993 by linkage analysis in several families (Ben Othmane and others 1993). Subsequently, all known families with significant linkage to 1p36 were shown to carry mutations in \textit{MFN2} (Zuchner and others 2004). Additional clinical symptoms are associated with specific CMT2A alleles, including optic atrophy, white matter changes in the CNS, and early onset stroke (Zuchner and others 2006). Thus far, about 60 mutations have been reported in MFN2 (http://www.molgen.ua.ac.be/cmtmutations/). This makes \textit{MFN2} the second most common gene in CMT after \textit{PMP22}, which is responsible for CMT1A. Mutations in \textit{MFN2} are now known to account for ~15-30\% of all familial
CMT2 cases, and specific mutations are known to cause CMT2A with optic atrophy (McCorquodale and others 2011; Zuchner and Vance 2006; Zuchner and others 2006). In chapter 2 we describe a genetic screen for mutations in MFN2 in a cohort of CMT2A patients.

**Molecular functions of the mitofusins.**

MFN2 and its paralog, MFN1, reside in the outer mitochondrial membrane and have been shown to regulate mitochondrial network architecture by mediating fusion of mitochondria (Bach and others 2003; Chen and others 2003; Rojo and others 2002; Santel and Fuller 2001). Mitochondria represent a tubular and branched reticular network, which undergoes a dynamically regulated balance between fusion and fission reactions (Nunnari and others 1997; Yaffe 2003). It has been shown that MFN2 and MFN1 are directly involved in tethering of mitochondria (Koshiba and others 2004). MFN1 and MFN2 functions appear to overlap as Mfn1 can partially rescue Mfn2 mutants by complementing Mfn2 as a heterodimer (Detmer and Chan 2007). Both mitofusins have homologs in mammals while *Gallus gallus*, *Danio rerio*, while *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae* have a single homologous gene, *fuzzy onion* (*fzo*). Studies have shown that MFN2 is involved in apoptosis by co-localization with the pro-apoptotic protein Bax (Brooks and others 2007; Karbowski and others 2002; Neuspiel and others 2005). MFN2 regulates expression of OXPHOS components (Pich and others 2005); and its expression appears reduced in obesity (Bach and others 2003).

Mfn2 is essential for embryonic development, and *Mfn2* knockout (KO) mice have been reported to die in mid-gestation due to placental defects (Chen and others
2003). *Mfn2* +/- heterozygotes were reported to have a "normal" phenotype, and no signs of peripheral neuropathy (David Chan, personal communication). When the placental defect is rescued, *Mfn2* null mice eventually develop profound cerebellar degeneration, which may overshadow more subtle peripheral nerve related phenotypes. Bach *et al.* also demonstrated that repression of MFN2 reduced glucose oxidation, mitochondrial membrane potential, cell respiration, and mitochondrial proton leak in muscle cells (Bach and others 2003). One important finding is that embryonic fibroblasts from *Mfn2* homozygous knockout mice showed a dramatic decrease in mitochondrial mobility (Chen and others 2003; Chen, McCaffery, Chan 2007). Thus, MFN2 may also play a role in axonal transport. Mitochondrial mobility and transport are key elements to the functional health of the axons of neurons, particularly in the long peripheral nerves specifically affected in CMT.

**Pathogenicity of MFN2 mutations**

The central and critical roles of mitochondria lend themselves to a number of hypotheses regarding how mutations in *MFN2* might cause CMT2A. Recent research has shown that some clinically observed *MNF2* mutations, but not others, cause mitochondrial defects (Amiott and others 2008a; Baloh and others 2007; Guillet and others 2009). Failure to reconcile these results hinders the synthesis of working mechanism regarding how MFN2 function is altered in CMT2A. Complicating matters, mitochondrial phenotypes resulting from MFN2 overexpression appear to be highly dose-dependent so that experimental overexpression of both wildtype and disease alleles cause almost identical forms of perinuclear mitochondrial clustering (Amiott and others 2008a).
Reports using cell models and CMT2A patient fibroblasts suggest that MFN2 mutations do not consistently alter mitochondrial fusion, morphology, mtDNA maintenance, oxidative phosphorylation, or mitochondrial transport (Amiott and others 2008a; Baloh and others 2007; Loiseau and others 2007). In the first half of Chapter 3, we present our initial studies aimed at elucidating the role of CMT2A mutations on mitochondrial morphology. How these mutations, which in our hands and others, appear to have no consistently discernable pathogenic potential in experimental systems yet cause disease in humans is still unknown. While it might be true that this is a technical challenging subject to study, or that different MFN2 mutations have different pathogenic mechanisms, it remains an unsatisfying situation. An intriguing possibility is that MFN2 has additional functions outside of mitochondrial fusion that could explain its pathological role in CMT2A. The recent discovery that Mfn2 also resides in endoplasmic reticulum (ER) membranes and tethers the sections of the ER to mitochondria opened new opportunities for elucidating the pathogenesis of CMT2A (de Brito and Scorrano 2008).

**MFN2 tethers ER to mitochondria.**

From its initial description in drosophila in 2001, ER related roles for MFN2 were unexplored by investigators until de Brito and Scorrano’s work published seven years later (de Brito and Scorrano 2008; Merkwirth and Langer 2008). Besides observing a fragmented ER network in Mfn2 (KO) mouse embryonic fibroblasts (MEF), de Brito et al. demonstrate several lines of compelling evidence which suggest that defects in ER-mitochondria interfaces may be the underlying pathogenic deficit caused by mutations in MFN2 (de Brito and Scorrano 2008). In both untransfected Mfn2 KO MEFs and MEFs
transfected with MFN2 alleles lacking the Ras binding domain (RBD) or mutations within the RBD, regions of intimate contact between mitochondria and the ER are significantly reduced (de Brito and Scorrano 2008). These regions of contact are thought to form at specialized domains of the ER, called mitochondrial associated membranes (MAM). De Brito et al. found Mfn2 enriched 14-fold in the MAM as compared to the mitochondrial fraction. Likewise, it was demonstrated that Mfn2 is a key protein in the formation of the MAM through its ability to tether the MAM to the mitochondrial outer membranes via Mfn2:Mfn2 homodimers or Mfn1:Mfn2 heterodimers. The second half of Chapter 3 describes our attempts to replicate many of the findings previously reported by de Brito and Scarrano.

**Ca^{2+} handling within the MAM.**

Recent studies suggest that Ca^{2+} flux from ER to mitochondria is organized by the formation of protein complexes within the MAM (Merkwirth and Langer 2008; Mironov, Ivannikov, Johansson 2005; Mironov and Symonchuk 2006; Pizzo and Pozzan 2007; Rizzuto, Duchen, Pozzan 2004; Rutter 2006). The Ca^{2+}-release channel, inositol 1,4,5-trisphosphate receptor (IP_{3}R), is thought to be located within the ER membrane and conduct the ER Ca^{2+} out-flux. Within the MAM, the IP_{3}R is brought within close proximity to the mitochondrial bound voltage dependent anion channel (VDAC), which conducts Ca^{2+} influx into the mitochondria (Hayashi and others 2009). The low affinity of VDAC for Ca^{2+} ensures that at normal resting physiological states, mitochondrial Ca^{2+} uptake is low. Upon local transient increases in Ca^{2+} caused by Ca^{2+} release from the ER, the VDAC begins to conduct Ca^{2+} into the mitochondria. A chaperon protein, Grp75, was
shown to mediate the formation of a protein complex, which included VDAC and IP₃R, as demonstrated by co-immunoprecipitation (Szabadkai and others 2006). Thus, the MAM may form a critical microdomain in which local and transient increases Ca⁺² are confined spatially to domains enriched in mitochondrial VDAC, the major Ca⁺² sink following ER Ca⁺² release (Hayashi and others 2009; Rizzuto and others 1998). By disrupting the formation of this organized domain, one would expect to see decreased Ca⁺² uptake by the mitochondria and increased cytosolic Ca⁺². Indeed, disruption of the MAM associated with the absence of MFN2 leads to many of these predicated changes.

How mutations in MFN2 might affect Ca⁺² handling at the MAM-mitochondrial interface has yet to be investigated.

**MFN2 localizes to cellular compartments associated with phospholipid synthesis.**

The existence of MAMs was first described as a specialized membrane fraction separated from associated mitochondria by Percoll gradients. In essence, the MAM is “pulled down” in a crude mitochondrial pellet, and once separated from mitochondria using a Percoll gradient, retains a density and protein composition consistent with the ER. The MAM contains the enzymatic activity to synthesize phospholipids associated with the ER. When the isolated MAM fraction was re-associated with the mitochondrial fraction, the synthesis of phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) was observed (Vance 1990; Vance and Steenbergen 2005; Voelker 1989; Voelker 1991). While it was known that phospholipid synthetic enzymes phosphatidylserine decarboxylase (PISD), cardiolipin synthase and phosphatidylglycerol synthase resided within mitochondria, the majority of phospholipid synthesis was known
to reside in the ER. Exactly how mitochondrial and ER phospholipid synthesis pathways were connected was not well understood. With the identification of the MAM, the putative site of phospholipids exchange between mitochondria and the ER was established (Vance 1990). Thus, the MAM represents a domain of the ER in close proximity with the mitochondrial outer membrane mediated by mitochondria outer membrane proteins (Achleitner and others 1999; Schumacher, Choi, Voelker 2002; Shiao, Lupo, Vance 1995; Shiao, Lupo, Vance 1995; Shiao, Balcerzak, Vance 1998; Vance 1990; Voelker 1989). This intimate MAM-mitochondrial interface is thought to facilitate the movement of phospholipids precursor species and end products between the two organelles. Most well characterized is the movement of PS from the ER to the mitochondria (Achleitner and others 1999; Schumacher, Choi, Voelker 2002; Shiao, Lupo, Vance 1995; Shiao, Lupo, Vance 1995; Shiao, Balcerzak, Vance 1998; Vance 1990; Voelker 1989). From the MAM, PS is eventually translocated to the outer leaflet of the inner mitochondrial membrane where it is decarboxylated by PS decarboxylase (Pisd) to form PE (Voelker 1991). PE is then shuttled back to the ER where is feeds into other important synthetic pathways or remains within the mitochondrial membranes (van Meer, Voelker, Feigenson 2008; Vance and Vance 2009; Vance and Steenbergen 2005; Vance 2008). While redundant extra-mitochondrial PE synthetic pathways exist, the majority of cellular PE synthesis in cultured cells occurs within the mitochondria (Steenbergen and others 2005; Vance and Vance 2009; Vance 2008).
**Pisd knockout and Mfn2 knockout** mouse models have identical mitochondria phenotypes and embryonic lethalities.

The importance of the mitochondrial generated PE is confirmed by the embryonic lethality (E8-10) of the *phosphatidylserine decarboxylase (Pisd)* null mouse model, corroborating that extra-mitochondrial PE synthetic pathways cannot substitute for absent mitochondrial PE synthesis (Steenbergen and others 2005; Vance and Vance 2009). More importantly is the observation that *Pisd* KO MEFs display fragmented and round mitochondria of irregular diameter conspicuously reminiscent of *Mfn2* KO mice (Steenbergen and others 2005). The resulting ER morphology was not investigated or published. The *Pisd* KO embryos demonstrate placental defects and embryo involution slightly earlier then *Mfn2* KO embryos (Chen and others 2003; Steenbergen and others 2005). The placental defect stems from atrophy of the syncio-trophoblast layer, a multinucleated layer serving as the maternal-fetal blood barrier formed by fusion of individual surrounding trophoblasts in the implanting embryo. The synciotrophoblast defect is identical in *Pisd* and *Mfn2* KO models. Underscoring the importance of PE in placental formation is the identification of anti-PE antibodies in women with antiphospholipid syndrome with spontaneous and recurring abortions(Girardi 2010; McIntyre, Wagenknecht, Faulk 2003; Sanmarco and Boffa 2009). Anti-PE antibodies attack PE on the surface of trophoblasts, implying PE is present at high levels on the surface of trophoblasts and is most likely a prerequisite for the fusion of trophoblastic cells and the formation of the synciotrophoblast(Girardi 2010; Sanmarco and Boffa 2009). While the critical role of structural proteins in inducing membrane curvature is critical in membrane and vesicle traffic and formation is well known, the composition of
the lipid bilayer also influences the degree of membrane curvature and the energetics required to induce membrane curvature and fusion (Churchward and others 2008; Collins 2006; Jiang and Powers 2008; Shnyrova, Frolov, Zimmerberg 2008). PE, whose space filled shape is approximated as a cone, is known to induce negative curvature on the inner leaflet of the lipid bilayer and promote Ca$^{2+}$ triggered membrane fusion events (Churchward and others 2008). One might speculate that the percentage of PE in mitochondrial and ER membranes determine the extent of curvature required to form small diameter tubules characteristic of mitochondrial and ER reticular networks, or to facilitate membrane fusion. In the case of cellular fusion events, as in the formation of the synciotrophoblast layer, the cell may depend on increased mitochondrial synthesis of PE to supplement ER synthesis pathways.

Scorrano’s data suggest that Mfn2 mutations disrupt mitochondria-MAM contacts, and data from the Pisd KO mouse suggest that disrupting biosynthetic processes dependent on mitochondria-MAM contact formation lead to the same cellular phenotype. This would place MFN2 functionally upstream of critical biosynthetic pathways dependent on mitochondria-MAM contacts, implicating deficient phospholipid synthesis in the pathogenesis of CMT2A. In the extreme examples of Pisd or Mfn2 KO models, profound deficits of mitochondrial generated PE result in dramatic changes in the physical nature of cellular membranes, affecting organelle shape and ability to fuse. In the case of CMT2A, an autosomal dominant disease, deficits may be more subtle, and stem from abnormalities in how mitochondria phospholipid synthesis is dynamically regulated.
We describe how the hypotheses described above were tested in Chapter 4 and 5, in which we used a lipidomics approach to profile and quantify phospholipid species in cell models. Our initial studies focusing on Mfn2 null models are described in Chapter 4. We demonstrate that Mfn2 and Mfn1 are critical to maintenance of cellular PE levels when a major redundant PE pathway is absent. Additionally, several other lipid species, including ether linked PE (ePE), also known as ethanolamine plasmalogen, were reduced in specifically Mfn2 null cells under all experimental conditions. Chapter 5 describes investigations into how CMT2A mutations in MFN2 influence cellular phospholipid profiles. Using lymphoblasts from CMT2A patients and control, we identified deficiencies in ePE in CMT2A lymphoblasts and compared to controls.

These results point to a novel role for the mitofusins in the synthesis of mitochondrial and cellular phospholipids. The consistent deficiency in ePE in both Mfn2 null and CMT2A patient cells suggest deficiencies in this lipid species may be important in the pathogenesis of CMT2A.
Table 1.1. Phenotypes of Mfn1, Mfn2, and Pisd Knockout mouse models.

<table>
<thead>
<tr>
<th>KO Model</th>
<th>Lethality Phenotype</th>
<th>Lethality Time course</th>
<th>mitochondrial Morphology</th>
<th>ER Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>N/A</td>
<td>N/A</td>
<td>Elongated tubules extending radially to the cell periphery</td>
<td>Reticular</td>
</tr>
<tr>
<td>Mfn1</td>
<td>Developmental Defects (unspecified but not placental related)</td>
<td>E11.5-E12.5</td>
<td>Small uniform spheres approximately same diameter as tubules throughout cell</td>
<td>Reticular</td>
</tr>
<tr>
<td>Mfn2</td>
<td>Placental Insufficeny due to synciotrophoblast atrophy</td>
<td>E10.5-E11.5</td>
<td>Varying sized spheres and ovals</td>
<td>Fragmented</td>
</tr>
<tr>
<td>PSDC</td>
<td>Placental Insufficeny due to synciotrophoblastic cell abnormalities (maternal blood mingle with embryo)</td>
<td>E8-E10</td>
<td>Varying sized spheres and ovals</td>
<td>Not Reported</td>
</tr>
</tbody>
</table>
CHAPTER 2. MUTATION SCREENING OF MITOFUSIN 2 IN CHARCOT-MARIE TOOTH DISEASE TYPE 2 (CMT2A)

Summary.

Charcot-Marie-Tooth (CMT) disease is among the most common inherited neurological disorders. Mutations in the gene mitofusin 2 (MFN2) cause the axonal subtype CMT2A, which has also been shown to be associated with optic atrophy, clinical signs of first motor neuron involvement, and early onset stroke. Mutations in MFN2 account for up to 20-30% of all axonal CMT type 2 cases. To further investigate the prevalence of MFN2 mutations and to add to the genotypic spectrum, we sequenced all exons of MFN2 in a cohort of 39 CMT2 patients. We identified seven variants, four of which are novel. One previously described change was co-inherited with a PMP22 duplication, which itself causes the demyelinating form CMT1A. Another mutation was a novel in frame deletion, which is a rare occurrence in the genotypic spectrum of MFN2 characterized mainly by missense mutations. Our results confirm a MFN2 mutation rate of ~15 to 20% in CMT2.

Background.

Charcot-Marie-Tooth disease (CMT) is a common inherited neurological disorder with an estimated incidence of 1 in 1214 to 2500 individuals (Braathen and others 2011; Skre 1974). Classically, CMT is broadly divided into two types based on nerve conduction study findings (Dyck and Lambert 1968). CMT type 1 (CMT1) is associated with slowed nerve conduction velocities (<38m/s), which correlate with histopathological observations characteristic of a demyelinating peripheral neuropathy. CMT type 2
(CMT2) is characterized by normal nerve conduction velocities and low compound muscle action potentials (CMAP) reflecting selective degeneration of axons without myelin involvement (Zuchner and Vance 2006). Genetic studies have revealed 40 genes associated with inherited peripheral neuropathies (Mutation Database of Inherited Peripheral Neuropathies, http://www.molgen.ua.ac.be/cmtmutations/Home/Default.cfm). Duplications of *PMP22*, the cause of CMT type 1A (CMT1A), account for approximately 70% of CMT type 1 (Wise and others 1993). Mutations in the gene *MFN2* are the most common cause of CMT type 2 with frequencies ranging from 8% to 20% (Kijima and others 2005; Lawson, Graham, Flanigan 2005; Verhoeven and others 2006; Zuchner and others 2004). Specific alleles in CMT2A are associated with optic atrophy, clinical signs of first motor neuron involvement, and early onset stroke (Chung and others 2008; Zhu and others 2005; Zuchner and Vance 2006). In patients with a documented family history of CMT2 *MFN2* mutations were identified in 33% of 323 CMT patients (Verhoeven and others 2006). Thus, screening for mutations in *MFN2* is first step in the molecular diagnosis of CMT2. Here we report the results of a screen of 39 CMT2 patients for mutations in *MFN2*. We describe seven variants, four of which are novel. Furthermore, we examine and discuss the clinical phenotype-genotype spectrum in seven probands and discuss evidence for pathogenicity.

**Materials and Methods**

**Patients.** The 39 CMT patients screened are part of our ongoing collection of unrelated CMT families and isolated index patients. Available clinical information on these samples is given in Table 2.1. The sample is composed of 38 patients clinically
determined to have an axonal peripheral neuropathy and one patient with a mixed axonal and demyelinating type of CMT. The mode of inheritance was autosomal dominant for 29 familial cases, 6 were classified as sporadic with no evidence of family history of peripheral neuropathy, and 4 did not have sufficient family history to make a determination. Informed consent was obtained from all individuals, and the Institutional Review Board (IRB) at the University of Miami Miller School of Medicine approved the study.

**Analysis of MFN2.** Blood (~24ml) was collected in either EDTA or acid citrate dextrose tubes from participating individuals by venipuncture and DNA was extracted in the Biorepository of the Hussman Institute for Human Genomics. Oligonucleotide primers flanking each exon (and neighboring intronic sequences) of MFN2 were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and are available upon request. Exons and flanking intronic sequences were amplified on the Applied Biosystems (ABI) Veriti 96-well Fast Thermal Cyclers using a touchdown protocol. PCR purification was completed with QuickStepTM2 SOPE resin (Edge BioSystems). Sequencing was performed using ABI BigDye® Dye Terminator Cycle Sequencing Kit on an ABI 3730 sequencer (ABI). Sequence traces were analyzed using Sequencher® ver. 4.8 (Gene Codes Corporation). Each nucleotide variation identified was confirmed by completing PCR amplifications and subsequent bi-directional sequencing on fresh aliquots of sample DNA. All variants were screened for segregation in additional family members if available and were absent in 200 control samples. All mutations were analyzed with the PolyPhen-2 software that predicts the functional significance of protein changes
Results and Discussion.

Direct sequencing of the MFN2 gene in 39 CMT2 patients identified seven non-synonymous coding sequence variants in seven probands (2.2). Four mutations were novel disease variants. Available clinical information regarding the seven probands is summarized in Table 2.3 and family pedigrees can be found in Figure 2.1. Nerve conduction studies were available for five of the seven identified patients. Four of the five nerve conduction studies revealed median and ulnar nerve conduction velocities over 38m/s and low CAMP, which were classified as axonal type CMT (CMT2). The fifth nerve conduction study suggested a mixed axonal and demyelinating peripheral neuropathy (family 20004). In all cases, studies were performed in advanced disease, as nerve conduction studies of the leg were not recordable in all but one case (Table 2.3). Nerve biopsies were not performed. In our sample, the age of onset ranged from 1 to 77 years.

The most severe phenotype (family 20006) with severe disability by the age of 7 years was caused by a L92R variant. This finding is consistent with previous reports on changes in residues 92 and 94, which reside in the Ras-binding domain of MFN2 and are also associated with increased disease severity (Calvo and others 2009; Neusch and others 2007; Verhoeven and others 2006). The proband’s parents reported low birth weight, pes cavus, and difficulty walking due to “weak ankles” by the age of three years. Nerve conduction study results were not available but were reported to demonstrate an
axonal neuropathy. Problems with visual acuity were not reported. Beyond a diagnosis of Raynaud’s syndrome in the mother, the family history is negative for neuropathies or related conditions. As both parents were screened negative for the same change, the mutation likely arose \textit{de novo} (Figure 2.1A).

Two mutations identified in separate probands occurred on adjacent residues (R250, P251) within the conserved GTPase domain of \textit{MFN2}. The R250Q change has been previously reported as a causative variant (Verhoeven and others 2006). \textit{In silico} modeling of protein function rated the R250Q mutation as probably damaging. The 50-year-old proband of family 20023 was diagnosed with CMT at the age of 12 years and has a complicated medical history including degenerative disc disease, a ten-year history of type II diabetes, and hypertension. A cranial MRI study was unremarkable. At the time of exam, the patient was able to ambulate with the aid of a cane and complained of mild to severe numbness, tingling, and neuropathic pain in hands and feet. Neurologic exam and nerve conduction studies were both consistent with an axonal peripheral neuropathy with symptoms complicated by diabetic neuropathy. The lack of a detailed history for family 20023 limits determination of the mode of inheritance, although a reported neuropathy in the aunt of the proband suggests that the change is not sporadic (Figure 2.1B). Additional family members were not available for segregation analysis.

A change in the adjacent proline residue (P251R) was associated with a more severe form of CMT2A. The P251R change is predicted to be damaging but has not been previously described. A variant in the same residue (P251A) has been previously reported (Zuchner and others 2004). The 37-year-old female patient was diagnosed with a neuropathy at the age of 2 years and has been wheelchair dependent for the past 10 years.
Physical exam and nerve conduction studies are consistent with CMT2 and her medical history is negative for other conditions. The patient reports no family history of CMT (Figure 2.1C). Subsequent screening of the affected daughter revealed co-segregation of the change with the disease.

The M376L variant is predicted to be benign. However changes in the same residue have been previously described in multiple reports (Casasnovas and others 2010; Chung and others 2006; Engelfried and others 2006; Verhoeven and others 2006). The M376L change, clustering with a number of previously reported mutations outside of known conserved domains, was identified in the proband from family 20033(Chung and others 2006; Engelfried and others 2006; Verhoeven and others 2006). Family 20033 has a positive history of neuropathy symptoms in five generations consistent with an autosomal dominant inheritance pattern, though no additional family members were available for segregation analysis (Figure 2.1D). At the time of exam the 48-year-old female patient, diagnosed with CMT at the age of 25 years, ambulated with the aid of ankle foot orthotics yet has difficulty walking distances over ~20 meters. In addition to typical lower extremity findings, the proband reported numbness and neuropathic pain in hands and arms. Physical examination revealed significant thenar and hypothenar atrophy. A cranial MRI study was unremarkable. In addition to CMT the proband’s medical history included a diagnosis of carpal tunnel syndrome.

The R468H is predicated to be possibly damaging. The proband of family 20004 had a relatively mild CMT2 phenotype consistent with a previous report by Engelfried of the R468H change (Engelfried and others 2006). Her medical history includes hereditary elliptocytosis, diagnosed at the age of 10 years, with subsequent splenectomy and
cholecystectomy. She was clinically diagnosed with CMT at 40 years of age, but retrospectively reports a symptomatic history of CMT since childhood, including avoidance of running and trouble walking. The patient also reported a recent worsening of her ability to walk and balance, which she associated with the onset of menopause. Her neurological exam revealed mild pes cavus, hammer toe, and peroneal muscle wasting. Nerve conduction studies revealed both axonal and demyelinating features. Her father had a medial history of CMT-like symptoms described as abnormal gait and week ankles. Interestingly, he also had hereditary elliptocytosis. Although it appears the elliptocytosis cosegregates with CMT2A in this family, we are unaware of any previous reports of this association. One of the proband’s daughters showed suggestive signs of CMT at the age of 11, including high foot arches, hammer toe, and difficulty running. No additional family members were available for genetic tests. Nerve conduction studies revealed both axonal and demyelinating features. Interestingly, in review of additional clinical documents, we discovered she had been previously found to have a PMP22 duplication. However, the relatively mild course and late onset of disease contrast with previously reported case studies of patients co-inheriting PMP22 duplications with other neuromuscular disease alleles. For instance, co-inheritance of GJB1/Connexin-32, DMPK, and ABCD1, with PMP22 duplications leads to more severe neuromuscular symptoms and earlier ages of onsets consistent with a “double hit” hypothesis (Hodapp and others 2006). Moreover, the R468H variant was previously reported in a screen of 130 anonymous healthy blood donor samples (Engelfried and others 2006). However, the R468H change was recently indentified in a larger screen of CMT families in a patient
diagnosed with CMT1 (Braathen and others 2010). It is thus conceivable that the R468H change is not associated with strong causality, but reflects a rare modifying allele in CMT.

In family 20037 we indentified the V705I variant, which is predicted to be probably damaging and has been previously described in three CMT2 families (Figure 2.1E) (Braathen and others 2010; Engelfried and others 2006). The proband was diagnosis with CMT at the age of 12. At age of 31 the neurological examination revealed weakness and sensory loss in the lower extremities, but also significant involvement of the upper extremity. Additionally, the patient reported extensive neuropathic pain and paraesthesia in her hands and feet. The neuropathic pain localized to her shoulders and radiated to her hands. Significant loss of sensation was noted in the innervation area of the right trigeminal nerve (V1). The family history includes three maternal generations with CMT and a diagnosis of multiple sclerosis in the sister of the proband. No family members where available for segregation analysis.

The novel deletion R707_N709del identified in the proband from family 20013 represents only the second deletion described in MFN2 and clusters with two other known mutations in the C-terminus of MFN2 (Verhoeven and others 2006). This 9bp deletion results in the loss of 3 amino acid residues with the remainder of the protein remaining in frame. The proband is currently 13 years of age and was diagnosed at the age of 10 after a longstanding history of weakness and clumsiness in his lower limbs. His parents noted that he has always been a slow runner and in retrospect state that he has a mild steppage gait, however he refuses to use AFOs to correct his foot drop. Physical examination revealed pes cavus and foot deformities, but no evidence of scoliosis. He
had normal strength in the upper extremities with the exception of weakness in the interossei muscles and hyporeflexia. There was a moderate degree of muscle wasting in both legs with corresponding decreased strength and absent reflexes. The remainder of his motor examination in the lower limbs was normal. The proband had decreased thresholds to cold and pain sensation to the knees and slightly increased thresholds to vibration in the toes. The absence of a family history or clinical findings of neuropathy in the proband’s parents and sister as well as the negative genetic testing for the change in both parents suggests that the deletion is a de novo event (Figure 2.1G).

**Conclusion.**

In summary, the identification of seven *MFN2* mutations in our cohort of 39 unrelated CMT2 families corresponds to a prevalence of 17.9% in our sample set, although questions remain regarding the R468H variant. Exclusion of the R468H variant would reduce the prevalence to 15.8%. This is in agreement with previous reports for *MFN2* mutation frequency in CMT2(Kijima and others 2005; Lawson, Graham, Flanigan 2005). We have provided careful phenotypic descriptions, which will be valuable additions to a catalogue of pathogenicity of *MFN2*. Mutations in *MFN2* remain the primary cause of CMT2 and are thus of great importance in the molecular diagnosis of CMT2.
<table>
<thead>
<tr>
<th>Table 2.1. Sample Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Ethnicity</td>
</tr>
<tr>
<td>European</td>
</tr>
<tr>
<td>Hispanic</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Age of Onset</td>
</tr>
<tr>
<td>Range in years:</td>
</tr>
<tr>
<td>Average in years:</td>
</tr>
<tr>
<td>Clinical Phenotype</td>
</tr>
<tr>
<td>Demyelinating</td>
</tr>
<tr>
<td>Axonal</td>
</tr>
<tr>
<td>Mixed Axonal/ Demyelinating</td>
</tr>
<tr>
<td>Inheritance Pattern</td>
</tr>
<tr>
<td>Autosomal Dominant</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Sporadic</td>
</tr>
<tr>
<td>Patients with MFN2 mutations</td>
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Table 2.2. Mutations indentified in *MFN2*.

<table>
<thead>
<tr>
<th>Family</th>
<th>Exo</th>
<th>Mutation</th>
<th>Mutation</th>
<th>Protein Change</th>
<th>Consequence PolyPhen</th>
<th>Prediction</th>
<th>PolyPhen</th>
<th>Familial/</th>
<th>Reference/ Novel</th>
</tr>
</thead>
<tbody>
<tr>
<td>20006</td>
<td>4</td>
<td>missense</td>
<td>c.275T&gt;G</td>
<td>p.L92R</td>
<td>Damaging</td>
<td>2.97</td>
<td>sporadic</td>
<td>Novel</td>
<td></td>
</tr>
<tr>
<td>20023</td>
<td>8</td>
<td>missense</td>
<td>c.749G&gt;A</td>
<td>p.R250Q</td>
<td>Benign</td>
<td>1.366</td>
<td>familial</td>
<td>Verhoeven et al., 2006</td>
<td></td>
</tr>
<tr>
<td>20035</td>
<td>8</td>
<td>missense</td>
<td>c.752C&gt;G</td>
<td>p.P251R</td>
<td>Probably Damaging</td>
<td>2.956</td>
<td>familial</td>
<td>Novel</td>
<td></td>
</tr>
<tr>
<td>20033</td>
<td>11</td>
<td>missense</td>
<td>c.1126A&gt;C</td>
<td>p.M376L</td>
<td>Benign</td>
<td>0.663</td>
<td>familial</td>
<td>Novel</td>
<td></td>
</tr>
<tr>
<td>20013</td>
<td>18</td>
<td>deletion</td>
<td>c.2120delGGGAGAACC</td>
<td>p.R707_N709del</td>
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<td>N/A</td>
<td>sporadic</td>
<td>Novel</td>
<td></td>
</tr>
<tr>
<td>20037</td>
<td>18</td>
<td>missense</td>
<td>c.2566C&gt;A</td>
<td>p.V705I</td>
<td>Benign</td>
<td>0.059</td>
<td>familial</td>
<td>Engelfried et al., 2006</td>
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Table 2.3. Clinical characteristics of CMT2 patients with mutations in MFN2

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<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Sex</th>
<th>Age of Onset</th>
<th>Age at Diagnosis</th>
<th>Assistive devices/Orthotics</th>
<th>Co-morbidities</th>
<th>Lower Motor*</th>
<th>Upper Motor*</th>
<th>Sensory‡</th>
<th>Nerve</th>
<th>Distal CMAP (mV)</th>
<th>Distal Latency (ms)</th>
<th>NCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20006</td>
<td>L92R</td>
<td>M</td>
<td>3</td>
<td>7</td>
<td>AFO</td>
<td>low-birth weight</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>Med.</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<td></td>
<td></td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>20023</td>
<td>R250Q</td>
<td>M</td>
<td>12</td>
<td>50</td>
<td>Cane</td>
<td>degenerative disk disease, diabetes, hypertension</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>Med.</td>
<td>3.97</td>
<td>6.64</td>
<td>42.66</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Ulnar</td>
<td>2.34</td>
<td>4.1</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Tibial</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>20035</td>
<td>P251R</td>
<td>M</td>
<td>2</td>
<td>37</td>
<td>wheelchair</td>
<td>carpal tunnel</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>Med.</td>
<td>2.4</td>
<td>3.78</td>
<td>55</td>
</tr>
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<td>Ulnar</td>
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<td>2</td>
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<td></td>
<td></td>
<td>Tibial</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>20033</td>
<td>M376L</td>
<td>F</td>
<td>26</td>
<td>48</td>
<td>AFO</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>Med.</td>
<td>1.8</td>
<td>3.6</td>
<td>42.4</td>
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<td>Ulnar</td>
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<tr>
<td>20004</td>
<td>R468H</td>
<td>F</td>
<td>39</td>
<td>40</td>
<td>None</td>
<td>hereditary ellipocytosis, PMP22 duplication</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Med.</td>
<td>5.2</td>
<td>8.6</td>
<td>22</td>
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<td>Ulnar</td>
<td>3.5</td>
<td>17.6</td>
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<td>Tibial</td>
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<tr>
<td>20037</td>
<td>p.V705I</td>
<td>F</td>
<td>12</td>
<td>12</td>
<td>None</td>
<td></td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>Med.</td>
<td>N/A</td>
<td>N/A</td>
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<td>Ulnar</td>
<td>N/A</td>
<td>N/A</td>
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<td></td>
<td></td>
<td>Tibial</td>
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<td>N/A</td>
</tr>
<tr>
<td>20013</td>
<td>R707_N709del</td>
<td>M</td>
<td>5</td>
<td>10</td>
<td>AFO prescribed</td>
<td></td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>Med.</td>
<td>6.3</td>
<td>4.3</td>
<td>48.5</td>
</tr>
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<td></td>
<td>Ulnar</td>
<td>9.3</td>
<td>3.1</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Tibial</td>
<td>5</td>
<td>4.3</td>
</tr>
</tbody>
</table>

*Motor strength score: “-“ is normal (5/5), “+“ indicates scores of 4/5, ++ indicated scores of 2/5 to 3/5, and +++ indicates scores of 0/5 to 1/5

‡Sensory score: “+“ indicates sensory loss in lower extremities, “++“ indicates sensory loss in upper and lower extremities, “+++“ indicated upper and lower extremity sensory loss with additional sensory symptoms (parasethesia and neuropathic pain)
Figure 2.1. Pedigrees and corresponding sequence traces of probands (indicated with arrowhead) and families with identified with mutations in MFN2.
CHAPTER 3. INITIAL CELLULAR AND MOLECULAR INVESTIGATIONS OF MFN2.

Introductory Remarks

The pathogenicity of MFN2 mutations in CMT2A was first thought to stem from abnormalities in mitochondrial fusion, morphology and transport previously observed in Mfn2 KO mouse models (Chen and others 2003; Zuchner and Vance 2006). This hypothesis was actively being tested at the outset of this thesis by our laboratory and others. The first section of this chapter discusses the initial investigations completed prior to the report by de Brito et al. in 2008, which described novel roles of MFN2 in the ER (de Brito and Scorrano 2008). After the publication of de Brito’s work, our efforts shifted focus to molecular genetic studies of CMT2A with respect to putative MAM and ER related functions of MFN2. The second section of this chapter, our attempts to replicate and validate Scorrano’s novel findings are presented and discussed.

SECTION 1. TISSUE SPECIFIC EXPRESSION RATIOS OF MFN2 AND MFN1.

Background.

At least part of the perplexity surrounding the molecular genetics of CMT2A stems from the ubiquitous expression of Mfn2 and its paralog Mfn1 (Chen, McCaffery, Chan 2007; Chen and others 2010; Chen and others 2004; Eura and others 2003; Rojo and others 2002; Santel and Fuller 2001; Soriano and others 2006; Zorzano 2009; Zorzano, Liesa, Palacin 2009; Zorzano and others 2010). As the mitofusins are expressed at detectable levels in most cell and tissue types tested thus far, it is unclear why only peripheral axons are affected in CMT2A. Furthermore, it was demonstrated by Detmer et
that CMT2A mutations in \textit{Mfn2} did not limit its ability to tether and fuse with Mfn1, indicating that Mfn1 could form heterooligomeric complexes with mutant Mfn2 and conceivably rescue potential deficits in mitochondrial fusion (Detmer and Chan 2007). Specifically the authors suggest that “… tissues with low Mfn1 expression are vulnerable in CMT2A and that methods to increase Mfn1 expression in the peripheral nervous system would benefit CMT2A patients (Detmer and Chan 2007).” To investigate if the relative levels of MFN2 and MFN1 mRNA could potentially explain cell or tissue specific vulnerability to mutations in MFN2, we sought to establish the ratio of MFN2 to MFN1 in different CNS regions. While peripheral nerve tissue was not available, we believed differences in MFN2 to MFN1 ratios between CNS tissues would clarify how much MFN2/MFN1 expression ratios varied between tissues.

\textbf{Materials and Methods.}

RNA was isolated from multiple CNS regions using the Qiagen RNeasy Mini Kit per manufacturer’s instructions (Qiagen, Valencia, CA). Complimentary DNA (cDNA) was made from purified mRNA using SuperScript III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). The MFN2 (Hs00208382_m1), MFN1 (Hs00966851_m1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH Endogenous Control (FAM / MGB Probe, Non-Primer Limited)) Applied Biosystems International (ABI, Carlsbad, California) TaqMan assay were used to quantify relative levels of MFN1 and MFN2 from cDNA from human post mortem CNS regions. RT-PCR was performed on an ABI 7900 in TaqMan Fast Universal PCR Master Mix per the assay kit instructions. Cycling data
was analyzed using ABI RQ Manager Ver 1.2, and is reported as relative values as compared to whole brain cDNA levels.

**Results and Discussion.**

Examination of MFN2 and MFN1 expression levels indicate that MFN2 is more highly expressed in all CNS tissues examined (Figure 3.1 and Table 3.1). The ratio of MFN2 to MFN1 ranged from the lowest level, a two fold difference in the cerebellum, to the highest ratiometric differences of ~12 in the cerebellar cortex. The average of all other regions examined was ~7 fold difference in MFN2 to MFN1 ratios, MFN2 always being more highly expressed. It is interesting to note that the cerebellar cortex has the highest MFN2 to MFN1 ratio (Figure 3.1). *Mfn2* KO mice rescued from placental defects that would normally result in embryonic lethality go on to develop profound cerebellar defects stemming from specific degeneration of Purkinje cells (Chen, McCaffery, Chan 2007). Perhaps the Purkinje cells of the cerebellar cortex have an increased vulnerability to loss of MFN2 because MFN1 levels are comparatively low. Peripheral nerve cDNA was not available, but the same observation may be true in peripheral nerves as well. However, the lack of a peripheral neuropathy phenotype in viable *Mfn2* KO mouse models suggest more than MFN2 to MFN1 ratios underlie the CMT2A phenotype. In all regions examined MFN2 mRNA exists at relatively higher levels than MFN1, where the magnitude of difference spans from ~2 to ~12 fold. Thus, MFN2 is more highly expressed than MFN1 in all regions but the ratio of MFN2 to MFN1 may have critical importance in determining vulnerability to MFN2 mutations.
Future studies should include peripheral nerve tissue to examine MFN2 to MFN1 ratios in CMT2A relevant cell types.

SECTION 2. CELL-BASED MITOCHONDRIAL MORPHOLOGY STUDIES OF CMT2A MUTATIONS.

Background.

Both *Mfn1* and *Mfn2* KO models clearly demonstrate that loss of either mitofusin results in an extensively fragmented mitochondria network (Chen and others 2003). To better understand how mutations in MFN2 affect mitochondria morphology, we sought to quantify morphological changes in the mitochondrial network in cells expressing both wildtype and CMT2A alleles of MFN2 in cell models.

Materials and Methods.

Mammalian expression plasmid constructs (pcDNA3.1/CT-GFP-TOPO®, Invitrogen Part K4810-01) in which green fluorescent protein (GFP) was fused to the C-termini of wild type and mutant MFN2 alleles were created. Construct sequences were verified by Sanger sequencing. MFN2-GFP fusion constructs were transfected into COS7 cells, fixed in 3.7% formaldehyde 18-24 hours post transfection, and mitochondria visualized with primary rabbit anti-TOMM20 antibodies and secondary goat anti-rabbit conjugated to Cy3. Nuclei were stained with DAPI during coverslip mounting. Stained cells were visualized using a Zeiss 710 confocal microscope. The resulting mitochondria morphologies were quantified by two blinded microscopists, based on four cellular
phenotype categories (Figure 3.2A). Percentages of cells with mitochondria in each morphological class from separate biologic triplicate measurements of N\geq30 cells were averaged.

**Results and Discussion.**

Transfection of MFN2-GFP constructs into COS7 cells caused an overwhelming collapse of the mitochondrial network even when wild type *MFN2* is exogenously expressed (Figure 3.2A- panel “cluster”). The expression level of MFN2 driven by a cytomegalovirus (CMV) promoter in this experimental approach far exceeds endogenous levels and results in an abnormal perinuclear mitochondrial clustering phenotype (Figure 3.2A- panel “cluster”). No consistent change in the resulting mitochondrial morphology exists apart from one R418X mutant, which lacks a transmembrane domain and fails to localize to the mitochondria (Figure 3.2B). Thus mutations in *MFN2* do not appear to affect its ability to induce the perinuclear aggregation of mitochondria, which presumably stems from MFN2’s role tethering mitochondria. In the case of the R418X mutation resulting in a truncation, lack of appropriate mitochondrial membrane targeting eliminates MFN2’s ability to induce aggregation of mitochondria. However, these results offer no quantifiable differences between known CMT2A causing mutations in *MFN2* that are single amino acid changes (Figure 3.2B). This experimental approach is limited by the mitochondrial collapse caused by over-expression of MFN2 and our inability to distinguish tether of mitochondria from fusion. Thus this and other planned experimental approaches employing overexpression of MFN2 (not discussed) were abandoned.
SECTION 3. REPLICATION OF FINDINGS SUPPORTING MAM-MITOCHONDRIA TETHERING BY MFN2.

Background.

The original observation which led de Brito et al. to examine MFN2’s role in ER-mitochondria tethering was a fragmented ER network in Mfn2 KO MEFs (de Brito and Scorrano 2008). Using an ER targeting YFP over-expression fusion construct, it was reported that ER morphology was fragmented in ~75% of Mfn2 KO MEFs. De Brito et al. also described reduced juxtaposition of ER-mitochondria and reported 40% reduction in ER-mitochondria colocalization in Mfn2 KO (de Brito and Scorrano 2008). This observation was specifically shown to be true in Mfn2 KO MEFs but not in Mfn1 KO MEFs. The specificity of the effect bolstered the hypothesis that ER-mitochondria contacts require tethering mediated by Mfn2. To investigate the specific role of Mfn2 in tethering MAM to mitochondria, we repeated ER-mitochondrial contact analysis on wildtype and Mfn2 and Mfn1 KO MEFs.

The existence of MAMs was first described as a specialized membrane fraction separated from associated mitochondria by Percoll gradients. In essence, the MAM is “pulled down” in a crude mitochondrial pellet, and upon separation from mitochondria using a Percoll gradient, retains a density and protein composition that is consistent with its ER origins. A critical experiment in testing the hypothesis that MFN2 tethers mitochondria to the ER is demonstrating that MFN2 is located in the MAM fraction. de Brito et al. report a 14 fold enrichment of Mfn2 in the MAM fraction. This fact comes as a surprise, since in previous immunohistochemistry and confocal analysis, endogenous Mfn2 and overexpressed MFN2-GFP constructs, both localize exclusively to the mitochondria (unpublished data). As this observation had not been reported in the 8 years
in which Mfn2 KO MEFs had been studied, we sought to replicate this finding in the same Mfn2 KO MEF cells received as a gift from D.C. Chan.

Materials and Methods.

ER Morphology Analysis.

To validate this finding, we used a similar commercially available ER targeting construct which expresses a dsRED fused to the ER targeting sequence of calreticulin at the 5’ end and an ER retention sequence (KDEL) sequence at the 3’ end (pDsRed2-ER Vector, Clontech, Part #632409). Images were captured in confocal planes where nuclear circumference was maximal with a 63X objective on a Zeiss 710 confocal microscope. ER morphology was quantified using Image Tool 3.0 (http://ddsdx.uthscsa.edu/dig/itdesc.html), where cells were scored as having a reticular ER when major axis was >5 \( \mu \text{m} \) and elongation index (major axis/minor axis) was >4 for more than 50% of the identified objects per cell as described by de Brito et al. (de Brito and Scorrano 2008). As a secondary method of ER visualization, the ER of wildtype and Mfn2 KO MEFs was labeled with ER-tracker Red (ER-Tracker™Red (BODIPY® TR glibenclamide) Invitrogen, Part # E34250) at 500nM for 10 minutes prior to live cell imaging. Connectivity analysis was performed on the identical images. Auto-thresholded images were converted to binary images and skeletonized using ImageJ. Connectivity scores were produced using the binary connectivity plugin (http://www.dentistry.bham.ac.uk/landinig/software/software.html) on skeletonized images. The connectivity score represents how may pixels are connected to a center pixel.
in a 3x3 array, so that a score of 0 represents an isolated pixel, 1 represent the end of a line, 2 represent a continuous line, 3 represents the intersection of 3 lines, *et cetera.*

**ER-mitochondrial contact analysis.**

Wildtype, *Mfn2* and *Mfn1* KO MEFs grown on untreated glass coverslips were transfected with ER-dsRed (pDsRed2-ER Vector, Clontech, Part #632409) and mito-GFP (pAcGFP1-mito vector, Clontech, Part #632432) constructs. Eighteen to 24 hrs post-transfection, cells were fixed with 3.7% formaldehyde and counter-stained with the nuclear stain DAPI. Z-stack images were captured using a 63X objective on a Zeiss 710 confocal microscope. ER-mitochondrial contacts were defined as areas where dsRed and GFP signals colocalize in reconstructed z-projection stacks of images as previously described. The ImageJ colocalization plugin, Just Another Colocalization Plugin (JACoP) was used to calculate Mander’s coefficients using Costes’ approach for thresholding and generation of p values based on randomized image pixel arrangements, as well for calculation of Pearson’s and general overlap coefficients as previously described (Bolte and Cordelieres 2006).

**Subcellular fractionation.**

To confirm Mfn2 localizes to the MAM fraction, we attempted to isolate MAM fractions and analyze the purified fraction using western blots for MFN2 and organelle markers. While this MAM isolation protocol was first described using mouse liver, we intended to perform the same analysis on MEF cells, in hopes to examine how loss of *Mfn2* might alter the MAM fractions. While Scorrano reports using $10^9$ MEF cells as starting material for the MAM isolation, which equates to ~150 10cm plates, a separate group led by Coldberg-Poley at George Washington University published a protocol for
the isolation of using cultured cells using $\sim 10^8$ cells (Bozidis, Williamson, Colberg-Poley 2007; de Brito and Scorrano 2008). The following protocol is adapted and derived largely from Bozidis et al. among other previously published MAM isolation protocols (Bozidis, Williamson, Colberg-Poley 2007; de Brito and Scorrano 2008; Vance 1990; Wieckowski and others 2009). Briefly, $\sim 75\text{-}80\%$ confluent MEF cells were washed twice with PBS, scraped of culture plates, pelleted by centrifugation at 600xg for 5 minutes, and resuspended and washed in PBS before repelleting at 600xg for 5 minutes. The cells were then homogenized in with 10 strokes with a motorized Teflon pestle and glass mortar at 500 rpm. Where indicated, the gall bladder was dissected away from liver tissue harvested from adult male BALB/c mice sacrificed by cerival dislocation. Mouse liver was washed twice in PBS, and livers where homogenized in a 5mL Teflon pestle and glass mortar at 1500 rpm. Both cell based and liver homogenates were spun twice at 600xg to remove debris and nuclei. The supernatant was spun at 10,300xg at 4C for 10 minutes, and repeated twice or until a pellet was no longer visible. The ER and cytosol fractions were collected by spinning the resultant supernatant for 1 hour at 100,000xg in a swinging bucket rotor (SW-40). The resulting pellet yielded ER or light membrane fractions and a small ($\sim 500uL$) the supernatant was retained as the cytosol fraction. The crude mitochondrial pellet was resuspended in 0.5mL of sucrose homogenation buffer, and gently loaded on top of 10mL of 30% Percoll in a 14x89mm ultraclear tube before ultracentrifugation at 95,000xg for 65 minutes. The MAM fraction was located above the multibanded mitochondria as a distinct white band. Both the MAM and the mitochondria where removed by puncturing the tube sidewall with a 3mL 20G syringe. The MAM fraction was diluted in 10mL mannitol buffer and repelleted by centrifugation at
100,000xg for 1 hour. The top mitochondrial bands (~3mL) were collected and diluted two fold in mannitol buffer and were pelleted by centrifugation at 6300xg for 10 minutes at 4C. The protein concentration of all collected fractions was quantified using a micro-BCA protein assay kit (Thermo scientific, Rockford IL, #23235). Equal amounts of protein (10-20ug) were used to normalize loading of each fraction on a NuPAGE 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, #NP0323BOX). After separation by electrophoresis, protein was transferred to nitrocellulose membranes using a semi-dry Owl-HEPE-I transblotting system (Thermo scientific, Rockford IL). Blocking, washing and primary and secondary antibody steps were performed using the SNAP ID western blotting system (Millipore, Billerica, MA).

Results and Discussion.

Upon casual visual inspection, the ER of Mfn2 KO MEFs was indistinguishable from the wildtype ER morphology (Figure 3.3A). We found that neither Mfn2 KO nor wildtype MEFs met “reticular” criteria as reported by de Brito et al. (de Brito and Scorrano 2008) (Figure 3.4A). Both wildtype and Mfn2 KO cells have similarly robust signal from ER-dsRed over expression. The wide dynamic range of dsRED labeling between the central and peripheral ER made morphometric object analysis difficult. The extensively labeled central or perinuclear ER limits effective image analysis, as details of the peripheral ER segments are lost when the central ER is appropriately thresholded. When automatic thresholding is used, as reported in Scorrano’s methods, the central ER becomes a single large object, while much of the peripheral detail is lost. Repeating the analysis with different global and local thresholding parameters did not alter morphological measures. When the image analysis is performed as described in the de
Brito’s original paper, only a very small fraction (<<50%) of the ER object identified are classified as reticular in both wildtype and MFN2 KO (Figure 3.4). It is unclear why our observations differ so greatly from the images and quantifications previously published. While our construct contains a dsRED fusion protein, the construct used by de Brito contained YFP based fusion protein, yet both are produced and available from Clontech Inc. and have identical ER targeting sequences. To ensure that overexpression of ER-dsRED was not limiting our ability to effectively quantify the ER network, the same quantification scheme was used on cells stained with ER-tracker Red dye. Again, no difference in ER morphology was observed either visually or when quantified as described above (Figure 3.3B and Figure 3.4B). The fact that even wildtype ER morphologies did not meet normal morphometric parameters for “reticular” morphology indicated this is not an effective methodology for quantifying this organelle’s morphology (Figure 3.4B).

We adopted another morphometric approach to quantify the connectivity of ER morphology based on methods previously described for mitochondria. Connectivity analysis scores how many pixels in a skeletonized image are directly juxtaposed or connected, and on this physical basis the connectivity is inversely related to fragmentation (Figure 3.5 inset) (Pon and Schon 2007). Applying connectivity analysis to cells labeled with ER-Tracker red dye reveals no significant differences in connectivity and branching between Mfn2 KO and wildtype MEF cells (Figure 3.5). We do not, in contrast to de Brito et al’s report, observe a fragmented ER morphology in the absence of Mfn2.
Analysis of z-projection images revealed reductions in ER-mitochondrial colocalization in $Mfn2$ KO MEFs similar to those previously described (Table 3.2 and Figure 3.5) (de Brito and Scorrano 2008). Specifically, the mitochondrial (mito-GFP) Mander's coefficient, which represents the percent of mito-GFP that colocalized with ER-dsRED, quantified a ~40% decrease in the mitochondria’s overlap with the ER (Figure 3.5). This decrease is identical to Mander’s coefficient values previously (de Brito and Scorrano 2008). The corresponding Mander’s coefficient representing the proportion of ER-dsRED signal colocalizing with mito-GFP did not significantly change though showed a decreased trend. It is difficult to interpret exactly why the mito-GFP Mander's coefficient would change independent of the ER-dsRED Mander’s coefficient. On one hand, the ER is a much larger organelle so that changes in the small proportion of the ER overlapping with the mitochondria are insignificant when compared to the summed ER signal. Alternatively, since changes in ER morphology are not observed the change in the mitochondrial Mander’s coefficient may reflect the fragmented mitochondrial network without changes in ER morphology. Other more general measures of colocalization, such as Pearson’s and Overlap coefficients demonstrate a ~32% decrease in ER-mitochondria contacts. While these observations are consistent with values previously reported for $Mfn2$ KO MEFs, significant reductions are also observed in $Mfn1$ KO MEFs in Pearson’s, Overlap and the Mander’s coefficients. This comes as a surprise and conflicts with the previous report (de Brito and Scorrano 2008). Furthermore, it weakens the argument that Mfn2 has specific ER-mitochondria tethering roles. The reduction in ER-mitochondria contacts and overlap may stem from other more general aspects of mitochondrial fragmentation. Specifically, in both $Mfn2$ and $Mfn1$ KO MEFs,
mitochondria were shown to have greatly reduced mobility. This lack of mobility may limit the ability of mitochondria to be brought into contact with ER membranes. Likewise, a number of more general mechanisms relating to mitochondrial shape in \textit{Mfn2} and \textit{Mfn1} KO MEFs could potentially explain the reduction in ER-mitochondrial contacts. Alternatively, there may be unidentified roles for Mfn1 in establishing ER-mitochondrial tethering. Regardless, these results indicate no role specific for Mfn2 in mediating ER-mitochondria contacts that is not shared by Mfn1.

While the originally the MAM was described using mouse liver as a starting material, a cell based protocol was published by Coldperg-Poley \textit{et al.} (Bozidis, Williamson, Colberg-Poley 2007). The Coldperg-Poley method required less cells but a more extensive crude mitochondria pelleting step. Additionally, this protocol describes a quantal or discontinuous mitochondrial banding pattern generated in the Percoll gradient for which the physical basis of is unknown. We contacted the authors of this protocol and received considerable assistance in setting up the protocol. The mitochondria banding pattern observed in wildtype MEFs was drastically altered in \textit{Mfn2} and \textit{Mfn1} KO cells, where the discrete banding pattern was interrupted by the presence of tubular fragments which did not adhere to horizontal planes of density along the gradient (Figure 3.7). Additionally, unlike wildtype cells, there was no clear spatial separation between the light MAM and denser mitochondrial fractions (Figure 3.7). This observation is consistent with a disrupted MAM subcompartment in \textit{Mfn2} KO MEFS. A more vertically compressed mitochondrial banding pattern is observed in \textit{Mfn1} KO MEFs than in wildtypes. The wildtype band spacing appears intact in \textit{Mfn2} KO MEFs, yet appears much fainter. Likewise, the presence of tubular speckles that defy the vertical density
gradient are larger and more apparent in Mfn2 KO MEFs, but also present in small numbers in sizes in Mfn1 KO MEFS. While Mfn1 KO MEFs produce altered mitochondria banding, a clear MAM fraction band is apparent with distinct spatial separation from mitochondria bands similar to wildtype cells. Additional steps were taken to ensure that the difference in banding patterns were not simply due to a potentially lower mitochondrial mass in Mfn2 KO MEFs. Since these initial experiments were performed with the starting cell masses controlled, we performed a second experiment in which the mass of the crude mitochondrial pellet, which is loaded on the Percoll gradient, was controlled. Again, when crude mitochondrial pellet mass was controlled for, Mfn2 KO MEFs produce abnormal mitochondrial banding patterns as compared to wildtype MEFs.

To investigate the presence of Mfn2 in the MAM fraction, ER, cytosolic, MAM and mitochondria fractions were collected and separated by gel electrophoresis and analyzed by western blotting. While Mfn2 was present at the highest levels in the mitochondrial fraction, Mfn2 was also present in the MAM fraction at low levels in both wildtype and Mfn1 KO MEFs. In Mfn1 KO MEFs, Mfn2 also appears at appreciably levels in the ER fraction, suggesting Mfn2 is properly localized to the ER, but may not be associating with mitochondria in the MAM (Figure 3.9). Triplicate measurements indicate Mfn2 is present at levels ~20% (21.7±13.7% N=3) of those in mitochondria. This contrasts to Scorrano’s work, which indicated a 14 fold enrichment of Mfn2 in the MAM fraction. Several other important observations must be considered. First, the positive marker for the MAM fraction, the long-chain acyl-CoA synthetase (FACL4), was not detectable in any subcellular fraction in any cell lines tested (data not shown).
This could be due to lack of expression of this protein in these cell lines. Alternatively, FACL4 may be lowly expressed, so that the amount of cellular material analyzed was insufficient for FACL4 detection. Likewise, confirmation of the mitochondrial fraction with the outer mitochondrial membrane protein VDAC1 and the inner mitochondrial protein COXIV suggested another potentially confounding phenomena. While the COXIV was present in the mitochondrial fraction, VDAC1 appears in both the MAM and mitochondria fractions. This suggests that there may be mitochondrial outer membrane contamination in the MAM fraction. The outer mitochondria membrane could have been detached and separated with the MAM fraction in the Percoll gradient leaving the denser inner mitochondrial membrane to migrate at high densities. Only a partial separation of OMM from the mitochondria could explain this observation, since both Mfn2 and VDAC1 are also present at higher relative levels in the mitochondrial fraction. Alternatively, the presence of OMM proteins in the MAM fraction may represent a confluence or continuousness between the MAM membranes and the OMM, such that certain OMM proteins are enriched at sites which maybe continuous with MAM membranes. While these cell based results indicate that Mfn2 along with other OMM proteins may be present in the MAM fraction, the lack of positive MAM fraction identity limits interpretation of these data.

Increasing the size of the cell based MAM/mitochondrial isolation protocol described above requires industrial scale cell culture equipment for a single experiment (~150 10cm plates per cell line per replicate or a total of ~1,350 10cm plates for triplicate experiments). To overcome potential yield problems in positively isolating MAM fractions from cultured cells, mouse liver was used to further investigate the presence of
Mfn2 in the MAM. Mouse liver tissue was originally used to identify and describe the MAM subcompartment, and FACL4 expression in this tissue had been previously confirmed. Indeed, mouse liver from wildtype male BALB/c mice confirmed the presence of Mfn2 in a FACL4 positive MAM fraction. Similar to the cell based studies, the OMM protein TOMM20 was also present in the MAM fraction, though the IMM protein COXIV was not. This result could be interpreted as OMM contamination in the MAM fraction, yet de Brito et al. and others have published the same finding without comment. Interestingly, Mfn1 is excluded from the MAM fraction while other OMM proteins (TOMM20 and Mfn2) are present. The differential occurrence of OMM proteins in the MAM suggests that it is more than simple contamination from the OMM, but potentially portions of the OMM enriched in specific OMM proteins. However, it is not possible to determine whether these proteins are enriched in an OMM fraction that separates and contaminates the MAM fraction or if these proteins are localizing in a pure MAM fractions.

Conclusion.

These initial exploratory studies yielded novel information regarding the study of MFN2. First, it appears that MFN2 is more highly expressed than MFN1 in all human post mortem CNS tissues tested, with the cerebellar cortex having the highest MFN2 to MFN1 mRNA expression ratio. This finding suggests that while MFN2 and MFN1 may have overlapping functions in mitochondrial fusion, they are differentially regulated in different tissues. With respect to CMT2A, while peripheral nerve tissue was not available for analysis, it is clear that different CNS tissue have different ratios of MFN2 to MFN1 expression. It is interesting that Mfn2 null mice rescued embryonically go on to develop
profound Purkinje cell degeneration, since our MFN2/MFN1 mRNA ratio results would predict the cerebellar cortex to have highest dependence on MFN2 expression. It is still unclear why the rescued Mfn2 null mice do not develop CMT2A like phenotypes.

The cellular models of Mfn2 alleles suggest that CMT2A mutations in MFN2 do not inhibit its ability induce perinuclear mitochondrial clustering when overexpressed. The one exception if the R418X truncating mutation, which prevent Mfn2 from localizing in the OMM. Since mitochondrial morphology is a balance between fusion and fission events, the overexpression of wildtype or CMT2A alleles of MFN2 similarly disrupt normal tubular and reticular mitochondria morphology. The resulting perinuclear clustering of mitochondria limits interpretation of how mutations in MFN2 might alter its function. A more sophisticated means of experimentally controlled expression of mutant forms of MFN2 would possible provide a more accurate CMT2A disease model.

We attempted replicating three critical results reported by de Brito et al. concerning a role for Mfn2 in mediating MAM-mitochondria contacts. Loss of Mfn2 does not alter ER morphology when quantified as previously described or using alternate approaches. While ER-mitochondria contacts are reduced in Mfn2 KO MEFs, similar reductions in colocalization are observed in Mfn1 KO MEFs. This finding suggest that the reduction in ER-mitochondria overlap may be due to more generally to a fragmented and static mitochondrial network and not specific ER tethering functions of Mfn2. Isolation of the MAM fraction in cell lines lacking Mfn2 and Mfn1 suggest that the MAM subcompartment in Mfn2 KO MEFs is non-existent or has physical properties that prevent its isolation and separation from mitochondria. In both Mfn2 and Mfn1 KO MEFs, mitochondria also appear to have irregular densities that alter how they appear
when separated on continuous density gradient. The lack of a discernable MAM membrane in Mfn2 KO MEFs is consistent with a role for Mfn2 in MAM formation. Likewise, Mfn2 is present at low levels in the MAM fraction, at a fraction of mitochondrial levels which conflicts with the original observation of a 14 fold enrichment. In addition to Mfn2, TOMM20 but not Mfn1 was present in the MAM fraction, suggesting that either specific OMM proteins may be enriched in the MAM fraction, or that segments of the OMM enriched in specific proteins contaminant the MAM fractions. Cumulatively, failure to replicate many of the original observations while finding that loss of Mfn1 may also reduce ER-mitochondria overlaps conflict with the Mfn2 tethering model proposed by de Brito. However, these results do indicate a role for Mfn2, albeit unclear, in defining the MAM subcompartment.
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<th>ΔΔ Ct stdev</th>
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Table 3.2- Measures of ER-mitochondria colocalization in wildtype, *Mfn2* and *Mfn1* KO MEFs.

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<td>0.755 ±0.027</td>
<td>0.335 ±0.063</td>
<td>0.544 ±0.016</td>
</tr>
<tr>
<td><em>Mfn2 KO</em></td>
<td>0.482 ±0.052**</td>
<td>0.515 ±0.072**</td>
<td>0.250 ±0.115</td>
<td>0.329 ±0.012***</td>
</tr>
<tr>
<td><em>Mfn1 KO</em></td>
<td>0.597 ±0.163*</td>
<td>0.635 ±0.170**</td>
<td>0.293 ±0.060</td>
<td>0.436 ±0.152***</td>
</tr>
</tbody>
</table>
Figure 3.1. RT-PCR relative expression values of MFN2 in CNS regions. RT-PCR reveals relative expression values of MFN2 that are on average ~7 fold higher than MFN1 over CNS regions examined. Expression levels of each region are reported using GAPDH as an endogenous control and whole adult brain as the relative index.
Figure 3.2. Initial morphology analysis in MFN2 overexpressing cell lines. (A) COS7 cells overexpressing wildtype or mutant alleles of MFN2 fused to GFP were fixed and mitochondria were visualized by staining with rabbit anti-mouse TOMM20 antibodies and visualized with Cy3 goat anti rabbit secondary antibodies. The resulting mitochondria morphologies were divided into the four phenotypes, “Cluster”, “punctate cluster”, “punctate”, and “tubular”. (B) While 100% of untransfected cells have mitochondria classified as tubular, overexpression of wildtype or all single point mutations in MFN2 resulted in an overwhelming collapse of the mitochondrial network around the nucleus. The only apparent difference was observed in the R418X mutation, which causes a truncation and eliminates the mitochondrial transmembrane domain of MFN2. Thus, CMT2A point mutations in MFN2 do not appear to limit its ability to form perinuclear aggregates when overexpressed.
Figure 3.3 – ER morphology in wildtype and MFN2 KO MEFs. (A) Confocal images of live wildtype and Mfn2 KO MEFs expressing an ER targeting dsRED fusion protein demonstrate no obvious difference in ER morphology. (B) Confocal images of live wildtype and Mfn2 KO MEFs stained with ER-tracker red dye also demonstrate no obvious difference in ER morphology. The insets below reveal a robust reticular peripheral ER network in both cell lines.
Figure 3.4. Object analysis of ER in wildtype and MFN2 KO MEFS. Object analysis of both MFN2 (n=10) and wt MEF (n=10) cells transfected with ER-dsRed (A) or stained with ER-tracker (B) demonstrate no significant differences in terms of elongation or major axis length. Points represent an individual segment of the ER from 10 separate cells. De Brito and Scarrano used the cut of values of an elongation value of greater than 4 (horizontal line) and a major axis length greater than 5μm (vertical line). Very (10-20) few of the indentified ER objects meet “reticular” criteria (shaded areas) from all 20 cells using the ER-dsRed plasmid. While still no cells were scored “reticular” using ER-tracker, the staining method allowed increased measures of elongation and major axis length.
Figure 3.5. Connectivity analysis of wildtype and Mfn2 KO MEFs reveals no differences in ER network connectivity. Thresholded images of cells stained with ER-tracker were skeletonized to quantify the degree of connectivity (top inset). The 3D bar graph shows the individual data for 10 wildtype and 10 Mfn2 KO cells, and reveals low cell to cell variability. The graph on the bottom right demonstrates that there are no significant differences between the connectivity of wildtype and Mfn2 KO cells.
Figure 3.6. Measures of colocalization reveal a decrease in the colocalization of ER and mitochondria in Mfn2 and Mfn1 KO MEFs. Values are reported as percentage of wildtype values over biological triplicates. Similar to previous reports, the second Mander’s coefficient, which represents the fraction of mitochondria that colocalizing with the ER is significantly decreased in both Mfn2 and Mfn1 KO MEFs. Other common measures of colocalization also reveal decreases in Mfn2 and Mfn1 KO MEFs.
Figure 3.7. Ultracentrifugation of crude mitochondrial pellets over a self-generating Percoll gradient. Separation of MAM from mitochondria and produce a mitochondrial banding pattern in wildtype MEFs that appears disrupted in Mfn2 and Mfn1 KO MEFs.
Figure 3.8- Ultracentrifugation of equal masses of crude mitochondrial pellets. Separation of equal masses of crude mitochondrial pellets over 30% Percoll gradients confirms the altered mitochondria banding pattern and irregular tubular speckles is not due to different crude pellet masses.
Figure 3.9. Western blots of total lysates (TL), ER, MAM and mitochondria (mt) indicate that Mfn2 is present in the MAM and mitochondria fractions from wildtype and Mfn1 KO MEFs. Overexposure of blots probed for Mfn2 reveals Mfn2 is present in the MAM fraction at ~10-20% of levels observed in the mitochondrial fraction. To ensure Mfn2 signal in the MAM fraction is not contamination from mitochondria, the same blot was probed with the VDAC1 and COXIV. While the MAM fraction does not include the inner mitochondrial membrane protein COXIV, the presence of the outer mitochondrial membrane (OMM) VDAC1 suggest that OMM proteins, including Mfn2 may be just contamination from the OMM.
Figure 3.10. Western blots of Cytosol (cyto), ER, MAM and mitochondrial fractions from mouse liver homogenates. Organelle fractions were separated by gel electrophoresis and analyzed by western blotting to confirm the presence of Mfn2 in the MAM fraction. Mfn2 is present in the MAM fraction at 21.7% of levels observed in mitochondria. Both the OMM protein Mfn1 and IMM protein COXIV are not present in the MAM fraction, suggesting that Mfn2 presence is not the result of general contamination from the OMM. TOMM20, an OMM protein is present in the MAM, at levels similar to those in the mitochondrial fraction. The MAM marker FACL4 and the ER marker Calnexin confirm the identity of the ER and MAM fractions.
CHAPTER 4. LOSS OF MITOFUSINS DISRUPTS CELLULAR PHOSPHATIDYLETHANOLAMINE

Summary.

The outer mitochondrial membrane proteins Mfn1 and Mfn2 mediate mitochondrial fusion by tethering outer mitochondrial membranes. It was reported that Mfn2 also tethers mitochondria to the endoplasmic reticulum (ER) at a subdomain of the ER referred to as the mitochondrial associated membrane (MAM). MAM-mitochondria interfaces facilitate the transport of phosphatidylserine (PS) from the MAM to mitochondria where it is decarboxylated to form phosphatidylethanolamine (PE). Thus, Mfn2 is in a unique position to regulate mitochondrial synthesis of PE by controlling the movement of PS into mitochondrial membranes. We hypothesized that absence of Mfn2 would lead to decreased mitochondrial synthesis of PE. Since PE is a fusogenic and curvature inducing phospholipid, a mitochondrial deficit in PE would be an attractive mechanistic explanation for the mitochondrial fusion and morphological abnormalities observed in Mfn2 null models. To investigate putative phospholipid related functions of the mitofusins, we employed a mass spectrometry approach to quantify and profile cellular phospholipids in the absence of Mfn1 and Mfn2. Our data indicates that loss of either Mfn1 or Mfn2 is associated with a decrease in mitochondrial synthesis of PE by MAM-mitochondrial dependent pathways. Suppression of the major redundant cellular PE synthesis pathway led to specific decreases of PE in Mfn2 and Mfn1 null cells but not in wildtype cells. Loss of either mitofusin also altered the acyl chain composition of PE and phosphatidylcholine (PC) populations. These results demonstrate the importance of
both *mitofusins* in MAM-mitochondria dependent phospholipid synthesis pathways and suggest a novel functional role for mitofusins in generating specific cellular subpopulations of PE and PC.

**Background.**

Mitofusin 1 and 2 (Mfn1 and Mfn2) reside in the outer mitochondrial membrane and have been shown to regulate mitochondrial network architecture by mediating tethering and fusion of mitochondria (Bach and others 2003; Chen and others 2003; Rojo and others 2002). Mitochondria normally form a tubular and branched reticular network dynamically regulated by a balance of fusion and fission events (Santel and Fuller 2001). Absence of either *Mfn1* or *Mfn2* results in a fragmented mitochondrial network (Chen and others 2003). Zuchner et al. previously described mutations in the gene *mitofusin 2* (*MFN2*) as the cause of the major autosomal-dominant, axonal form of Charcot-Marie-Tooth neuropathy (CMT2A) (Zuchner and others 2004). CMT type 2 (CMT2) is characterized by chronic axonal degeneration of peripheral nerves leading to the loss of functional nerve fibers. The finding that mutations in *MFN2* cause CMT2A led to investigations focused on deficiencies of mitochondrial fusion and transport, specifically in the context of long axonal processes affected in CMT (Baloh and others 2007; Misko and others 2010). While some experimental work supports disrupted mitochondrial transport in the etiology of CMT2A, other studies on CMT2A patient fibroblasts and cell models suggest abnormal mitochondrial fusion and dynamics do not underlie the etiology of this disease (Amiott and others 2008b; Baloh and others 2007; Detmer and Chan 2007; Misko and others 2010).
De Brito and Scorrano reported a novel role for Mfn2 in tethering the endoplasmic reticulum (ER) to mitochondria (de Brito and Scorrano 2008). In Mfn2 null mouse embryonic fibroblasts (MEFs) regions of contact between mitochondria and the endoplasmic reticulum (ER) are significantly reduced (de Brito and Scorrano 2008). These regions of contact are thought to form specialized subdomains of the ER, called mitochondrial associated membranes (MAM) (Vance 1990). Mfn2 was enriched 14-fold in purified MAM fractions as compared to mitochondrial fractions (de Brito and Scorrano 2008). Besides observing a fragmented ER network in Mfn2 knockout (KO) mouse embryonic (MEF) cells de Brito and Scorrano presented several lines of evidence which suggest that the underlying pathogenic mechanism in CMT2A stems from disrupted ER-mitochondria tethering (de Brito and Scorrano 2008).

The MAM represents a sub-domain of the ER in close association with the mitochondrial outer membrane (Achleitner and others 1999; Schumacher, Choi, Voelker 2002; Shiao, Lupo, Vance 1995; Shiao, Lupo, Vance 1995; Shiao, Balcerzak, Vance 1998; Vance 1990; Voelker 1989). Thorough investigations have demonstrated that the spatial proximity of MAM-mitochondria contacts is essential for effective Ca$^{2+}$ exchange between these two organelles (Hayashi and others 2009; Jouaville and others 1999; Rizzuto and others 1993; Rizzuto and others 1998; Rizzuto, Duchen, Pozzan 2004; Szabadkai and others 2006). Initial studies of MAM-mitochondria interfaces focused on the localization of lipid precursors, end products, and synthesis enzymes within these two organelles (Vance 1990; Voelker 1989; Voelker 1991). The movement of phosphatidylserine (PS) from the ER to mitochondria and its subsequent decarboxylation to PE by the enzyme PS decarboxylase (Pisd) has been well characterized and is known
to depend on the existence of an outer mitochondrial membrane protein (Figure 4.1) (Achleitner and others 1999; Choi, Wu, Voelker 2005; Schumacher, Choi, Voelker 2002; Shiao and Vance 1995; Shiao, Lupo, Vance 1995; Shiao, Balcerzak, Vance 1998; Vance and Steenbergen 2005; Voelker 1989; Voelker 1991). Intriguingly, Pisd KO MEFs display fragmented and round mitochondria of irregular diameter conspicuously reminiscent of Mfn2 KO MEFs (Steenbergen and others 2005). The morphologic similarities between Mfn2 and Pisd KO MEFs suggest that these gene products may function in the same mitochondrial PE synthesis pathway (Figure 4.1). As PE has curvature inducing and fusogenic biophysical characteristics, a deficiency in PE would be an attractive mechanism contributing to the morphological and fusion defects observed in these experimental models (Churchward and others 2008; Seddon 1990; Siegel and Kozlov 2004).

In addition to PE synthesis via the PS decarboxylation pathway, cells retain another major PE synthesis pathway, known as the Kennedy or CDP-ethanolamine pathway (Kennedy 1956). While the spingosine-1-phosphate (S1P) lyase pathway can generate ethanolamine from sphingosine precursors, the CDP-ethanolamine pathway relies on ethanolamine derived from the diet (Houweling and others 1992; Shiao and Vance 1995; Van Veldhoven and Mannaerts 1991). In the case of cultured cells, free ethanolamine for use in the CDP-ethanolamine pathway can be supplemented in culture media (Shiao and Vance 1995; Voelker and Frazier 1986). The CDP-ethanolamine pathway resides in the ER separated spatially from the MAM-mitochondrial based PS decarboxylation pathway (Figure 4.1). The CDP-ethanolamine and PS decarboxylation pathways produce the large majority of cellular PE, but the contribution of each pathway
differs depending on cell or tissue type as well as the availability of free ethanolamine (Bleijerveld and others 2007; Houweling and others 1992; Shimada, Morita, Sugiyama 2003; Steenbergen and others 2005).

Here we report the how the absence of Mfn1 or Mfn2 alters phospholipid profiles of whole cell and mitochondrial enriched fractions. While it has been suggested that Mfn2 is a critical component in the movement of PS from the ER to mitochondria, no reported studies have investigated how loss of Mfn2 affects cellular or mitochondrial phospholipid levels (Hailey and others 2010). We employed a mass spectrometry based phospholipidomic approach to test the hypothesis that loss of Mfn2 ablates tethering of MAM to mitochondria and results in deficiencies of the fusogenic phospholipid PE (Figure 4.1). Our results indicate an important role for both mitofusins in the mitochondrial generation of PE specifically when cells are deprived of ethanolamine. Furthermore, we report how interruption of MAM-mitochondria associations resulting from loss of either Mfn2 or Mfn1 alters the fatty acyl chain composition of PE and PC.

**Materials and Methods.**

**Cell culture.**

The Mfn2 null (Mfn2 KO), Mfn1 null (Mfn1 KO), and wildtype MEF cells lines were a kind gift from Dr. David Chan (California Institute of Technology, CA). MEF cells were grown in 10% FBS Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA). Where indicated, a 20mM ethanolamine (Sigma, E0135) stock solution was added to media to a final concentration of 20μM or 200μM for a period of 10 days prior to lipid
or imaging analysis. To reliably induce autophagy, normal media from 75-80% confluent MEFs was replaced for 2 hours with Hanks Buffered Saline Solution as previously described for these specific MEF cells lines (Invitrogen, Carlsbad, CA)(Hailey and others 2010). To obtain mitochondria enriched fractions, a bench-top mitochondrial isolation kit was used as directed by the manufacturer (Mitosciences Eugene, Oregon).

**Lipid extraction.**

Phospholipids were extracted from whole cells and organelle fractions from MEF cells using a modified Bligh-Dyer procedure described elsewhere(Hanahan 1997). Briefly, phospholipids from cells or mitochondria are extracted using a chloroform: methanol: cell or organelle (1:2:0.8 vol/vol/vol) solvent system. Butylated hydroxytoluene (B1378, Sigma, St Louis, MO) was added to all chloroform solutions (0.01%) to protect from oxidation. The solvent mixture is mixed and allowed sit for 30 minutes prior to removal of the lower organic layer and three washes with one volume of chloroform. The combined lower layers were again washed with one volume of 1M KCl, and with 1 volume water. The extract containing the phospholipid extract was dried down under a stream of nitrogen and stored at -80°C under nitrogen prior to analysis.

**Thin Layer Chromatography.**

Dried-down phospholipid extracts were resuspended in 0.01% BHT chloroform solution based on extract mass to a final concentration of 5ug/uL. Equal masses of each extract were loaded onto activated HP-TLC plates (Merck, Darmstadt, Germany) along with phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and cardiolipin
standards. For TLC, the primary running solvent was chloroform:methanol: 25% ammonia (50:50:3 vol/vol/vol). Visualization of separated phospholipids was achieved using a modified Dittmers sprayed reagent described elsewhere (Ryu and MacCoss 1979). Spot were analyzed using migration length and densitometry application on Image J.

**Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) lipid profiling.**

An automated electrospray ionization-tandem mass spectrometry approach was used, and data acquisition and analysis and acyl group identification were carried out by the Kansas Lipidomics Research Center's Analytical Laboratory as previously described (Devaiah and others 2006). The lipid samples were dissolved in 1 mL chloroform and aliquots of 10-35 µL of extract was analyzed. Precise amounts of internal standards, obtained and quantified as previously described (Welti and others 2002). The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.4 ml. Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 µl/min. Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a
custom script and Applied Biosystems Analyst software. The first and typically every 11th set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the two internal standards on the same lipid class, except for PI, which was quantified in relation to a single internal standard. Ether-linked (alk(en)yl,acyl) lipids were quantified in comparison to the diacyl compounds with the same head groups without correction for response factors for these compounds as compared to their diacyl analogs. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the “internal standards only” spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each “internal standards only” set of spectra was used to correct the data from the following 10 samples. The data were corrected for and reported as the fraction of the sample analyzed. See supplemental methods for a more detailed description of the ESI-MS/MS method.

**Analysis and classification of phospholipid species.**

Phospholipid levels were reported as nmoles detected normalized to the cell or mitochondrial pellet weight prior to lipid extraction and as a mole % of all phospholipid moles detected. The data is reported as the average of triplicate experiments. Significance was calculated using two tailed unpaired student t-tests based on hetero/homoscedasticity of sample populations.
Mitochondrial morphology analysis.

MEF cells were grown in 10cm tissue culture plates and split into 8-well glass bottom plates (Lab-Tek/Thermo Sciences, Rochester, NY 155411) one day before staining and imaging. Cells were incubated with 50nM MitoTracker Deep Red (Invitrogen, Carlsbad CA) for 10 minutes, washed, and fixed in 3.7% formaldehyde prior to imaging using a Zeiss LSM-710 confocal microscope (Zeiss, Germany). Images were auto-thresholded and objects were identified and morphology analyzed using the Analyze Particles function in ImageJ (version 1.43j NIH, http://rsb.info.nih.gov/ij, Bethesda MD).

Results.

Thin layer chromatography reveals decreases in PE in Mfn2 and Mfn1 KO MEFs.

To quantify levels of PE and PS in the absence of Mfn2 and Mfn1, lipids were extracted from wildtype, Mfn1 and Mfn2 KO MEFs, and equal masses of extracted lipid were separated by high performance thin layer chromatography (TLC). Phospholipids were detected based on phospho-moiety chemistry, compared to phospholipid standards, and quantified by densitometry. Significant differences in PE levels were observed between Mfn2 and Mfn1 MEFs and wildtype MEFs when analyzed as raw values (decreased 38.7% and 37.2%, Figure 4.2) or normalized to PC spots to control for potential loading errors (decreased 51.7% and 50.6%, Figure 4.2). Levels of PS and cardiolipin were low to undetectable and limited comparative quantification by densitometry (data not shown). Additionally, the relative migration or retention factor (Rf) of PE was altered in Mfn1 and Mfn2 KO lipid extracts, while the Rf values of PC were unchanged (Figure 4.2). To confirm the identity of the PE spot, a ninhydrin based amine detection method was used to validate PE and PS spots as previously described.
Absence of Mfn2 or Mfn1 results in altered levels of multiple phospholipid classes as quantified by ESI-MS/MS.

To further characterize the quantitative and qualitative changes observed in PE in the absence of Mfn2 and Mfn1, we performed an ESI-MS/MS phospholipid screen on lipid extracts from wt, Mfn1 and Mfn2 KO MEFs cultured under conditions consistent with previously published studies (Chen and others 2003; de Brito and Scorrano 2008; Hailey and others 2010). Confirming our TLC data, mass spectrometry results revealed a significant decrease in PE in Mfn2 KO MEFs as compared to wildtype MEFs when reported as nmoles normalized to cell pellet weight (34.6% decrease, Table 4.1, Figure 4.3.) and as a molar percent of all phospholipids detected (51.6% decrease, Table 4.2, Figure 4.3). Significantly lower levels of PE are also observed in Mfn1 KO MEF lipid extracts when analyzed as percent of all phospholipids detected (54.0% decrease, Table 4.2 and Figure 4.3). PE is also decreased in mitochondria enriched fractions from Mfn2 and Mfn1 MEF cells (Table 4.3 and 4.4). In addition, increases in PS are observed when quantified as nmol per cell pellet weight in Mfn1 and Mfn2 KO MEF whole cell lipid extracts (97.8% and 131.5% increases, Table 4.1 and Figure 4.3). No increases in PS are observed in mitochondrial enriched fractions from Mfn2 and Mfn1 KO MEFs (Tables 4.3 and 4.4). Other changes include increases in lyso-phosphatidylcholine (lyso-PC), PC, ether linked-phosphatidylcholine (ePC), sphingomyelin (SM) and dihydrosphingomyelin
(DSM) observed in whole cell extracts from Mfn2 KO MEFs (Table 4.1). Many of the same trends are observed in Mfn1 KO MEFs but do not achieve statistical significance (Table 4.1).

**Restoration of the CDP-ethanolamine pathway rescues PE abnormalities associated with loss of mitofusins.**

The concomitant rise in PS and drop in PE levels is consistent with the hypothesis that loss of either mitofusins disrupt phospholipid exchange at MAM-mitochondrial contact sites (Figure. 1). Lack of sufficient free ethanolamine in routinely used culture media (Dulbecco’s Modified Eagle Media supplemented with 10% fetal bovine serum) limits the contribution of the redundant CDP-ethanolamine pathway which requires free ethanolamine as a substrate (Shiao and Vance 1995; Voelker 1984; Voelker and Frazier 1986). Most cultured mammalian cells derive the majority of PE from the decarboxylation of PS by Pisd and thus have an increased dependence on MAM-mitochondrial interactions. From the data reported in the section above, it can be concluded that the presence of both mitofusin proteins is required for normal PE levels in the ethanolamine deprived state in which MEFs are regularly cultured. However, these routine culture conditions contrast with normal conditions in mammals where the concentration of circulating free ethanolamine is approximately 20μM (Lipton and others 1990; Shiao and Vance 1995; Voelker and Frazier 1986). Physiologic ethanolamine concentrations can be achieved by supplementing media in which free ethanolamine is provided solely by serum (10% FBS yields ~2.0 μM ethanolamine). To better understand the role of Mfn1 and Mfn2 under physiologic concentrations of ethanolamine, we repeated the ESI-MS/MS phospholipid screen in media supplemented with ethanolamine. We choose to supplement with 20μM ethanolamine (final concentration of ~22μM) to
mimic physiological conditions and also included a 200μM ethanolamine supplemental condition to ensure free ethanolamine would not limit the potential rescue of PE levels (Lipton and others 1990).

The addition of both 20μM and 200μM ethanolamine to Mfn1 and Mfn2 KO MEF cultures similarly rescued all differences in phospholipid levels as compared to wildtype MEFs (Tables 4.5 and Figure 4.3). In addition to normalizing levels of PE and PS, the restoration of the CDP-ethanolamine synthesis pathway also normalized nmol/g levels of lyso-PC, PC, ePC, DSM and SM (Table 4.5). The complete rescue of phospholipid class profiles by restoration of the normally dominant PE synthesis pathways suggests that the changes in multiple phospholipid classes observed (lyso-PC, PC, ePC, SM and DSM) in the absence of ethanolamine stem from a specific deficiency in PE and not from additional lipid-related functions mediated by the mitofusins. Thus, the presence of both mitofusins is required for the cell to sustain normal PE levels and cellular phospholipid profiles during ethanolamine deprivation.

**Ethanolamine supplementation does not rescue fragmented mitochondrial network associated with the loss of either mitofusins.**

Previous work on the Pisd-null mouse models suggests that PE synthesized by Pisd within mitochondria is critical in shaping tubular and reticular mitochondrial morphology (Steenbergen and others 2005). Homozygous Pisd-null MEFs exhibit fragmented mitochondria very similar to both Mfn2 and Mfn1 KO MEFs (Steenbergen and others 2005). Additionally, PE is a fusogenic and curvature inducing phospholipid (Churchward and others 2008; Jiang and Powers 2008). Thus, its absence may at least partially account for the profound mitochondrial fusion and morphology defects observed in Pisd, Mfn2 and Mfn1 null models. Restoration of the major alternative
PE synthesis pathway completely corrected whole cell PE levels in the absence of either mitofusin. However, it was unclear if CDP-ethanolamine derived PE could effectively rescue the fragmented mitochondrial morphology which putatively stems from a mitochondrial PE deficit. To address this question, wildtype and $Mfn2$ KO MEFs cultured with and without ethanolamine supplementation were stained with mitotracker-deep red (Invitrogen, Carlsbad, CA) and fixed prior to confocal imaging and morphometric analysis using ImageJ. Morphology analysis was able to quantify significant differences between wildtype and $Mfn2$ KO MEFs. However, the addition of 20μM and 200μM ethanolamine did not alter the mitochondrial morphology of wildtype or $Mfn2$ KO MEFs (Figure 4.8).

**PE demands under starvation accentuate phospholipid abnormalities in Mfn1 and Mfn2 KO MEFs.**

Autophagosomes formation can be reliably induced in MEFs by two hours of serum starvation (Hailey and others 2010). Recent work suggest that mitochondrial generated PE is essential in the biogenesis of autophagosomes (Hailey and others 2010). Consistent with our hypothesis, autophagosomes do not form in $Mfn2$ KO MEFs following serum starvation. (Hailey and others 2010). To investigate the effect of autophagy in $Mfn2$ and $Mfn1$ KO MEF lipid profiles, we induced autophagy by serum starvation and analyzed lipid extracts from whole MEF cells using ESI-MS/MS. In wt MEFs, the induction of autophagy caused a 15.2% reduction in total PE (Table 4.2). In both $Mfn1$ and $Mfn2$ KO MEFs, the induction of autophagy precipitated a 63.2% and a 41.2% increase in total PE levels, opposite of the observed effect in wt MEFs (Table 4.2). Examination of subgroups of PE reveals that wt MEF mitochondrial $PE_{Pisd}$ levels
decrease while $\text{PE}_{\text{CDP-Etn}}$ levels remain unchanged (Figure 4.4). Conversely, in both $Mfn1$ and $Mfn2$ KO MEFs, $\text{PE}_{\text{CDP-Etn}}$ levels are specifically increased while $\text{PE}_{\text{Pisd}}$ levels remain unchanged (Figure 4.4). Induction of autophagy also causes opposite changes in PS in $Mfn2$ and $Mfn1$ KO MEFS as compared to wt MEFS, but does not alter levels of PC or PI (Table 4.2).

**Loss of mitofusins decreases specific subpopulations of PE in the absence of ethanolamine.**

In addition to quantitative data on each phospholipid class, ESI-MS/MS provides more specific data regarding the summed carbon number and unsaturated bonds of fatty acyl components. Therefore we could investigate how the loss of Mfn1 and Mfn2 altered the distribution of specific subpopulations of PE composed of various numbers of fatty acyl carbons and unsaturated bonds. While information regarding the specific identity of either acyl chains is lost when considering the summed carbon number and bonds of each phospholipid, a trend was apparent in the profile of PE species (Figure 4.4). While total PE levels are decreased in $Mfn1$ and $Mfn2$ KO MEFS, it is apparent that this trend stems primarily from decreases in PE species with 4 or more unsaturated bonds. Likewise, the opposite trend was noted for PE species with 3 or fewer unsaturated bonds (Figure 4.4). This observation may reflect the known substrate preferences of Pisd for PS species containing highly unsaturated (>4 unsaturated bonds) acyl species with longer (>20 carbons) in the sn-2 position (Bleijerveld and others 2007). More specifically, Pisd has been previously reported to preferentially synthesize PE(18:0-20:4), PE (18:1-20:4), and PE (16:0-20:4), which would be included under the subpopulations of PE(36:4), PE(38:4), and PE(38:5) (Bleijerveld and others 2007; Salvador, Lopez, Giusto 2002;
Schuiki and others 2010). For a more detailed examination of these particular PE subpopulations, we chose to define “Pisd PE” as the sum of PE(36:4), PE(38:4), and PE(38:5). Species included under “Pisd PE” have been previous reported as predominant species of membrane phospholipids, so that contributions by other less typical fatty acyl chain combinations likely make only a small contribution to these subpopulation (Bleijerveld and others 2007). Likewise, the CDP-ethanolamine pathway preferentially synthesizes PE species with less saturated and shorter acyl chain composition, consisting of PE(16:0/18:1), PE(16:0/18:2), PE(18:0/18:1), PE(18:0/18:2), PE(18:1/18:1), and PE(18:1/18:2) (Bleijerveld and others 2007; McMaster and Bell 1997). These CDP-ethanolamine derived PE species would be included in subpopulations of PE(34:1), PE(34:2), PE(36:1), PE(36:2), and PE(36:3). A graphical explanation of the subpopulation breakdown can be found in Figure 4.5.

Application of this classification scheme reveals that while total PE levels are decreased in the absence of either mitofusin when starved of ethanolamine, specific decreases in the Pisd-PE subpopulation account for the majority of the decrease (Figure 4.5). Conversely, CDP-ethanolamine derived PE subpopulations actually increase, and complete PE compensation is conceivably prevented by lack of free ethanolamine as a substrate (Figure 4.5). Indeed, the restoration of the CDP-ethanolamine pathway by the addition of 20μM or 200μM ethanolamine to culture media abolishes the differences in PE subpopulations observed in ethanolamine deprivation (sub-population data not shown). Thus, in the absence of the CDP-ethanolamine pathway loss of either mitofusin
appears to limit the synthesis of PE catalyzed by Pisd. Yet when ethanolamine is present, the CDP-ethanolamine pathway can effectively compensate for PE subpopulations normally synthesized by Pisd.

**Changes in specific subpopulations of PC reflect changes in PE.**

Similar to the observations for PE, the loss of Mfn1 or Mfn2 and the absence of ethanolamine specifically decreased levels of PC species with 4 or more unsaturated bonds (Figure 4.4). Much like PE, PC is synthesized by redundant pathways in the cell (Figure 4.1). The CDP-choline pathway originally described by Kennedy et al, is located in the ER and dependent on a CDP intermediate (CDP-choline)(Kennedy and Weiss 1956). A second PC synthesis pathway is catalyzed within the MAM by phosphatidylethanolamine N-methyltransferase (PEMT)(Cui and others 1993). Pemt converts PE to PC, and also has distinct substrate preferences which yield the PC species PC(18:2/20:4), PC(18:1/20:4), PC(18:0/20:4), PC(18:0/22:6) and PC(18:1/22:5)(Cui and others 1993; DeLong and others 1999; Ridgway and Vance 1988). Conversely the CDP-choline pathway preferentially yields PC species PC(16:0/18:1), PC(18:0/18:2), and PC(18:1/18:1)(Cui and others 1993; DeLong and others 1999; Ridgway and Vance 1988). When PC subpopulations are stratified into “Pemt PC” and “CDP-Choline” sub-populations, a significant decrease in Pemt derived PC species is apparent, with the increase in total PC species being accounted for by CDP-choline derived species and PC species falling outside this classification scheme (Figure 4.5). Thus, in addition to limiting the generation of Pisd derived PE sub-populations during ethanolamine deprivation, loss of either mitofusin also reduces levels of PC sub-populations known to be preferentially synthesized by Pemt. The specific decrease in both PC and PE sub-
populations dependent on MAM-mitochondria interactions suggest that loss of either mitofusins disrupt phospholipid exchange between these organelles, leading to alterations in the acyl chain makeup of cellular PE and PC. While overall levels of phosphatidylinositol (PI) are not altered in Mfn2 and Mfn1 KO MEFs under ethanolamine deprivation, a similar trend is observed for many PI subpopulations with 4 to 5 or more unsaturated bonds (Figure 4.4).

We considered whether this decrease in highly unsaturated acyl components of PE and PC is specific to these three phospholipid classes or represent a decrease in unsaturated fatty acyl components of all phospholipid species. The unsaturation index is a measure of the global saturation status of a lipid population and can be calculated by multiplying the % mole (percentage of all phospholipid detected) of a specific phospholipid species by the unsaturated bonds present per fatty acyl carbon (Dole and others 1959). In wildtype cells, the unsaturation index increases as a function of decreasing ethanolamine media concentration (Figure 4.6). This effect could represent a cellular shift from the CDP-ethanolamine pathway to the Pisd synthesis pathway which preferentially produces PE with more unsaturated acyl components in the sn-2 position (Bleijerveld and others 2007; Salvador, Lopez, Giusto 2002). This trend appears to be reversed in Mfn1 and Mfn2 KO MEFs, where the unsaturation index is unchanged or decreases with decreasing media ethanolamine (Figure 4.5). When the unsaturation index of PE and PC populations is considered separately from the total unsaturation index, it is readily apparent that PE and PC species are responsible for the decrease in the overall unsaturation index (Figure 4.6, upper curves). Indeed, all other remaining phospholipids contribute a minor increase in the unsaturation index (Figure 4.6, lower
curves). Hence, the absence of either mitofusin limits the cell's ability to increase highly unsaturated acyl chain containing species of PE and PC during ethanolamine deprivation.

**Discussion.**

*Mfn1* and *Mfn2* KO MEFs were used in the original investigations that described mitofusin’s roles in determining mitochondrial morphology and tethering MAM to mitochondria (Chen and others 2003; de Brito and Scorrano 2008). Using the same model cell lines, we report how loss of Mfn2 and Mfn1 alter cellular and mitochondrial phospholipid profiles. Numerous studies of phospholipid synthesis pathways localized to MAM-mitochondria interfaces indicate that the transfer of lipid species between MAM and mitochondria requires sufficient spatial proximity (Achleitner and others 1999; Choi, Wu, Voelker 2005; Schumacher, Choi, Voelker 2002; Shiao, Lupo, Vance 1995; Shiao, Balcerzak, Vance 1998; Vance and Vance 2008; Voelker 1989; Voelker 1991). The tethering and proximal anchoring of MAM to mitochondria have been shown to be carried out by Mfn2, which has analogous functions in the tethering and fusion of mitochondria (de Brito and Scorrano 2008).

The transport of PS from MAM to mitochondrial membranes has been extensively characterized, and has been shown to depend on the presence of an outer mitochondrial membrane protein (Kobayashi and Arakawa 1991; Schuiki and others 2010; Schumacher, Choi, Voelker 2002; Shiao, Lupo, Vance 1995; Shiao, Balcerzak, Vance 1998; Steenbergen and others 2005; Vance and Vance 2009; Vance 1990; Vance and Steenbergen 2005; Vance 2008; Voelker 1991). Once in the inner mitochondrial membrane, PS is decarboxylated by Pisd to yield PE. PE is a phospholipid known to
induce membrane curvature and enhance the fusogenic potential of membranes (Churchward and others 2008; Seddon 1990). The morphological and fusion defects of mitochondria observed in the absence of either mitofusin could be explained by a deficiency in PE. Interestingly, Pisd KO MEFs contain fragmented and irregularly shaped mitochondria similar to that observed in Mfn2 KO MEFs (Steenbergen and others 2005). Both Pisd KO and Mfn2 KO MEFs also demonstrate similar placental defects and embryonic lethality time courses (Chen and others 2003; Steenbergen and others 2005). In both cases, the placental defect stems from atrophy of the syncytiotrophoblast layer, a multinucleated layer serving as the maternal-fetal blood barrier formed by fusion of individual trophoblasts around the implanting embryo. Underscoring the importance of PE in placental syncytiotrophoblast formation is the identification of anti-PE antibodies in women with antiphospholipid syndrome with spontaneous and recurring abortions (Girardi 2010; McIntyre, Wagenknecht, Faulk 2003; Sanmarco and Boffa 2009). Together these observations putatively place Mfn2 functionally upstream of the MAM-mitochondrial dependent Pisd based PE synthesis pathway (Figure 4.1). Our results demonstrate that loss of Mfn2 is associated with increases in cellular PS and deficiencies in mitochondrial and cellular PE levels consistent with this model.

Our results also indicate that not only Mfn2 but also Mfn1 maybe critical for the effective movement of phospholipids at MAM-mitochondrial interfaces. Since specifically Mfn2 and not Mfn1 was shown to tether MAM to mitochondria, the fact that similar reductions in PE are seen in Mfn1 comes as a surprise (de Brito and Scorrano 2008). Perhaps Mfn1 also has unidentified tethering roles. It is possible that the observed deficiencies are a general consequence of a fragmented mitochondrial network, which is
associated with severely decreased mitochondrial mobility in *Mfn1* and *Mfn2* KO MEFs (Chen and others 2003). A loss of mobility likely reduces the frequency of interaction between mitochondria and ER membranes, thereby limiting MAM-mitochondria related functions. While Mfn2 may be specifically required for MAM-mitochondria tethering and the import of PS, loss of Mfn1 may limit the distribution of newly formed PE by disrupting fusion of PE rich MAM-associated mitochondria with PE deficient mitochondria. In this model, Mfn2 would be required for inter-organelle PS transport, while Mfn1 is required for intra-organelle distribution of PE or PS throughout the mitochondrial network. Regardless of the mechanism, our data represent the first reported phospholipidomic study of cells with reduced MAM-mitochondria interactions. Future work will have to determine the exact mechanism by which both mitofusins influence MAM-mitochondria dependent lipid synthesis.

Redundant PE synthesis pathways exist independent of MAM-mitochondria interactions. By supplementing media with ethanolamine we demonstrated that restoration of the CDP-ethanolamine pathway completely rescues phospholipid abnormalities associated with loss of either mitofusin. However, much like the *Pisd* null MEFs, addition of free ethanolamine does not rescue fragmented mitochondrial network in *Mfn2* KO MEFs (Steenbergen and others 2005). The failure of the CDP-ethanolamine derived PE to rescue fragmented mitochondrial network in *Mfn2* KO MEFs in light of restoring whole cell PE levels can be explained by several mechanisms. One possibility is that mitochondrial PE must be locally generated by Pisd and that CDP-ethanolamine derived PE does not effectively localize to mitochondrial membranes. Alternatively, while restoration of the CDP-ethanolamine pathway corrects cellular PE levels, deficits in
specific subpopulations of PE with unique biochemical and biophysical properties may not be effectively compensated. Likely both the location and the specific species of mitochondrial synthesized PE have important consequences in determining mitochondrial morphology and function.

These mechanistic hypotheses warrant attention. To our knowledge all published studies of Mfn2 and Mfn2 KO MEFS have used routine cell culture conditions which provide ~10% of physiologic concentrations of ethanolamine (Chen and others 2003; Chen and others 2010; de Brito and Scorrano 2008; Hailey and others 2010). Interestingly, a recent report has indicated the formation of autophagosomes following 2 hours of serum starvation depends on mitochondrial synthesis of PE and hence the formation of MAM-mitochondrial contacts mediated by Mfn2 (Hailey and others 2010). In this study, Mfn2 KO MEFs were shifted from routinely used media in which ethanolamine is provided by serum at final concentration of ~2μM to media containing no serum and hence 0 μM ethanolamine. It was concluded that the lack of autophagosome formation in Mfn2 KO MEFs in response to serum starvation was due to a lack of mitochondrial PE synthesis. However, serum starvation without ethanolamine supplementation in the absence of Mfn2 would eliminate both major PE synthesis pathways. It is interesting to consider whether the addition of ethanolamine during serum starvation would result in the same observation in Mfn2 KO MEFs. It seems likely that PE supplied from a restored CDP-ethanolamine pathway would effectively increase cellular levels of PE, but would not provide PE of the correct species or subcellular location to restore autophagosome biogenesis in Mfn2 KO MEFs. With respect to the animal, low levels of free ethanolamine resulting from lack of dietary ethanolamine may
reflect periods of starvation, and act as an early molecular trigger in establishing appropriate PE levels for autophagosome formation (Vance and Vance 2008). It is interesting to consider how the availability of phospholipid substrates like ethanolamine and choline might modulate cellular phospholipid profiles to coordinate a cellular response to starvation. The MAM-mitochondria based Pisd and Pemt pathways may serve as more than just back-up pathways when dietary choline and ethanolamine are low, but produce spatially and functionally distinct cellular phospholipid species that reflect the cells metabolic state.

Physiologic ethanolamine concentrations are considered to be ~20μM, yet little is known about the dynamics of serum ethanolamine concentrations or what ethanolamine levels exist within specific tissues (Houweling and others 1992; Lipton and others 1990). There is large (~50 fold) range for differences in the serum ethanolamine concentrations between bovine, hamster and rat (Lipton and others 1990; Sundler and Akesson 1975; Zelinski and Choy 1984). There are also large (~100 fold) differences in the kinetics of ethanolamine uptake between cell types, such as glia and neurons (Massarelli and others 1986). While serum concentrations of ethanolamine may hover close to 20μM, the application of a protein kinase C agonist (phorbol ester) to cultured glia cells results in the extracellular release of free ethanolamine (McNulty, Sayner, Rumsby 1991; McNulty and others 1992). The regulated release of ethanolamine by glia cells suggest local ethanolamine concentration may be more dynamic that what the limited reports of ethanolamine serum measurements reveal. As mutations in MFN2 cause CMT2A, a disease which specifically affects peripheral axons, it is interesting to consider how free
ethanolamine might be dynamically regulated within the peripheral nerve. Perhaps mutations in MFN2 cause cell specific vulnerabilities to low ethanolamine concentrations and account for the extraordinary cell specificity of the CMT2A.

Besides potential insight into the pathogenic mechanisms behind CMT2A, our results also suggest that mitofusin mediated MAM-mitochondrial interactions have specific effects on important and potentially bioactive lipid subpopulations. When deprived of ethanolamine, we observed a decrease in PE and PC species containing 4 or more unsaturated bonds both Mfn1 and Mfn2 KO MEFs. Our results are consistent with a previous report of increased dietary ethanolamine in rats significantly decreasing levels of PE and PC species containing highly unsaturated fatty acids, which the authors attribute to PE’s influence on delta-5- and delta-6-desaturase activities (Shimada, Morita, Sugiyama 2003). Likewise, the MAM resident long-chain-fatty-acid CoA ligase 4 (ACSL4), commonly used as a MAM marker, preferentially catalyzes fatty acyl-CoA esters containing arachidonic acid (Cao and others 1998). Disruption of ER- mitochondria contacts may decrease the activity of ACSL4 and subsequently result in similar subpopulation changes in PE, PC and PI (Golej and others 2011). Even though our PE and PC classification scheme is limited to making generalizations regarding the acyl content of these phospholipids, PE and PC subpopulations enriched in the sn-2 position with polyunsaturated fatty acids made up primarily of arachidonic acid (20:4 n-6), eicosapentaenoic acid (20:5 n-3) docosapentaenoic acid (22:5 n-3/6), and docosahexaenoic acid (22:6 n-3). PE, PC, and PI species composed of these highly unsaturated fatty acids are targeted by phospholipases which free component fatty acids and provide substrate for synthesis of bioactive eicosanoid molecules. Differences in
how glia, endothelial cells and neurons synthesize these critical fatty acid components suggest that mutations in *MFN2* might alter how these fatty acids are transferred and/or metabolized in the neuro-vascular or neuro-glia unit (Moore, Yoder, Spector 1990; Moore and others 1991). Interestingly, decreases in arachidonyl (20:4 n-6) containing species of PE, PC and PI have been reported in the sciatic nerve of rodent models of diabetic neuropathy and Schwann cells cultured in hyperglycemic (Doss and others 1997; Head and others 2000; Kuruvilla and Eichberg 1998; Zhu and Eichberg 1993). Thus, beyond disrupting PE synthesis, disruption of MAM-mitochondrial interfaces in the absence of Mfn2 may have consequences in the arachidonic acid composition of membrane phospholipids and alter downstream eicosanoid signaling.

**Conclusions**

In conclusion, our results demonstrate the presence of both mitofusins is required for cells to shift to MAM-mitochondria based synthesis of PE when cells are deprived of ethanolamine. In addition to deficiencies in PE, specific sub-populations of PE and PC containing highly unsaturated acyl components appear to be specifically decreased. However, all of the observed phospholipid deficiencies can be rescued by restoration of the redundant CDP-ethanolamine pathway, confirming the original deficiencies stem from a specific deficiency in PE. Deficiencies in mitochondrial and cellular PE levels in the absence of Mfn1 or Mfn2 point to novel cellular roles for Mfn1 and Mfn2 which may directly relate to roles in defining mitochondrial morphology. More detailed mechanistic studies of how Mfn2 and Mfn1 mediate and perhaps regulate MAM-mitochondria interactions will further our understanding of the MAM sub-compartment and how lipid
synthesis might be coupled with other MAM related processes such Ca\(^{+2}\) exchange. Additionally, the identification of decreased subpopulations of PE and PC offer an alternative route of investigation in addition to the current models of the pathogenesis of CMT2A. Future work focusing on the molecular genetics of CMT2A will be essential to determine whether mutations in \(Mfn2\) alter phospholipid profiles.
Table 4.1. Lipid profiles of whole cell lipid extracts from wildtype, Mfn2 and Mfn1 KO MEFs, reported as nmol/g cells

<table>
<thead>
<tr>
<th>Lipid Profile</th>
<th>Wildtype MEF Average ± StdDev</th>
<th>Mfn2 KO MEF Average ± StdDev</th>
<th>Mfn1 KO MEF Average ± StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>0.009 ± 0.007</td>
<td>0.027 ± 0.007</td>
<td>0.021 ± 0.010</td>
</tr>
<tr>
<td>PC</td>
<td>1.737 ± 0.231</td>
<td>2.894 ± 0.442</td>
<td>2.911 ± 1.240</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>0.187 ± 0.107</td>
<td>0.436 ± 0.111</td>
<td>0.426 ± 0.160</td>
</tr>
<tr>
<td>ePC</td>
<td>0.236 ± 0.028</td>
<td>0.446 ± 0.085</td>
<td>0.721 ± 0.388</td>
</tr>
<tr>
<td>Lyso-PE</td>
<td>0.004 ± 0.001</td>
<td>0.004 ± 0.001</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>PE</td>
<td>0.790 ± 0.058</td>
<td>0.587 ± 0.104</td>
<td>0.578 ± 0.202</td>
</tr>
<tr>
<td>Cer-PE</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>ePE</td>
<td>0.095 ± 0.016</td>
<td>0.074 ± 0.023</td>
<td>0.101 ± 0.039</td>
</tr>
<tr>
<td>PI</td>
<td>0.384 ± 0.185</td>
<td>0.593 ± 0.107</td>
<td>0.588 ± 0.216</td>
</tr>
<tr>
<td>PS</td>
<td>0.184 ± 0.097</td>
<td>0.364 ± 0.067</td>
<td>0.426 ± 0.124*</td>
</tr>
<tr>
<td>ePS</td>
<td>0.008 ± 0.005</td>
<td>0.015 ± 0.003</td>
<td>0.018 ± 0.005</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.021 ± 0.009</td>
<td>0.033 ± 0.010</td>
<td>0.031 ± 0.012</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.017 ± 0.008</td>
<td>0.022 ± 0.003</td>
<td>0.022 ± 0.012</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-phosphatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS. * p<0.05, **p<0.01, and ***p<0.001 as compared to wildtype.
# Table 4.2. Lipid profiles of whole cell lipid extracts from wildtype, Mfn2 and Mfn1 KO MEFs, reported percentage of all phospholipids detected

<table>
<thead>
<tr>
<th></th>
<th>Wildtype MEF Average ± StdDev</th>
<th>Mfn2 KO MEF Average ± StdDev</th>
<th>Mfn1 KO MEF Average ± StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>0.23 ± 0.17</td>
<td>0.48 ± 0.05</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>PC</td>
<td>47.57 ± 2.61</td>
<td>52.78 ± 1.02*</td>
<td>49.33 ± 1.91</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>4.84 ± 2.30</td>
<td>7.85 ± 0.75</td>
<td>7.36 ± 0.43</td>
</tr>
<tr>
<td>ePC</td>
<td>6.49 ± 0.50</td>
<td>8.11 ± 0.51*</td>
<td>11.87 ± 3.77</td>
</tr>
<tr>
<td>Lyso-PE</td>
<td>0.12 ± 0.01</td>
<td>0.07 ± 0.01*</td>
<td>0.06 ± 0.00*</td>
</tr>
<tr>
<td>PE</td>
<td>22.05 ± 4.91</td>
<td>10.67 ± 0.10*</td>
<td>10.15 ± 1.37*</td>
</tr>
<tr>
<td>Cer-PE</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>ePE</td>
<td>2.70 ± 0.95</td>
<td>1.34 ± 0.22*</td>
<td>1.76 ± 0.29</td>
</tr>
<tr>
<td>PI</td>
<td>10.02 ± 3.67</td>
<td>10.82 ± 1.07</td>
<td>10.20 ± 0.69</td>
</tr>
<tr>
<td>PS</td>
<td>4.78 ± 2.01</td>
<td>6.61 ± 0.30</td>
<td>7.70 ± 1.93</td>
</tr>
<tr>
<td>ePS</td>
<td>0.20 ± 0.10</td>
<td>0.27 ± 0.00</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.55 ± 0.18</td>
<td>0.60 ± 0.16</td>
<td>0.56 ± 0.19</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.46 ± 0.15</td>
<td>0.40 ± 0.04</td>
<td>0.36 ± 0.08*</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-phophatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS. * p<0.05, **p<0.01, and ***p<0.001 as compared to wildtype.
Table 4.3. Lipid profiles of mitochondrial enriched fractions from wildtype, Mfn2 and Mfn1 KO MEFs, reported as nmol/g mitochondrial pellet

<table>
<thead>
<tr>
<th></th>
<th>Wildtype MEF Average ±</th>
<th>Mfn2 KO MEF Average ± StdDev</th>
<th>Mfn1 KO MEF Average ± StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>0.011 ± 0.006</td>
<td>0.025 ± 0.006*</td>
<td>0.051 ± 0.035</td>
</tr>
<tr>
<td>PC</td>
<td>4.050 ± 0.335</td>
<td>3.238 ± 0.747</td>
<td>3.062 ± 1.455</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>1.905 ± 0.080</td>
<td>1.318 ± 0.357*</td>
<td>1.635 ± 1.068</td>
</tr>
<tr>
<td>ePC</td>
<td>0.827 ± 0.205</td>
<td>0.975 ± 0.192</td>
<td>1.206 ± 0.865</td>
</tr>
<tr>
<td>Lyso-PE</td>
<td>0.012 ± 0.001</td>
<td>0.012 ± 0.003</td>
<td>0.019 ± 0.013</td>
</tr>
<tr>
<td>PE</td>
<td>1.450 ± 0.163</td>
<td>0.929 ± 0.192*</td>
<td>1.056 ± 0.590</td>
</tr>
<tr>
<td>Cer-PE</td>
<td>0.004 ± 0.000</td>
<td>0.000 ± 0.000*</td>
<td>0.001 ± 0.001*</td>
</tr>
<tr>
<td>ePE</td>
<td>0.196 ± 0.017</td>
<td>0.144 ± 0.036</td>
<td>0.203 ± 0.149</td>
</tr>
<tr>
<td>PI</td>
<td>0.520 ± 0.030</td>
<td>0.594 ± 0.146</td>
<td>0.598 ± 0.391</td>
</tr>
<tr>
<td>PS</td>
<td>0.441 ± 0.124</td>
<td>0.389 ± 0.051</td>
<td>0.430 ± 0.142</td>
</tr>
<tr>
<td>ePS</td>
<td>0.020 ± 0.006</td>
<td>0.019 ± 0.003</td>
<td>0.024 ± 0.009</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.034 ± 0.008</td>
<td>0.027 ± 0.009</td>
<td>0.029 ± 0.014</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.073 ± 0.008</td>
<td>0.029 ± 0.005**</td>
<td>0.045 ± 0.021</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-phosphatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS. * p<0.05, **p<0.01, and ***p<0.001 as compared to wildtype.
Table 4.4. Lipid profiles of mitochondrial enriched fractions from wildtype, Mfn2 and Mfn1 KO MEFs, reported percentage of all phospholipids detected

<table>
<thead>
<tr>
<th></th>
<th>Wildtype MEF Average ±</th>
<th>Mfn2 KO MEF Average ± StdDev</th>
<th>Mfn1 KO MEF Average ± StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>0.12 ± 0.06</td>
<td>0.33 ± 0.01*</td>
<td>0.56 ± 0.12*</td>
</tr>
<tr>
<td>PC</td>
<td>42.41 ± 1.95</td>
<td>42.04 ± 1.26</td>
<td>38.19 ± 4.26</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>19.98 ± 0.69</td>
<td>16.98 ± 1.00*</td>
<td>18.51 ± 2.84</td>
</tr>
<tr>
<td>ePC</td>
<td>8.63 ± 1.83</td>
<td>12.72 ± 0.42*</td>
<td>13.69 ± 2.68</td>
</tr>
<tr>
<td>Lyso-PE</td>
<td>0.12 ± 0.01</td>
<td>0.16 ± 0.01*</td>
<td>0.21 ± 0.04*</td>
</tr>
<tr>
<td>PE</td>
<td>15.20 ± 1.62</td>
<td>12.10 ± 0.26*</td>
<td>12.55 ± 0.62</td>
</tr>
<tr>
<td>Cer-PE</td>
<td>0.04 ± 0.01</td>
<td>0.00 ± 0.00***</td>
<td>0.01 ± 0.01**</td>
</tr>
<tr>
<td>ePE</td>
<td>2.05 ± 0.11</td>
<td>1.87 ± 0.24</td>
<td>2.25 ± 0.51</td>
</tr>
<tr>
<td>PI</td>
<td>5.45 ± 0.10</td>
<td>7.69 ± 0.24***</td>
<td>6.88 ± 0.82*</td>
</tr>
<tr>
<td>PS</td>
<td>4.65 ± 1.46</td>
<td>5.14 ± 0.70</td>
<td>5.87 ± 1.96</td>
</tr>
<tr>
<td>ePS</td>
<td>0.21 ± 0.07</td>
<td>0.26 ± 0.03</td>
<td>0.32 ± 0.08</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.36 ± 0.10</td>
<td>0.34 ± 0.09</td>
<td>0.37 ± 0.07</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.77 ± 0.09</td>
<td>0.38 ± 0.04*</td>
<td>0.58 ± 0.16</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-phophatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS. * p<0.05, **p<0.01, and ***p<0.001 as compared to wildtype.
Table 4.5. Lipid profiles of whole cell lipid extracts from wildtype, Mfn2 and Mfn1 KO MEFs cultured in physiologic ethanolamine (22.5uM ethanolamine), reported as nmol/g cells

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Wildtype MEF + Average ± StdDev</th>
<th>Mfn2 KO MEF + Average ± StdDev</th>
<th>Mfn1 KO MEF + Average ± StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>0.028 ± 0.013</td>
<td>0.031 ± 0.017</td>
<td>0.024 ± 0.012</td>
</tr>
<tr>
<td>PC</td>
<td>3.461 ± 1.519</td>
<td>3.436 ± 1.643</td>
<td>4.085 ± 2.036</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>0.559 ± 0.223</td>
<td>0.663 ± 0.308</td>
<td>0.726 ± 0.434</td>
</tr>
<tr>
<td>ePC</td>
<td>0.424 ± 0.196</td>
<td>0.335 ± 0.170</td>
<td>0.506 ± 0.245</td>
</tr>
<tr>
<td>Lyso-PE</td>
<td>0.014 ± 0.006</td>
<td>0.014 ± 0.007</td>
<td>0.011 ± 0.006</td>
</tr>
<tr>
<td>PE</td>
<td>1.310 ± 0.558</td>
<td>1.329 ± 0.543</td>
<td>1.464 ± 0.660</td>
</tr>
<tr>
<td>Cer-PE</td>
<td>0.001 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>0.001 ± 0.001</td>
</tr>
<tr>
<td>ePE</td>
<td>0.149 ± 0.061</td>
<td>0.120 ± 0.048</td>
<td>0.259 ± 0.121</td>
</tr>
<tr>
<td>PI</td>
<td>0.619 ± 0.245</td>
<td>0.631 ± 0.280</td>
<td>0.804 ± 0.382</td>
</tr>
<tr>
<td>PS</td>
<td>0.278 ± 0.151</td>
<td>0.343 ± 0.193</td>
<td>0.239 ± 0.161</td>
</tr>
<tr>
<td>ePS</td>
<td>0.014 ± 0.008</td>
<td>0.012 ± 0.008</td>
<td>0.007 ± 0.006</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.049 ± 0.023</td>
<td>0.031 ± 0.023</td>
<td>0.030 ± 0.018</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.033 ± 0.018</td>
<td>0.030 ± 0.017</td>
<td>0.027 ± 0.011</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-phophatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS. * p<0.05, **p<0.01, and ***p<0.001 as compared to wildtype.
Table 4.6. Lipid profiles of whole cell lipid extracts from wildtype, Mfn2 and Mfn1 KO MEFs cultured in physiologic ethanolamine (22.5uM ethanolamine), reported as percent of all phospholipids detected

<table>
<thead>
<tr>
<th></th>
<th>Wildtype MEF + Average ±</th>
<th>Mfn2 KO MEF + Average ± StdDev</th>
<th>Mfn1 KO MEF + Average ± StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>0.40 ± 0.07</td>
<td>0.44 ± 0.05</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>PC</td>
<td>49.82 ± 0.91</td>
<td>49.07 ± 0.86</td>
<td>49.88 ± 0.11</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>8.17 ± 1.05</td>
<td>9.59 ± 0.67</td>
<td>8.49 ± 1.58</td>
</tr>
<tr>
<td>ePC</td>
<td>6.02 ± 0.48</td>
<td>4.75 ± 0.28*</td>
<td>6.24 ± 0.17</td>
</tr>
<tr>
<td>Lyso-PE</td>
<td>0.20 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.14 ± 0.01*</td>
</tr>
<tr>
<td>PE</td>
<td>18.92 ± 0.20</td>
<td>19.48 ± 1.35</td>
<td>18.41 ± 1.69</td>
</tr>
<tr>
<td>Cer-PE</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>ePE</td>
<td>2.16 ± 0.05</td>
<td>1.77 ± 0.14*</td>
<td>3.22 ± 0.16*</td>
</tr>
<tr>
<td>PI</td>
<td>9.03 ± 0.45</td>
<td>9.07 ± 0.77</td>
<td>9.95 ± 0.42</td>
</tr>
<tr>
<td>PS</td>
<td>3.93 ± 0.65</td>
<td>4.67 ± 1.03</td>
<td>2.61 ± 0.95*</td>
</tr>
<tr>
<td>ePS</td>
<td>0.19 ± 0.04</td>
<td>0.16 ± 0.05</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.69 ± 0.05</td>
<td>0.40 ± 0.15*</td>
<td>0.35 ± 0.06*</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.46 ± 0.07</td>
<td>0.41 ± 0.06</td>
<td>0.35 ± 0.05</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-phophatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS.* p<0.05 as compared to wildtype.
Table 4.7. Lipid profiles of whole cell lipid extracts from wildtype, Mfn2 and Mfn1 KO MEFs cultured in supra-physiologic ethanolamine (200uM ethanolamine), reported as nmol/g cells

<table>
<thead>
<tr>
<th></th>
<th>Wildtype MEF + Average ±</th>
<th>Mfn2 KO MEF + Average ± StdDev</th>
<th>Mfn1 KO MEF + Average ± StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>0.040 ± 0.025</td>
<td>0.025 ± 0.007</td>
<td>0.022 ± 0.016</td>
</tr>
<tr>
<td>PC</td>
<td>4.803 ± 2.241</td>
<td>3.150 ± 0.565</td>
<td>3.445 ± 2.139</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>0.777 ± 0.325</td>
<td>0.584 ± 0.108</td>
<td>0.639 ± 0.397</td>
</tr>
<tr>
<td>ePC</td>
<td>0.581 ± 0.318</td>
<td>0.284 ± 0.050</td>
<td>0.441 ± 0.284</td>
</tr>
<tr>
<td>Lyso-PE</td>
<td>0.020 ± 0.008</td>
<td>0.013 ± 0.003</td>
<td>0.009 ± 0.006</td>
</tr>
<tr>
<td>PE</td>
<td>1.855 ± 0.570</td>
<td>1.346 ± 0.382</td>
<td>1.154 ± 0.665</td>
</tr>
<tr>
<td>Cer-PE</td>
<td>0.001 ± 0.001</td>
<td>0.000 ± 0.000</td>
<td>0.001 ± 0.000</td>
</tr>
<tr>
<td>ePE</td>
<td>0.207 ± 0.056</td>
<td>0.124 ± 0.033</td>
<td>0.215 ± 0.142</td>
</tr>
<tr>
<td>PI</td>
<td>0.863 ± 0.297</td>
<td>0.642 ± 0.093</td>
<td>0.704 ± 0.425</td>
</tr>
<tr>
<td>PS</td>
<td>0.394 ± 0.212</td>
<td>0.316 ± 0.089</td>
<td>0.422 ± 0.241</td>
</tr>
<tr>
<td>ePS</td>
<td>0.018 ± 0.011</td>
<td>0.011 ± 0.003</td>
<td>0.016 ± 0.009</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.069 ± 0.039</td>
<td>0.026 ± 0.008</td>
<td>0.027 ± 0.016</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.045 ± 0.029</td>
<td>0.023 ± 0.004</td>
<td>0.022 ± 0.012</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-phophatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS.* p<0.05 as compared top wildtype.
Table 4.8. Lipid profiles of whole cell lipid extracts from wildtype, Mfn2 and Mfn1 KO MEFs cultured in supra-physiologic ethanolamine (200uM ethanolamine), reported percentage of all phospholipids detected

<table>
<thead>
<tr>
<th></th>
<th>Wildtype MEF + Average ± StdDev</th>
<th>Mfn2 KO MEF + Average ± StdDev</th>
<th>Mfn1 KO MEF + Average ± StdDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>0.39 ± 0.10</td>
<td>0.38 ± 0.09</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>PC</td>
<td>49.10 ± 2.06</td>
<td>48.19 ± 0.47</td>
<td>48.23 ± 0.42</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>8.05 ± 0.25</td>
<td>8.96 ± 0.81</td>
<td>8.95 ± 0.50*</td>
</tr>
<tr>
<td>ePC</td>
<td>5.82 ± 0.70</td>
<td>4.34 ± 0.08*</td>
<td>6.13 ± 0.23</td>
</tr>
<tr>
<td>Lyso-PE</td>
<td>0.21 ± 0.03</td>
<td>0.19 ± 0.02</td>
<td>0.12 ± 0.01**</td>
</tr>
<tr>
<td>PE</td>
<td>19.79 ± 2.90</td>
<td>20.37 ± 2.93</td>
<td>16.43 ± 0.53</td>
</tr>
<tr>
<td>Cer-PE</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>ePE</td>
<td>2.23 ± 0.38</td>
<td>1.88 ± 0.22*</td>
<td>2.97 ± 0.15*</td>
</tr>
<tr>
<td>PI</td>
<td>9.09 ± 0.66</td>
<td>9.91 ± 1.01</td>
<td>9.92 ± 0.26*</td>
</tr>
<tr>
<td>PS</td>
<td>3.99 ± 1.16</td>
<td>4.85 ± 1.01</td>
<td>6.02 ± 0.24</td>
</tr>
<tr>
<td>ePS</td>
<td>0.18 ± 0.07</td>
<td>0.17 ± 0.03</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.68 ± 0.09</td>
<td>0.39 ± 0.06*</td>
<td>0.38 ± 0.10*</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.44 ± 0.09</td>
<td>0.36 ± 0.00</td>
<td>0.33 ± 0.03</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-phophatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS.* p<0.05 as compared to wildtype. * p<0.05, **p<0.01, and ***p<0.001 as compared to wildtype.
Figure 4.1. Two major PE synthesis pathways exist in mammalian cells. It has been reported that Mfn2 mediates tethering of an ER sub-domain known as the mitochondria associated membrane (MAM) to outer mitochondrial membranes. By mediating MAM-mitochondria associations, Mfn2 is in a position to regulate the transport of PS to mitochondria, in a mechanism known to involve an outer mitochondrial membrane protein. We hypothesized that the movement of PS from MAM to mitochondrial membranes depends on the presence of Mfn2. Thus, loss of Mfn2 would result in decreased mitochondrial PE levels. The CDP-ethanolamine (CDP-Etn) pathway is a redundant PE synthesis pathway that resides in the ER. The CDP-ethanolamine pathway does not rely on ER interactions with mitochondria but is dependent on extracellular ethanolamine. Supplementation or removal of extracellular ethanolamine from media can modulate the contribution of the CDP-ethanolamine and Pisd based pathway. Additionally, substrate preferences of Pisd and the CDP-ethanolamine enzymes (Pyct2) result in the generation of different PE species, which we refer to as PE$_{Pisd}$ or PE$_{CDP-Etn}$. Similarly, two PC synthesis pathways share many of the same features as PE synthesis. We refer to the PC species synthesized by Pemt within the MAM as PC$_{Pemt}$, and PC synthesized within the ER by Pcyt1 as PC$_{CDP-Cho}$. Sub-population of PC and PE represent preferences of the corresponding pathways and are not exclusively synthesized. However, we suggest they can be used to investigate the contribution of MAM-mitochondria dependent and independent PE and PC synthesis pathways. Specifically, we hypothesized that in the absence of ethanolamine, loss of Mfn2 would lead to specific decreases in PE$_{Pisd}$ sub-subpopulations.
Figure 4.2. TLC and MS/MS analysis of phospholipids reveals changes in the abundance of PE in both Mfn1 and Mfn2 KO MEFs. (A) Phospholipids were separated by TLC, levels of PE were quantified by densitometry and normalized to PC to control for potential loading errors (N=3 for each condition). Significant decreases are observed in both Mfn2 KO and Mfn1 KO lipid extracts. (B) While PE species from Mfn1 and Mfn2 KO MEFs migrated within the range expected for PE as determined by known standards, the center of PE spots from Mfn1 and Mfn2 KO MEF migrated significantly shorter distances (retention factor (Rf)), indicating a qualitative change in the chemistry of these PE populations. No changes in the abundance or Rf values of PC were observed (see supplemental data). (N=3 for each experimental condition; * p<0.05, ***p<0.005).
Figure 4.3. Phospholipidomic analysis of whole cell Mfn2 and Mfn1 KO MEFs reveal decreases in PE and increases in PS which are rescued by the addition of 20μM ethanolamine. Phospholipid species from whole cell lipid extracts from wildtype (wt), Mfn2 KO (2KO) and Mfn1 KO (1KO) MEFs cultured in regular media (-ETN) and in 20μM ethanolamine (+ETN) were analyzed using ESI-MS/MS and reported as nmol/g cell pellet (A,C) or as a % of all phospholipids detected (B,D). (N=3 for each experimental condition; * p<0.05, ***p<0.005).
Figure 4.4. Induction of autophagy results in inverse changes in the relative abundance of PE in Mfn1 and Mfn2 KO MEFs. While autophagy induced by 2 hour serum starvation specifically decreases PE preferentially synthesized by mitochondria (PE_{Pisd}) in wt MEFs, the reverse effect is observed in Mfn1 and Mfn2 KO MEFs. In Mfn2 and Mfn1 KO MEFs, starvation induced autophagy does not significantly alter PE_{eACMS}, yet increases PE_{SMDT}. (N=3 for each experimental condition; * p<0.05, **p<0.01, ***p<0.005).
Figure 4.5. Subpopulation analysis of PE, PC, and PI. Analysis of subpopulations of PE, PC, and PI in wt, Mfn2, and Mfn1 KO MEFS deprived of ethanolamine reveal a reciprocal relationship between decreasing levels of species with 4 or more unsaturated bonds and increasing levels of those with 3 or fewer unsaturated bonds. Red indicates a decrease, green indicated an increase, while the presence of arrows indicates statistically significant differences (p<0.05).
Figure 4.6. Stratification of subpopulations of PE and PC based on acyl-chain composition demonstrate decreases in levels of species preferentially synthesized by Pisd and Pemt in Mfn1 and Mfn2 KO MEFs deprived of ethanolamine. (A) We stratified total PE and PC into sub-populations that represent species with acyl chain composition preferentially generated by MAM-mitochondria based (Pisd and Pemt) and ER based (CDP-Etn/Cho) pathways. The graph (B and C) error bars indicate standard deviation of each sub-population reported as nmol/mg cell and the percentage values indicated the fraction of PE or PC made up of each sub-population. Both PE_{Pisd} (B) and PC_{Pemt} (C) species reported as nmol/g cell pellet and as a percent of PE or PC are decreased in Mfn2 (2KO) and Mfn1(1KO) KO MEFs deprived of ethanolamine. Likewise, PE and PC species derived from CDP-ethanolamine and CDP-choline are increased in Mfn2 and Mfn1 KO MEFs (N=3 for each experimental condition; * p<0.05, **p<0.01, ***p<0.005).
Figure 4.7. Unsaturation index as a function of media ethanolamine concentration. (A) Analysis of the unsaturation index (UI) of whole cell lipid extracts from wildtype (wt), Mfn2 KO and Mfn1 KO MEFS reveals an increase in the UI of wildtype lipid extracts as media ethanolamine concentration decreases that is not apparent when either mitofusin is absent. (B) When PE and PC are considered separately from all other phospholipid species (upper curve), it is apparent that the lack of UI increase in the absence of Mfn1 or Mfn2 is due to a decrease in the UI of PE and PC populations. All other species contribute a small increase in UI (lower curve). (N=3 for each experimental condition; * p<0.05)
Figure 4.8. Morphological analysis demonstrates the addition of ethanolamine does not alter mitochondrial morphology in wt of Mfn2 MEFs. Relative frequency of mitochondrial shape in WT and Mfn2 KO MEF cells. Roundness, as measured by ImageJ is \(4 \times \left(\frac{\text{Area}}{\pi \times \text{Major axis}^2}\right)\), where shapes approach a perfect circle as they approach 1. (A) shows distribution for WT cells in 2.2 μM Etn and in 200 μM Ethanolamine. (B) shows distribution for Mfn2 KO in 2.2 μM Etn cells and in 200 μM. (Frequencies average of 5 cells in 3 triplicate experiments). Average measures of multiple parameters analyzed by ImageJ demonstrated no significantly differences in any morphological measures in either cell line with the addition of ethanolamine.
CHAPTER 5. PHOSPHOLIPID PROFILING OF CMT2A PATIENT LYMPHOBLASTS AND PLASMA.

Summary and Background.

We previously reported how the absence of Mfn2 altered phospholipid profiles in whole cell and mitochondrial enriched lipid extracts from mouse embryonic fibroblasts. Specifically, we demonstrated an important role for Mfn2 in the mitochondrial synthesis of the fusogenic phospholipid phosphatidylethanolamine (PE). When the primary PE synthesis pathway is limited by low extracellular ethanolamine Mfn2 KO cells cannot maintain normal cellular PE levels. Thus, Mfn2 is required for redundant MAM-mitochondrial PE dependent synthesis pathways. Secondly, we showed PE and phosphatidylcholine PC enriched in highly unsaturated fatty acids preferentially synthesized at the MAM-mitochondria interface were also be reduced in Mfn2 KO MEFs. Lastly, our results indicated that ether linked PE (ePE), also known as ethanolamine plasmalogen is decreased in Mfn2 null cells independent of extracellular ethanolamine.

Interestingly, PE, ePE and PC are known to be important cellular pools of arachidonic acid (20:4ω6)(Perez-Chacon and others 2009). These are specifically targeted by lipases, which free arachidonic(Perez-Chacon and others 2009). Arachidonic acid (20:4) is synthesized from dietary linoelic acid (18:2) and is the precursor for over 20 prostaglandin species that regulate hemodynamics, inflammation and immunity(Fan and Chapkin 1998). Decreases in ACMS, have been reported in the sciatic nerve of models of diabetic neuropathy and Schwann cells cultured in hyperglycemic conditions(Kuruvilla and Eichberg 1998; Miinea and
others 2002). It is thought that ACMS depletion results in downstream abnormalities in the ratios of prostacyclin/thromboxane species, resulting in chronic vasoconstriction of the peripheral nerve blood vessels, chronic nerve ischemia, and eventual neuronal injury (Kuruvilla and Eichberg 1998; Miinea and others 2002).

Early clinical investigations by Dyck et al. identified significant reductions in linoelcic acid, the precursor of arachidonic acid, in serum from CMT2 patients (Yao, Ellefson, Dyck 1976; Yao and Dyck 1978). Thus, beyond disrupting PE synthesis, disruption of MAM-mitochondrial interfaces in the absence of Mfn2 may have consequences in the arachidonic acid composition of membrane phospholipids.

To investigate how CMT2A mutations influence cellular phospholipid profiles, we profiled cellular phospholipids of CMT2A patients and control lymphoblasts with and without ethanolamine supplementation. We hypothesized that mutations in MFN2 would result in decreased levels of PE. More specifically, we proposed that PE enriched in polyunsaturated fatty acids would be specifically reduced. We also proposed that abnormalities in the arachidonic acid composition of PE and PC would be reflected in CMT2A patient plasma levels of the arachidonic acid precursor linoelcic acid, an observation previously reported in CMT2 patients (Yao, Ellefson, Dyck 1976). To explore this relationship, we also screened CMT2A and control patient plasma fatty acid profile to investigate potential abnormalities in linoelcic acid.
Materials and Methods.

Cell Culture.

The Hussman Institute for Human Genomics had three CMT2A patient lymphoblast lines available, the oldest collecting ~30 years ago (1989). Additionally, four control lymphoblast lines were selected as well. Of the seven lines available, three controls and two CMT2A patient lines were viable after prolonged freezing. Both CMT2A patient lymphoblasts carried the R280H mutations, but are from independently collected families. The R280H mutation is a frequent MNF2 mutation associated with a pure CMT2 clinical phenotype and has been reported in the literature multiple times. The controls were collected from non-affected family members from CMT families, and age and sex matched to the resulting the CMT2A cases. Nonadherent immortalized lymphoblasts were grown in 15% FBS RPMI 1640 media (Invitrogen, Carlsbad, CA). Where indicated, a 20mM ethanolamine (Sigma, E0135) stock solution was added to media to a final concentration of 20μM for a period of 10 days prior to lipid analysis.

Lipid Extraction.

Phospholipids were extracted from whole cells and organelle fractions from MEF cells using a modified Bligh-Dyer procedure described elsewhere (Hanahan 1997). Briefly, phospholipids from cells or mitochondria are extracted using a chloroform: methanol: cell or organelle (1:2:0.8 vol/vol/vol) solvent system. Butylated hydroxytoluene (B1378, Sigma, St Louis, MO) was added to all chloroform solutions (0.01%) to protect from oxidation. The solvent mixture is mixed and allowed sit for 30 minutes prior to removal of the lower organic layer and three
washes with one volume of chloroform. The combined lower layers were again washed with one volume of 1M KCl, and with 1 volume water. The extract containing the phospholipid extract was dried down under a stream of nitrogen and stored at -80°C under nitrogen prior to analysis.

**Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) lipid profiling.**

An automated electrospray ionization-tandem mass spectrometry approach was used, and data acquisition and analysis and acyl group identification were carried out by the Kansas Lipidomics Research Center's Analytical Laboratory as previously described (Devaiah and others 2006). The lipid samples were dissolved in 1 mL chloroform and aliquots of 10-35 µL of extract was analyzed. Precise amounts of internal standards, obtained and quantified as previously described (Welti and others 2002), The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.4 ml. Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 µl/min. Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software. The first and typically every 11th set of mass spectra were acquired
on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the two internal standards on the same lipid class, except for PI, which was quantified in relation to a single internal standard. Ether-linked (alk(eny)l,acyl) lipids were quantified in comparison to the diacyl compounds with the same head groups without correction for response factors for these compounds as compared to their diacyl analogs. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the “internal standards only” spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each “internal standards only” set of spectra was used to correct the data from the following 10 samples. The data were corrected for and reported as the fraction of the sample analyzed.

**Analysis and classification of phospholipid species.**

Phospholipid levels were reported as nmoles detected normalized to the cell or mitochondrial pellet weight prior to lipid extraction and as a mole % of all phospholipid moles detected. The data is reported as the average of triplicate experiments. Significance was calculated using two tailed unpaired student t-tests based on hetero/homoscedasticity of sample populations.
Selection of patient plasma.

All available genotyped CMT2A patient plasma samples were used in this study. Best efforts to sex and age match control samples were made using unaffected controls from CMT families. Pertinent patient sample information can be found in Table 5.3.

Gas Chromatography- Flame Ionization Detection (IGC-FID).

Plasma analysis was carried out by Kronos Science Laboratory (Phoenix, AZ). With the addition of internal standard and chemical reagents, the lipids in the plasma cells are hydrolyzed and converted to methyl esters. The fatty acid methyl esters (FAME) obtained are extracted, purified and reconstituted in hexane for GC-FID measurement. One uL of FAME extracts are separated via gas chromatography on a Supelco SP-2560 (100m x 0.25mm, film thickness 0.2μm) capillary column with H2 as the carrier gas. Total GC run time is 25 minutes per sample.

Results.

PE levels are normal in CMT2A patient lymphoblasts in media with reduced and physiologic levels of ethanolamine.

Our previous phospholipid profile results from Mfn2 KO MEFs led us to hypothesize that mutations in MFN2 would also result in a reduction in cellular levels of PE. To test this hypothesis, we performed the same mass spectrometry phospholipid screen (ESI-MS/MS) on cells derived from CMT2A patients. To assess how exogenous ethanolamine influences cellular PE levels, lymphoblasts were grown separately in media deprived of ethanolamine (~3μM ethanolamine, or 15% fetal bovine serum) and 20μM ethanolamine.
Mass spectrometry results indicate PE levels in CMT2A lymphoblasts do not significantly differ from controls (Table 5.1 and Figure 5.1). This observation holds true with and without physiologic ethanolamine levels and when PE levels are reported as a percent of all phospholipids detected and as nmole/ug values (Figure 5.1). This is in striking contrast to the results we observed in Mfn2 null cells, which levels are PE are roughly half of control levels when cells are deprived of exogenous ethanolamine (Figure 4.2). Likewise, supplementing Mfn2 KO MEF media with physiologic levels of ethanolamine rescued this PE deficiency (Figure 4.2). Our results concerning CMT2A patient lymphoblasts indicate mutations in MFN2 do not lead to low cellular levels of PE or alterations in acyl chain composition of PE subpopulations (supplemental data). Additionally, supplementing media with ethanolamine does not alter the phospholipid profiles of CMT2A or control lymphoblast (Tables 5.1 and 5.2).

**ePE levels are reduced in CMT2A patient lymphoblasts with and without ethanolamine supplementation.**

While our hypothesis was specifically focused on Mfn2 related changes in PE, the phospholipid screen indicated that other ethanolamine derived phospholipids, was significant lower in CMT2A lymphoblast cells (Table 5.1 and 5.2, Figure 5.1). Decreases in ePE did not appear to be dependent on extracellular ethanolamine levels, as ethanolamine supplementation did not alter ePE levels in either group. Both nmole/ug (~35-48%) and percent values (~26-33%) of ePE are decreased (Figure 5.1). Examination of ePE acyl composition also revealed that decreased in ePE stem mostly from ePE species containing acyl chains with four or more unsaturated bonds.
When ePE subpopulations with four or more unsaturated bonds is considered separate from other ePE species, it become apparent that ePE species with four or more unsaturated bonds are responsible for the overall ePE decrease (Figure 5.1). Species of ePE with less than four unsaturated double bonds show decreasing trends, yet are not statistically significant (Figure 5.1).

To examine the distribution of ePE levels over the multiple measurements, we examined the ranges of ePE levels of control and CMT2A lymphoblasts. When expressed as a percent of all phospholipids detected, the lowest control ePE levels is still greater than the highest CMT2A level (Figure 5.2). When considering species of ePE that contain acyl chain groups with four or more unsaturated bonds, the gap between control and CMT2A lymphoblasts increases further (Figure 5.2). When reported as a percent value or as nmol/ug, the lowest control values fall outside of the highest CMT2A values. The distribution of these ePE in this small sample set suggests ePE levels may even have utility as a clinical marker in CMT.

We also reexamined phospholipid data from the Mfn2 and Mfn1 null MEF models for changes in PE. While changes in ePE are not significantly different when expressed as nmol/ug values, decreases in the percent levels of ePE are present specifically in Mfn2 KO MEFs in the presence and absence of ethanolamine supplementation (Table 4.4, 4.6, 4.8, and Figure 5.3). As ePE levels in MEF cells appear to be ~50% of lymphoblast levels, changes in the MEF lines may approach limits of detection. However, deficiencies in ePE appear specific to loss of Mfn2, as the absence of Mfn1 actually results in an increase is ePE. Interestingly, the deficiency in ePE appears independent of extracellular ethanolamine, much like
CMT2A patient lymphoblasts. Changes in ePE are the single consistent phospholipid change observed in both Mfn2 null models and CMT2A patient lymphoblasts.

**PC and ePC have inconsistent abnormalities in CMT2A patient lymphoblasts.**

In addition to changes in ePE, other changes in choline-derived phospholipids are significantly altered. PC percentage levels are slightly increased (~4.4%) in CMT2A lymphoblasts when media is supplemented with ethanolamine (Figure 5.4). However, the same change does not appear when results are presented as nmol/ug. This change likely reflects a compensatory or reciprocal increase related to decreases in ePE and ePC levels.

Levels of ePC appear decreased under several conditions. While generally decreased in CMT2A lymphoblasts, ePC levels are significantly different when reported as a percent of phospholipid detected in CMT2A supplemented with 20uM ethanolamine (Figure 5.4). In the other instance, nmole/ug levels of ePE appear to be decreased in CMT2A cells deprive of ethanolamine (Figure 5.4). It is unclear exactly why ePC levels appear to be intermittently decreased, other than to suppose a very small deficiency exists in CMT2A that approaches the detection limit.

**Nervonic acid is elevated in CMT2A patient plasma.**

To investigate potential lipid related changes in the plasma of CMT2A patients, nine control and nine CMT2A patient plasma samples were analyzed by gas chromatography- flame ionization detection (GC-FID). No abnormally low or high levels of any fatty acyl species were detected, using the Kronos patient serum database as a reference (Kronos Science, Phoenix, AZ). Levels of nervonic acid were significantly elevated in the plasma of CMT2A patient plasma (Table 5.4). By
measures of uM and ug/mL, nervonic acid is elevated 38.8% and 47.6% respectively. No other changes in naturally occurring fatty acyl species were detected in CMT2A patient lymphoblasts.

**Trans fatty acids are reduced in CMT2A patient plasma.**

In addition to detecting elevations in nervonic acid, significant decreases were observed in elaidic and linoelaidic acid species (Table 5.4). Decreases were observed in all measured values (percent weight, uM, ug/mL and %mole). Mean changes in elaidic acid (C18:1w6t) were significantly decreased by ~50% in CMT2A patient lymphoblast. Average values of linoelaidic acid (C18:2 w6t) were reduced by ~25% in CMT2A patient plasma. Together, these changes account for decreases in total trans fatty acyl uM and ug/mL levels of ~40-50% (Table 5.4). When the individual control and CMT2A patient plasma trans fatty acids levels are examined, it is clear that there is a large overlap in their distribution (Figure 5.5)

**Discussion**

The previous chapter indentified profound decreases in cellular and mitochondrial levels of PE in Mfn2 KO MEFs deprived of ethanolamine. Deficiencies in PE were revered when physiologic levels of ethanolamine were supplemented in the media. These data led us to propose that deficiencies in PE would also result from mutations in MFN2. To test this hypothesis, we performed a mass spectrometry based phospholipid screen on control and CMT2A patient lymphoblasts cells. Our results indicate that PE is not decreased in CMT2A patient cells. Furthermore, supplementation of media with physiologic levels of ethanolamine does not alter
cellular phospholipid profiles of control or CMT2A lymphoblast cells. These data together suggest that mutations in MFN2 do not result in the many of the same lipid changes observed in *Mfn2* null models.

The one exception is ether-linked PE or ethanolamine plasmalogens (ePE). In both CMT2A patient lymphoblasts and in *Mfn2* KO MEFs, levels of ePE are decreased as compared to controls. Furthermore, this decrease appears to be specific to *Mfn2* and not *Mfn1* null cells, and is independent of extracellular ethanolamine concentration. Thus, beyond disrupting phospholipid synthesis pathways, particularly the movement of PS from the MAM to mitochondrial membranes, MFN2 may have additional roles in ePE synthesis that are specifically disrupted by CMT2A mutations.

ePE, also known as ethanolamine plasmalogen, has a very similar structure as PE but like all ether plasmalogens contains a vinyl ether bond at the *sn-1* position instead of ester bond. ePE is a major lipid species and can account for up to 60% of brain ethanolamine glycerophospholipids (Han, Holtzman, McKeel 2001). Human brain ePE levels increase to peak levels at age of 30-40 years, then decline with age (Davison and Thompson 1981; Rouser and Yamamoto 1968). Much like PE, the *sn-1* position include shorter and more saturated acyl chains, were as the *sn-2* position is enriched in longer polyunsaturated fatty acids like arachidonic acid (Hawthorne and Ansell 1982). The first steps of ePE synthesis occur within the peroxisome, and diseases stemming from peroxisome dysfunction are associated with low levels of ePE (Farooqui, Horrocks, Farooqui 2000; Farooqui and Horrocks 2001). Interestingly, MFN2 expression is driven by the transcription factor peroxisome
proliferator-activated receptor-γ coactivator (PGC)-1α (Soriano and others 2006; Zorzano 2009; Zorzano and others 2010).

ePE has many relevant functions within neural and glia cells. ePE has a unique biophysical structure that has a tendency to form inverse hexagonal structures that influence membranes fluidity and fusion as well as how membranes interact with integral membrane proteins (Farooqui and Horrocks 2001; Lohner 1996). Likewise, ePE is a critical component of synaptic vesicles and is required for their fusion with the synaptic plasma membrane (Breckenridge and others 1973). The vinyl ether bond also makes plasmalogens vulnerable to oxidative attack by free radicals such that plasmalogens serve to protect cellular membranes from oxidative stress (Calzada, Bruckdorfer, Rice-Evans 1997; Hahnel, Beyer, Engelmann 1999; Zoeller and others 1999; Zommara and others 1995).

Much like PE, ePE serves as a reservoir for arachidonic acid and other eicosanoid precursors (Neuberger and van Deenen 1981). Through the action of phospholipase A2 (PLA2), the sn-2 acyl component, arachidonic acid or long chain ω-3 fatty acids are freed which can then be directed toward synthesis of eicosanoid species (Nagan and Zoeller 2001). Interestingly, it is species of ePE with four or more double bonds which are enriched in arachidonic acid and long chain ω-3 fatty acids that appear to be most decreased in CMT2A patient lymphoblasts. Thus, a specific deficiency in ePE enriched in arachidonic acid and other long change ω-6 and ω-3 fatty acids may have importance in the pathogenesis of CMT2A.

Lastly, ePE has some unique characteristics with regards to cellular transport. Intercellular cholesterol efflux and intracellular cholesterol transport are both
influenced by cellular levels of ePE (Mankidy and others 2010; Munn and others 2003). Notably, while all phospholipids can be synthesized within neuronal as well as axonal compartments, cholesterol can only be synthesized within the soma and thus requires transport down the axon (Vance, Campenot, Vance 2000). Species of ePE with 3 or more unsaturated bonds have a strong influence on the esterification of cholesterol in cell models (Mankidy and others 2010). Interestingly, Dyck et al described significantly lowered rates of cholesterol esterification in CMT2 patient plasma (Yao, Ellefson, Dyck 1976). The transport of ePE is also unique in that it may be transported and differentially unloaded to the axolemma and even to opposing myelinating membranes (Ledeen and Haley 1983; Toews and others 1988). A central neuron specific knock out of model of Pex5 drastically decreases CNS levels of plasmalogens (Bottelbergs and others 2010; Hulshagen and others 2008). Marked axonal degeneration was observed across multiple CNS regions. Axon injury appeared multifocal with clearly visible accumulation of debris between the axonal and inner myelin sheath membrane (Hulshagen and others 2008). It remains to be determined what role ePE might have in the peripheral nervous system. Interestingly, treatment of ePE deficient cell models with ePE precursors (1-alkyl-2-acyl glycerols) restored ePE to normal (Mankidy and others 2010). Furthermore, treatment with ePE precursors could restore specific ePE species enriched in polyunsaturated fatty acids with 3 or more unsaturated bonds. Such a strategy should be investigated if ePE levels are found to be deficient in forthcoming CMT2A mouse models or in tissue and cells from more CMT2A patients.
Original investigations into lipid abnormalities in hereditary neuropathies identified significant decreases in linoelic acid (18:2ω-6) in serum triglycerides and cholesterol esters from CMT2 patients (Yao, Ellefson, Dyck 1976). Linoelic acid is the precursor of arachidonic acid (20:4ω-6). We have previously described decreases in phospholipid groups enriched in arachidonic acid in the absence of Mfn2. Thus, we sought to determine linoelic acid levels in plasma from CMT2A patients with MFN2 mutations. Our results indicate that there are no differences in the linoleic acid content of plasma lipids.

Nervonic acid (C24:1ω-9) levels are increased in CMT2A patient plasma. Reductions in sphingomyelin containing nervonic acid levels have been reported in type 1 diabetes models and multiple sclerosis (Fox and others 2011; Sargent, Coupland, Wilson 1994). Dyck et al. also reported significantly higher levels of nervonic acid in serum sphingolipids from CMT1, CMT2, Dejerine-Sottas disease (HMSN III) and Fredrick Ataxia patients (Yao and Dyck 1978). The commonality of this finding in multiple neuropathies suggests elevated nervonic acid levels are likely a secondary feature of axonal degeneration in CMT2A.

Additionally there are significant changes in trans fatty isomers of linoleic acid (linoelaidiac acid) and oleic acid (elaidiac acid) in plasma from CMT2A patients. The analytical method we employed discriminates between cis and trans isomers, yet the mass spectrometry method employed by Dyck et al. did not (Yao, Ellefson, Dyck 1976; Yao and Dyck 1978). Likewise, our analysis profiled the acyl chain components of all plasma lipids, where as the previous reports separated each lipid class prior to analysis. Regardless, it is unclear why trans fatty acids would be
decreased in CMT2A, though we believe it may be a benign consequence of altered lipid and cholesterol handling in CMT2A.

**Conclusions**

Investigations using CMT2A patient cell lines and plasma have added to our understanding of how mutations in MFN2 alter cellular phospholipid profiles. The original phospholipid abnormalities surrounding profound PE deficiencies in *Mfn2* KO cells overshadowed deficiencies in ePE which are independent of extracellular ethanolamine. Numerous reports have described biological roles for ePE which appear relevant to CMT2A. Future work should reexamine ePE levels in tissues from CMT2A mouse models and CMT2A patient cell lines and tissues. Most promisingly, treatment with ePE precursors may hold promising in restoring ePE levels *in vivo* should ePE levels prove a critical component of CMT2A pathogenesis.
<table>
<thead>
<tr>
<th></th>
<th>~3uM ETN</th>
<th></th>
<th>~23uM ETN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTRL</td>
<td>CMT2A</td>
<td>CTRL</td>
</tr>
<tr>
<td>Lyso PC</td>
<td>0.156 ±0.021</td>
<td>0.143 ±0.040</td>
<td>0.161 ±0.012</td>
</tr>
<tr>
<td>PC</td>
<td>46.106 ±2.028</td>
<td>47.854 ±0.400</td>
<td>45.07 ±0.758</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>9.952 ±0.554</td>
<td>8.658 ±1.272</td>
<td>9.808 ±0.032</td>
</tr>
<tr>
<td>ePC</td>
<td>8.091 ±1.207</td>
<td>7.216 ±0.938</td>
<td>7.942 ±0.092</td>
</tr>
<tr>
<td>LysoPE</td>
<td>0.078 ±0.011</td>
<td>0.071 ±0.012</td>
<td>0.078 ±0.033</td>
</tr>
<tr>
<td>PE</td>
<td>21.22 ±1.550</td>
<td>22.295 ±0.774</td>
<td>21.53 ±2.638</td>
</tr>
<tr>
<td>PE-Cer</td>
<td>0.028 ±0.011</td>
<td>0.035 ±0.044</td>
<td>0.033 ±0.013</td>
</tr>
<tr>
<td>ePE</td>
<td>4.172 ±0.284</td>
<td>2.822 ±0.187**</td>
<td>4.339 ±0.133</td>
</tr>
<tr>
<td>PI</td>
<td>5.919 ±1.842</td>
<td>5.899 ±0.807</td>
<td>6.379 ±2.273</td>
</tr>
<tr>
<td>PS</td>
<td>3.446 ±0.582</td>
<td>4.306 ±1.110</td>
<td>3.79 ±1.165</td>
</tr>
<tr>
<td>ePS</td>
<td>0.151 ±0.041</td>
<td>0.195 ±0.036</td>
<td>0.169 ±0.062</td>
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<tr>
<td>Phosphatidic acid</td>
<td>0.303 ±0.220</td>
<td>0.194 ±0.099</td>
<td>0.325 ±0.220</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.377 ±0.050</td>
<td>0.312 ±0.018</td>
<td>0.376 ±0.030</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide-
phophatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS. * p<0.05, **p<0.01, and ***p<0.001 as compared to control.
Table 5.2  Lipid profiles of whole cell lipid extracts from control and CMT2A patient lymphoblast reported as nmoles per μgram of cell pellet.

<table>
<thead>
<tr>
<th></th>
<th>~3uM ETN</th>
<th></th>
<th>~23uM ETN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTRL</td>
<td>CMT2A</td>
<td>CTRL</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>Std Dev</td>
<td>Average</td>
</tr>
<tr>
<td>Lyso PC</td>
<td>0.014 ± 0.004</td>
<td>0.010 ± 0.003</td>
<td>0.011 ± 0.002</td>
</tr>
<tr>
<td>PC</td>
<td>4.065 ± 0.992</td>
<td>3.185 ± 0.004</td>
<td>3.304 ± 0.388</td>
</tr>
<tr>
<td>SM and DSM</td>
<td>0.877 ± 0.213</td>
<td>0.577 ± 0.084</td>
<td>0.808 ± 0.088</td>
</tr>
<tr>
<td>ePC</td>
<td>0.695 ± 0.058</td>
<td>0.483 ± 0.063*</td>
<td>0.452 ± 0.055</td>
</tr>
<tr>
<td>LysoPE</td>
<td>0.007 ± 0.000</td>
<td>0.005 ± 0.001</td>
<td>0.008 ± 0.002</td>
</tr>
<tr>
<td>PE</td>
<td>1.866 ± 0.491</td>
<td>1.484 ± 0.050</td>
<td>1.772 ± 0.279</td>
</tr>
<tr>
<td>PE-Cer</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.000</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>ePE</td>
<td>0.361 ± 0.056</td>
<td>0.189 ± 0.013*</td>
<td>0.317 ± 0.044</td>
</tr>
<tr>
<td>PI</td>
<td>0.494 ± 0.038</td>
<td>0.392 ± 0.056</td>
<td>0.459 ± 0.166</td>
</tr>
<tr>
<td>PS</td>
<td>0.291 ± 0.016</td>
<td>0.286 ± 0.073</td>
<td>0.281 ± 0.088</td>
</tr>
<tr>
<td>ePS</td>
<td>0.013 ± 0.001</td>
<td>0.013 ± 0.002</td>
<td>0.011 ± 0.005</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.028 ± 0.023</td>
<td>0.013 ± 0.007</td>
<td>0.026 ± 0.014</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.033 ± 0.009</td>
<td>0.021 ± 0.001</td>
<td>0.025 ± 0.005</td>
</tr>
</tbody>
</table>

Lyso-PC, lysophosphatidylcholine; ePC, ether linked PC; Lyso-PE, lysophosphatidylethanolamine; Cer-PE, ceramide- phosphatidylethanolamine; ePE, ether-linked PE; ePS, ether linked PS. * p<0.05, **p<0.01, and ***p<0.001 as compared to control.
<table>
<thead>
<tr>
<th>Table 5.3 Plasma Sample Characteristics</th>
</tr>
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<tbody>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Ethnicity</td>
</tr>
<tr>
<td>European</td>
</tr>
<tr>
<td>Age at Collection</td>
</tr>
<tr>
<td>Range in years:</td>
</tr>
<tr>
<td>Average in years:</td>
</tr>
<tr>
<td>Average years since Collection</td>
</tr>
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</table>
Table 5.4. Significantly different results from gas chromatography/flame ionization detector (GC/FID) analysis of plasma from CMT2A patients and controls.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CTRL VALUE</th>
<th>STD DEV</th>
<th>CASE VALUE</th>
<th>STD DEV</th>
<th>p Value</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:1w9t ELAIDIC (M%)</td>
<td>0.54 ± 0.17%</td>
<td></td>
<td>0.26 ± 0.12%</td>
<td></td>
<td>0.00095</td>
<td>48.2%</td>
</tr>
<tr>
<td>C18:1w9t ELAIDIC (ME)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.48 ± 5.36 ug/mL</td>
<td></td>
<td>9.41 ± 4.40 ug/mL</td>
<td></td>
<td>0.00302</td>
<td>53.8%</td>
</tr>
<tr>
<td>C18:1w9t ELAIDIC (UG/ML)</td>
<td>16.65 ± 5.11 ug/mL</td>
<td></td>
<td>8.96 ± 4.20 ug/mL</td>
<td></td>
<td>0.00302</td>
<td>53.8%</td>
</tr>
<tr>
<td>C18:1w9t ELAIDIC (WT%)</td>
<td>0.55 ± 0.18%</td>
<td></td>
<td>0.27 ± 0.12%</td>
<td></td>
<td>0.00115</td>
<td>48.5%</td>
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<tr>
<td>C18:2w6t LINOLELAIDIC (M%)</td>
<td>0.07 ± 0.02%</td>
<td></td>
<td>0.05 ± 0.02%</td>
<td></td>
<td>0.00995</td>
<td>71.2%</td>
</tr>
<tr>
<td>C18:2w6t LINOLELAIDIC (ME)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.32 ± 0.25 ug/mL</td>
<td></td>
<td>1.87 ± 0.40 ug/mL</td>
<td></td>
<td>0.01108</td>
<td>80.6%</td>
</tr>
<tr>
<td>C18:2w6t LINOLELAIDIC (UG/ML)</td>
<td>2.21 ± 0.24 ug/mL</td>
<td></td>
<td>1.78 ± 0.38 ug/mL</td>
<td></td>
<td>0.0114</td>
<td>80.7%</td>
</tr>
<tr>
<td>C18:2w6t LINOLELAIDIC (UM)</td>
<td>7.88 ± 0.84 uM</td>
<td></td>
<td>6.35 ± 1.36 uM</td>
<td></td>
<td>0.01118</td>
<td>80.6%</td>
</tr>
<tr>
<td>C18:2w6t LINOLELAIDIC (WT%)</td>
<td>0.07 ± 0.02%</td>
<td></td>
<td>0.05 ± 0.02%</td>
<td></td>
<td>0.01862</td>
<td>72.7%</td>
</tr>
<tr>
<td>C24:1w9c NERVONIC (ME)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.14 ± 5.50 ug/mL</td>
<td></td>
<td>19.41 ± 5.26 ug/mL</td>
<td></td>
<td>0.02508</td>
<td>147.7%</td>
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<tr>
<td>C24:1w9c NERVONIC (UM)</td>
<td>36.78 ± 9.30 uM</td>
<td></td>
<td>51.07 ± 3 uM</td>
<td></td>
<td>0.02047</td>
<td>138.8%</td>
</tr>
<tr>
<td>TOTAL TRANS FATTY ACIDS (UG/ML)</td>
<td>18.96 ± 5.19 ug/mL</td>
<td></td>
<td>10.74 ± 4.52 ug/mL</td>
<td></td>
<td>0.0025</td>
<td>56.7%</td>
</tr>
<tr>
<td>TOTAL TRANS FATTY ACIDS (UM)</td>
<td>61.80 ± 24.65 uM</td>
<td></td>
<td>38.13 ± 4 uM</td>
<td></td>
<td>0.02812</td>
<td>61.7%</td>
</tr>
</tbody>
</table>
Figure 5.1. ePE levels are decreased in CMT2A patient lymphoblasts. (A and B) Levels of PE appear to not significantly differ in CMT2A patient lymphoblasts cultured with and without ethanolamine. Levels of ePE (C and D), and in particular ePE containing acyl chains with 4 or more unsaturated bonds (E and F) (C=C≥4) are decreased regardless of ethanolamine supplementation. Although ePE containing acyl chains with 3 or fewer unsaturated bonds trend to decrease, the differences are not significant as compared to wildtype.
Figure 5.2. Distribution of replicate measurements demonstrate ePE levels of CMT2A patient cells do not overlap with control values. Total ePE represented as % of all phospholipids detected (A) and subpopulations of ePE containing acyl chains with more than 4 unsaturated bonds represented as a % or as nmol/ug reveal a gap between the lowest control and the highest CMT2A levels.
Figure 5.3. ePE levels are also decreased in Mfn2 KO MEFs but not Mfn1 KO MEFs with and without ethanolamine supplementation. (A) When reported as nmole/ug, ePE is reduced in Mfn2 KO MEFs but is not significant. (B) When examined as % of all phospholipids detected, significant reductions in ePE are observed in Mfn2 KO MEFs only. Note that ePE levels are ~1/2 to 1/3 of those observed in human lymphoblasts, which may explain why more clear differences are observed in lymphoblasts.
Figure 5.4. Levels of ePC demonstrate a decreased trend CMT2A. (A and B) Levels of PC when analyzed as nmole/ug values show a decrease where as the opposite trend is observed when considered as a % of all phospholipids detected. (C and D) While ePC levels are consistently decreased in CMT2A patient lymphoblasts, this decrease meets significance in half the observations.
Figure 5.5- Distribution of trans fatty acid species in control and CMT2A patient plasma. While mean values of elaidic (A), linolelaidic (B) and total trans fatty acids (C) are significantly lower in CMT2A patient plasma, significant overlap in values exist.
CHAPTER 6. CONCLUSION.

Section 1. Discussion of the cellular and lipid biology of Mfn2

Background.

De Brito and Scarrano originally described a novel role for Mfn2 in tethering mitochondria to sections of the ER known as mitochondrial associated membranes (MAM) (de Brito and Scarrano 2008). We hypothesized that both loss of Mfn2 or mutations in MFN2 would disrupt normal ER-mitochondrial tethering, and interrupt cellular processes dependent on this interface. We specifically proposed that the transport of phosphatidylserine (PS) from the ER to mitochondrial membranes would be disrupted, and lead to a deficiency in cellular levels of PE. This hypothesis was based on several previously published observations.

While the critical role of structural proteins in inducing membrane curvature is critical in membrane and vesicle traffic and formation is well known, the composition of the lipid bilayer also influences the degree of membrane curvature and the energetics required to induce membrane curvature (Churchward and others 2008; Collins 2006; Jiang and Powers 2008; Shnyrova, Frolov, Zimmerberg 2008). PE shape is approximated as a cone. It is known induce negative curvature on the inner leaflet of the lipid bilayer and promote Ca^{2+} triggered membrane fusion events (Churchward and others 2008). One might speculate that the percentage of PE in mitochondrial and ER membranes determine the extent of curvature required to form small diameter tubules characteristic of mitochondrial and ER reticular networks. The formation of these tight diameter tubules could potentially be a prerequisite for transport down a narrow axon. In both Mfn2 KO
and *Pisd* KO MEFs, the fragmented mitochondria are round yet of varying diameters (Chen and others 2003; Chen, McCaffery, Chan 2007; Schumacher, Choi, Voelker 2002). In contrast, the fragmented mitochondria of *Mfn1* KO MEFs are round but of small uniform diameter (Chen and others 2003; Chen, McCaffery, Chan 2007; Schumacher, Choi, Voelker 2002). The large diameter mitochondria observed in *Mfn2* KO cerebellar Purkinje cells are excluded from small diameter dendrites by what appears to be their abnormal size (Chen, McCaffery, Chan 2007). These observations are consistent with the idea that MFN2 is critical in establishing the proper phospholipid composition for both the mitochondria and the ER to form tight diameter tubules making up reticular networks. As an autosomal dominant disease, CMT2A may result from a reduced rate phospholipid synthesis critical to maintaining reticular networks required for axonal transport. Interestingly, the acyl chain content of PS, which changes with age (decreasing percentages of docosahexaenoic acid (22:6n-3) which is vulnerable to oxidation) alters the activity of PS decarboxylase (Giusto and others 2002). Altered phospholipid profiles, including an increase in PS and decrease in PE, of brain and liver mitochondria are associated with aging (Modi, Katyare, Patel 2008). Normal changes in phospholipid metabolism and composition associated with aging may be magnified or accelerated by mutations in *MFN2*. The biophysical characteristics of PE may contribute not only to the membranes physical tendency to bend, curve or fuse, but may have important functional interactions, as will be described below.

Our results indicate Mfn2 does have important roles in establishing cellular phospholipid levels, but also indicate that the mechanism by which this occurs is not sufficiently explained by the model proposed by de Brito and Scorrano. Specifically, we
find that in disruption of ER-mitochondria contacts occurs in both Mfn1 and Mfn2 KO MEFS, which suggest a tethering role specifically for Mfn2 is not the case. Likewise, we find that almost all phospholipid abnormalities are the same in both Mfn1 and Mfn2 KO MEFs. This suggests that reduced ER-mitochondria contacts are the result of the static and fragmented mitochondrial network seen in both Mfn1 and Mfn2 KO MEFs. While these data suggest no specific role for Mfn2 in tethering MAM formation, it does suggest that normal mitochondria morphology is required for normal ER-mitochondria interactions. While loss of both Mfn1 and Mfn2 appear to similarly influence ER-mitochondria interactions and phospholipid profiles, Mfn2 does appear to be specifically enriched, along with other outer mitochondrial membrane proteins (TOMM20) in the MAM fraction. Mfn1 is not present in MAM fractions. Likewise, the MAM fraction appears specifically reduced in subcellular fraction preparations specifically in Mfn2 and not Mfn1 KO MEFs. While not enough to form a mechanistic hypothesis for ER related roles of Mfn2 and Mfn1, these results suggest these proteins have functions within mitochondria which influence the MAM. The simple tethering role for Mfn2 put forth by de Brito and Scarrano is not satisfactorily consistent with all of our data.

Our investigations using the Mfn1 and Mfn2 null cell models yielded some important observations with regard to the basic cell biology of the mitofusins, PE and a number of published observations using these same MEF cell lines. Below we describe several intriguing connections between PE levels and biological processes related to the mitofusins and the pathogenesis of axonal degeneration.
MFN2 may indirectly influence prohibitin function via reduction of mitochondrial PE levels.

Initially, prohibitins were associated with anti-proliferative activity with diverse molecular roles. Prohibitin 1 (PHB1) was first identified as “repressor of estrogen receptor activity” (REA), and found to co-regulate the estrogen receptor’s sensitivity to estrogen and anti-estrogen drugs. (Montano and others 1999). Prohibitin 2 (PHB2) was initially characterized as interacting with the IgM isotype of the B-cell receptors (BAP37), potentially integrating B-cell receptor signaling to appropriate proliferative responses (Terashima and others 1994). PHB1 and PHB2 form a ring like complex in the inner mitochondrial membrane and influence cell proliferation, as well as mitochondrial morphology and function (Merkwirth and others 2008; Merkwirth and Langer 2009). Structural studies in yeast reveal that phb1 and phb2 form a large (>1MDa) structure, forming a ring with a diameter of approximately ~20–25 nm (Merkwirth and Langer 2009; Osman and others 2009). The formation of this large heterooligomeric assembly is required for normal prohibitin complex (PHB1/2 complex) function. Knock out models of PHB1 and PHB2 demonstrate that loss of either gene eliminates the expression of the other, due to rapid degradation of uncomplexed protein. Each prohibitin contains a PHB domain, which is shared by the stomatin/prohibitin/flotillin/HflK (SPFH) family proteins, which are thought to partition functional membrane domains and associate with lipid rafts and specific membrane lipids (Mishra, Murphy, Murphy 2006; Nijtmans and others 2002; Osman and others 2009; Winter, Kamarainen, Hofmann 2007). As the inner mitochondrial membrane is the most protein rich lipid bilayer known, it has been suggested that the PHB1/2 complex’s ring like structure acts as a lipid organizer, even serving as a fence excluding membrane proteins from its lipid rich center (Osman and
others 2009). While a partitioning function remains to be demonstrated, several important studies report critical interactions between the PHB1/2 complex and phospholipid species (Birner and others 2003; Osman and others 2009).

Using a genetic interaction screen in yeast, Birner and colleagues first described interactions between the phb1/2 complex and PE (Birner and others 2003). Using temperature sensitive genetic constructs, they observed that phb1 and phb2 mutants survive when mitochondrial PE levels are normal. In the absence of the two yeast phosphotidylserine decarboxylase genes (mammals have only one), which eliminate mitochondrial PE synthesis, phb1 and phb2 mutants are no longer viable. Thus, the normally high levels of mitochondrial PE appeared to compensate for the loss of the phb1/2 complex—indicating the existence of a functional interaction between mitochondrial PE and the phb1/2 complex. In a recent report, this observation was corroborated, as both cardiolipin and PE were shown to have critical roles in the survival of prohibitin-deficient cells (Osman and others 2009). Using synthetic genetic arrays to define the genetic interactome of prohibitins, Osman et al identified interactions with a gene, genetic interactor of prohibitin 1 (GEP1), involved in import of PE into the inner mitochondrial membrane. When GEP1 was disrupted, resulting in a demonstrated decrease in mitochondrial PE, normally viable phb1 and 2 mutants died. These results are again indicative of interactions between prohibitin function and mitochondrial membrane PE levels. While Osman et al.’s results focus on reduction of mitochondrial PE via mutations in GEP1, which transports PE from the outer mitochondrial membrane to the inner mitochondrial membrane, they acknowledge that: “The identification of components mediating lipid import from the ER, transbilayer flipping across
mitochondrial membranes, or lipid transport across the intermembrane space of mitochondria has yet be accomplished.” (Osman and others 2009) Clearly, our hypothesized role of MFN2 providing substrate in the mitochondrial synthesis of PE via interactions with MAM places MFN2 functionally upstream of PHB1/2 complex function.

Prohibitin Complex Function: Optic Atrophy, Hereditary Spastic Paraplegia, and CMT genes converge on mitochondrial protease pathways.

The PHB1/2 complex has several identified functional roles, all of which hold special relevance to peripheral neuropathies. Human PHB1 has been shown to have critical roles in mtDNA copy number and organization. In PHB1 deficient cells, many mitochondria no longer contain mtDNA, with no effect on mitochondrial membrane potential (Kasashima and others 2008). Additionally, the remaining mtDNA appeared in the soluble cellular fraction, instead of the insoluble detergent fraction, suggesting a change in mtDNA organization in the absence of PHB1 (Kasashima and others 2008). It was concluded that the PHB1/2 complex most likely localizes mtDNA to the luminal side of the inner mitochondrial membrane, which influences mtDNA stability. PHB1 also influences mtDNA copy number through interactions with mitochondrial transcription factor A (TFAM) (Kasashima and others 2008). In yeast, the importance of PE in the mtDNA stabilizing role of Phb1 is highlighted as loss of PS decarboxylase (Pisd), the PE synthetic enzyme, induces the loss of mtDNA (Birner and others 2003). Additionally, mitochondria in Mfn2 KO neurons were observed to lack mtDNA (Chen, McCaffery, Chan 2007). More recently, skeletal muscle specific mitofusin knockout models were shown to have profound mtDNA depletion and rapidly accumulate mtDNA
mutations (Chen and others 2010). It is interesting to consider PE role in determining PHB and TFAM function could explain this recent observation (Figure 6.1).

In addition to its role in mtDNA organization, the PHB1/2 complex interacts with two other important proteins already associated with dominant optic atrophy and hereditary spastic paraplegia (HSP), OPA1 and paraplegin (SPG7) respectively (Merkwirth and others 2008; Merkwirth and Langer 2009). Two ATP dependent AAA-type protease complexes reside within the inner mitochondrial membrane, each with their catalytic domain exposed to opposite sides of membrane (Koppen and Langer 2007). The m-AAA protease projects its protease domain into the mitochondrial matrix (m), while the i-AAA protease projects in the intermembrane space (i). While still currently under debate, in humans, the m-AAA protease complex is thought to be made up of paraplegin and AFG3L2 and has been shown to proteolytically process matrix proteins (Koppen and Langer 2007). The PHB1/2 complex has been shown to act as chaperone to the m-AAA protease complex, reducing the protease’s activity, and thus influencing the turnover of mitochondrial proteins (Nijtmans and others 2002). Specifically, the PHB1/2 complex has been shown to influence the rate at which the m-AAA protease complex processes OPA1. In cell lacking PHB2, the distribution of OPA1 isoforms is altered and the mitochondria appear fragmented (Merkwirth and others 2008). It was previously known that the longer isoforms of OPA1 (L1 and L2) induce tubular mitochondrial morphology, while shorter isoforms (S3, S4, and S5) are associated with fragmented mitochondria (Duvezin-Caubet and others 2007; Guillery and others 2008; Ishihara and others 2006; Merkwirth and others 2008; Song and others 2007). When the long, but not the short, isoforms of OPA1
are overexpressed in PHB deficient cells, the fragmented mitochondrial morphology is rescued (Merkwirth and Langer 2009). This result suggests that the PHB1/2 complex is required in for normal OPA1 processing by the m-AAA protease. While OPA1 is the most studied protein processed by the \textit{m}-AAA protease, other matrix proteins are thought to be similarly processed, such as essential respiratory complex proteins (Koppen and Langer 2007; Song and others 2007; Steglich, Neupert, Langer 1999). As interactions between PE and the PHB1/2 complex have been demonstrated, mitochondrial membrane levels of PE may indirectly influence OPA1 processing along with other proteolytic processes (Birner and others 2003; Osman and others 2009). PE has also been shown to influence the activity of the \textit{i}-AAA protease (Nebauer and others 2007). Additional PE interacting mitochondrial proteases may influence general rates of mitochondrial protein turnover. This may have relevance to the pathology of CMT2A, in which the neurons with the longest axons degenerate. If nuclear encoded mitochondria proteins turnover exceed a threshold rate of protein synthesis, whether protein synthesis be from local (synaptic terminal) mRNA pools or not, mitochondrial dysfunction would likely results. Likewise, mitochondrial encoded protein synthesis may be reduced as mtDNA stability is decreases with falling levels of PE. By defining how mutations in \textit{MFN2} alter mitochondrial PE levels, and subsequently PHB1/2 complex function, we may begin to elucidate the initial steps in pathogenesis of CMT2A. These observations, along with the functions of \textit{OPA1} and \textit{paraplegin}, highlight the importance of mitochondrial proteolytic pathways within in the pathogenesis of CMT 9 (Figure 6.1).
Future directions.

The most compelling evidence to elucidate the role of mitochondrial generated PE in basic cell biology functions concerning PHB and related proteins would come from rescue experiments. By experimentally rescuing or increasing levels of PE in the context of mitofusin of Pisd null models, the definitive importance of PE would be established.

Several obstacles might make this particular experiment difficult. The first is the subcellular location of PE. While we were able to rescue whole cell levels of PE, the addition of ethanolamine to cell media did not rescue or alter mitochondrial morphology or dynamics. We believe that although cellular levels of PE are rescued with the addition of ethanolamine, local mitochondria membrane levels of PE may not be, and thus the rescue is not truly complete. Alternatively, mitochondrial PE levels are increased sufficiently which suggests PE does not play a critical role in defining mitochondrial morphology. However the mitochondrial morphology seen in Pisd null cell models suggests the later explanation is not likely true. One of the first follow up experiments to be performed should examine mitochondrial PE levels with and without ethanolamine supplementation. Limitations in the quality and quantity of mitochondrial fractions from the MEF cells prevented us from answering this question definitively. Therefore use the tissues (liver) from the placental Mfn2 KO rescue animal would be essential in answering this question. Secondly, the type of PE used to rescue effects might be important as well. As mitochondrial generated PE is enriched highly unsaturated fatty acids, the PE used for the proposed experiments would have to contain similar subpopulations of PE. Thus the intracellular targeting and the acyl content of PE used in rescue experiments may be critically important.
Another important consideration regarding basic physiology and the function of Mfn2 would be a more clear understanding or ethanolamine chemistry in humans. While wide variation in plasma ethanolamine concentration is observed across mammalian species, little is known about how ethanolamine is handled in human tissues (Shiao and Vance 1995). Some data indicates serum ethanolamine may be significant lower in human serum and be change with age. Future work concerning how ethanolamine is handled in different organs and tissues may aid in understanding how mutations in Mfn2 might cause cell specific vulnerability in disease.

Section 2. CMT2A and ePE species

The single consistent change observed between Mfn2 null models and CMT2A patient fibroblast in the presence and absence of ethanolamine supplementation are decreases in cellular levels of ePE. Changes in ePE are the most outstanding in the CMT2A patient fibroblast, and with the exception of ePC, all other phospholipid class remain unchanged. This contrasts with the mitofusin null models, which show dramatic changes in many phospholipid classes, with the most profound change occurring in PE. Unlike the other phospholipid class abnormalities, supplementation with ethanolamine did not rescue ePE levels in Mfn2 null or CMT2A cell lines. These data together suggest ePE may be an important component in CMT2A pathogenesis.

Unlike PE, ePE is not synthesized in the mitochondria but in peroxisomes. As ePE synthesis is not as well defined as other phospholipid synthesis pathways, it is difficult to speculate on how mutations in MFN2 or loss of Mfn2 would result in decreases in ePE. However, as MFN2’s expression is in part controlled by peroxisomal
proliferation transcription factors, so defects in MFN2 may disrupt unidentified peroxisomal functions. Alternatively, as ePE has been characterized as an antioxidant lipid, it is possible that decreases in ePE represent an increase in reactive oxidative species (ROS) burden in Mfn2 null and CMT2A cell lines. In this scenario, decreases in ePE would represent a phenomena secondary to increased ROS generation.

Ethanolamine plasmalogens have diverse roles in many cellular processes relevant to CMT2A, such as cholesterol transport, as a reservoir of arachidonic acid and eicosanoid precursors, and as an antioxidant. Future work must confirm if deficiencies in ePE in CMT2A are true in the forthcoming R94W MFN2 transgenic mouse model. Specifically, similar phospholipid screens should be performed on peripheral nerve tissue and liver at a minimum. If decreased ePE levels are confirmed, several biologic functions ePE hold promise. In our laboratory, work by Fan Zhang, PhD suggests abnormalities in cholesterol handling in Mfn2 KO MEFs, which may be caused by ePE deficiencies as previously reported. As cholesterol is not synthesized within the axon, its transport through the vasa nervorum or down the axon may represent an underlying vulnerability in the longest axons in the body. Again, the R94W mouse model holds the most promise for investigating intracellular transport and synthesis of cholesterol in CMT2A.

If ePE deficiencies are confirmed in CMT2A, supplementation of ePE precursors is of great promise. Supplementation of ePE precursor, known as 1-alkyl-2-acylglycerols, has been shown to restore ePE deficiencies in cell models. Not only are total ePE levels restored, but treatment with specific 1-alkyl-2-acylglycerols containing specific acyl chains restored specific populations of ePE. While investigated as a pharmacologic
means of modulating cholesterol levels in the context of cardiovascular disease, such a
strategy may hold promise if ePE levels are confirmed to be deficient in CMT2A.

Section 3. An alternate hypothesis for CMT type 2.

The pathogenesis of CMT2A and other “axonal” forms of hereditary peripheral
neuropathy have long been thought to be primary diseases of the axon. Likewise,
demyelinating forms, such as CMT1, are known to stem from primary abnormalities in
the myelinating Schwann cells of the peripheral nervous system. With the identification
of a growing number of CMT2 genes, CMT2 gene functional studies have tended to
focus on roles relevant to axonal transport. While deficiencies in axonal transport are an
attractive model for a disease that spares all but the longest cells of the body, little direct
evidence supports this idea. Here, we describe an alternate hypothesis regarding the
pathogenesis of axonal degeneration that stems from lipid and cholesterol related roles of
many of the known CMT2 genes.

MFN2 (CMT2A) as a regulator of vascular smooth muscle proliferation and
response to oxidized LDL.

Independent of work on MFN2 in controlling mitochondrial tethering and fusion,
MFN2 was identified in 2004 as a vascular smooth muscle hyperplasia suppressor gene
(HSG)(Chen and others 2004). A line of spontaneously hypertensive rats (SHR) were
developed as a model of vascular smooth muscle cells (VSMC) proliferation. Gene
expression profiling of SHR VSMC led to the identification of HSG, later discovered to
be Mfn2(Chen and others 2004). Expression studies of Mfn2 in wildtype VSMCs
revealed that down regulation of Mfn2 is a generalized response to proliferative stimuli
(PDGF, FGF, and ET-1) in VSMC. Subsequent experiments indicated that overexpression of Mfn2 in VSMC prevented restenosis of arteries following balloon angioplasty. Likewise, Mfn2 is progressively down regulated in ApoE-KO mouse arteries, especially in hyperplastic arterial atherosclerotic lesions. These effects were determined to be mediated by Mfn2s inhibition of the Ras–Raf–MEK–ERK1/2 signaling cascade which induced cell cycle arrest in the G0/G1 phases, thus blocking VSMC proliferation. Interestingly, the effects of Mfn2 appear to be independent of its mitochondrial targeting, but did depend on its Ras binding domain. Subsequent reports have also described Mfn2 as an important determinant of VSMC apoptosis(Guo and others 2007a). Specifically, Mfn2 is required for oxidative stress-mediated VSMC apoptosis. This notion is consistent with other reports which connect Mfn2 associations with Bax/Bcl-2 during apoptosis(Brooks and others 2007; Karbowski and others 2002; Neuspiel and others 2005). Additionally, adenoviral mediated expression of Mfn2 in rabbit VSMC was shown in inhibit VSMC proliferation caused by oxidized LDL (oxLDL) particles (Guo and others 2007b). Although published exclusively in Chinese, several reports have since examined how polymorphisms in Mfn2 associate with hypertension and cardiovascular disease in humans(Liu and others 2007; Wen and others 2005). These results highlight Mfn2 importance in the VSMC response to increased LDL and oxLDL levels, hallmark features of atherosclerosis.

Our data regarding the phospholipid profiles of CMT2A patient cells indicates that ethanolamine plasmalogens (ePE) are specifically reduced as compared to controls. ePE has defined roles as a membrane antioxidant that protects cholesterol in LDL s from oxidative damage(Esterbauer and others 1992; Jurgens and others 1995). Likewise, ePE
has been shown to be an important component of cholesterol transport, specifically from the plasma membrane or endocytic compartments to acyl-CoA cholesterol acyltransferases in the ER (Munn and others 2003). It is interesting to consider how deficiencies in ePE might fit into Mfn2 mediated protection against oxLDL injury.

**Dynamin 2 (DNM2/CMT2M).**

Ontological analysis of genome-wide association studies identified DNM2 as a candidate gene in the metabolism of oxidized LDL (Tsoi and others 2009). Functional studies of DNM2 and mutant forms of DNM2 clearly demonstrate DNM2 has critical roles in the clathrin mediated endocytosis of the LDL receptor. Specific CMT2M mutations significantly decrease LDL receptor endocytosis (Bitoun and others 2009). Thus, dynamin 2 has well characterized functions in cellular transport of cholesterol by mediating the initial steps of LDL receptor endocytosis.

**Rab7 (CMT2B) role in cholesterol and LDL transport.**

Early on Rab7 was identified as a key gene involved in atherosclerotic lesions (Gyun Kim and others 1998). Experimental models of cholesterol loading in rabbits showed Rab7 is elevated in liver cells (endothelial cells and hepatocytes surrounding the central veins) and in arteries (Kim and others 2002). Atherosclerotic plaques from rabbits and humans also show elevated levels of Rab7 in these tissues (Kim and others 2002).

Molecular studies have demonstrated that Rab7 regulates late endosomal processes but not early endocytic events (Cogli, Piro, Bucci 2009; Vitelli and others 1997). Cellular cholesterol accumulation increases Rab7 presence on membranes and reduces endosomal bidirectional motility (Lebrand and others 2002). Expression of the
Rab7 N125I mutant restores cholesterol loaded endosomes, while overexpression of wildtype Rab7 has the opposite effect. These data indicated that loading membranes with cholesterol influenced Rab7’s control of endosomal transport (Chen and others 2008; Lebrand and others 2002). Work regarding Niemann Pick disease type C (NPC) also indicates an important cholesterol related role for Rab7, as overexpression of Rab7 can rescue LDL accumulation caused by NPC1 mutations (Bergo and Young 2002; Choudhury and others 2002). These data point to important roles for Rab7 in how endocytosized cholesterol LDL is handled.

**HSP27 (CMT2L) and LDL.**

Small heat shock proteins are ubiquitously expressed, but are known to have critical roles in vascular smooth muscle cells (Salinthone, Tyagi, Gerthoffer 2008). Specifically, vessel wall release of HSP27 in plasma correlated inversely with atherosclerosis. HSP27 is considered a biomarker of atherosclerotic injury (Kardys and others 2008; Martin-Ventura and others 2004; Martin-Ventura and others 2006). Both within plaques and in plasma of patients with atherosclerosis, HSP27 levels are lower (Duran and others 2007; Martin-Ventura and others 2006). Like MFN2, HSP27 has anti-proliferative effects and may inhibit smooth muscle proliferation associated with atherosclerosis (Rayner and others 2008; Rayner and others 2010). HSP27 is also able to prevent the uptake of atherogenic lipids and reduces foam cell-induced inflammation by competing interactions with the scavenger receptor (SR-A) on macrophages (Rayner and others 2008). Thus the extracellular release of HSP27 from vascular smooth muscle cells may limit cholesterol uptake.
Vasa nevorum, CMT2 and cholesterol.

Of the many lipid synthesis pathways studied, it is thought that cholesterol is the only lipid that cannot be synthesized within axons and must be delivered to the axon through axonal transport or supplied extracellularly (via receptor mediated endocytosis)(de Chaves and others 1997; Karten and others 2002; Karten and others 2003; Posse De Chaves and others 2000; Vance and others 1994; Vance and others 1995; Vance, Campenot, Vance 2000). Taking this single consideration into account, one might speculate that long axons would have increased dependence on extracellular sources of cholesterol, namely by receptor mediated endocytosis of LDL particles. With CMT2A, CMT2B, CMT2L and CMT2M genes all having direct connections to cholesterol handling, it seems plausible that cholesterol transport may have a key role in axonal degeneration.

Original work by Peter Dyck described significant differences in the unsaturation index of cholesterol esters in CMT2(Yao, Ellefson, Dyck 1976). Likewise, the percentage of cholesterol linoleate showed a marked decrease in CMT2 that correlated with age and disease severity (Yao, Ellefson, Dyck 1976). In addition to CMT1, CMT2 patient serum studies revealed significant decreases in the rates of cholesterol esterification by the lecithin cholesterol acyltransferase (LCAT) enzyme(Yao, Ellefson, Dyck 1976). Our plasma lipid data revealed decreases in trans fatty acids in CMT2A patients. While the mechanism behind this observation in unclear, trans fats are thought to inhibit LDL receptors and increase plasma LDL levels. While there appears to be no trends to develop co-morbid atherosclerotic disease in CMT type 2, early onset stroke and white matter hyperintensities are associated with certain MFN2 mutations(Chung and others 2008).
Additional, long-term use of statins is associated with an increase the odds ratio of developing a polyneuropathy in non-CMT populations (Gaist and others 2002). Perhaps the axonal compartment’s requirement for cholesterol has unique characteristic that might explain a distal motor and sensory neuropathy, occasionally accompanied by stroke, deafness and optic atrophy.

When considering the anatomy of the peripheral axonal environment, the glia-neuro-vascular unit must be considered. Many of the CMT2 genes have roles described in VSMC, and the smooth muscle cells of the epineural arterioles are thought to regulate blood flow through the peripheral nerve (Kihara and Low 1990). Indeed higher than normal oxygen tension in peripheral nerve vessels causes epineural arteriole constriction via reactive oxidative species generation (Sakai and others 2007). A small study of sural nerve biopsies from controls, vasculitic neuropathy, neuropathy with microangiopathy, CMT2 (axonal neuropathy), and mixed axonal and demyelinating neuropathy revealed vascular changes only in the CMT2 biopsies (Mawrin, Schutz, Schroder 2001). These changes included correlative changes between the axon/myelin diameter and the number of epineural blood vessels and the endoneural blood vessel density. In models of peripheral nerve ischemia/reperfusion, compound muscle action potentials (CMAP), in which deficits are pathognomonic in CMT2, are the most sensitive measure of nerve injury and recovery (Iida and others 2003; Iida and others 2009). The observations suggest the initial insult in CMT type 2 may involve VSMC of the epineural arterioles.

Besides the possibility of VSMC of the vasa nervorum being the pathogenic site in CMT, both cholesterol and LDL transport between glia and neurons may prove to be important in CMT type 2. Many of the studies focused on cholesterol in the peripheral
nerve deal with nerve injury and models of Niemann Pick disease (de Chaves and others 1997; Karten and others 2002; Karten and others 2003). Future work will have to focus on elucidating how CMT genes within peripheral nerve glia and axons might be involved in lipid and cholesterol metabolism.

**Conclusions and future direction.**

Cumulatively the data regarding the functions of CMT2 genes suggest the glial, neuronal, or vascular transport and metabolism of cholesterol may be important in diseases pathogenesis. Yet, exactly how each CMT gene fits into a mechanistic picture is unclear. The significant number of connections to cholesterol and lipid related functions warrants attention and suggests popular and commonly accepted axonal transport hypothesis regarding CMT type 2 may be limiting. While the extreme length (L) of the axon has always thought to represent a vulnerability in distal hereditary neuropathies, the axon lipid membrane size is approximated as a multiple of length and axon radius squared (2πrL). Thus the axonal lipid membrane and its maintenance is a process that must require significant support from local glia and vascular cells. Investigators should consider these processes as they continue to elucidate the molecular and cellular mechanism behind axonal forms of CMT.
**Figure 6.1.** The transport of phospholipids from the ER to mitochondria occurs within the MAM and has consequences on matrix protein activity. (1) PS movement from MAM to mitochondria is dependent on MFN2 tethering. (2) PS is translocated to the IMM and consumed in the synthesis of PE by PSDC. (3) PE within the IMM forms organized lipid domains through interactions with the PHB complex. (4) The PHB interacts with lipid domains and negatively regulates \( m \)-AAA protease activity. (5) \( m \)-AAA protease processes long isoforms of OPA1 (L-OPA1), into short isoforms (S-OPA1). In the absence or reduced activity of MFN2, PE levels are reduced—thus altering the organized lipid domains interacting with PHB. (6) Loss of PE interaction with PHB alters PHB’s interactions with TFAM, which results in mtDNA instability. (7) Subsequently, matrix protease activity escapes regulation by PHB, and increases processing of proteins like OPA1. Mutations in MFN2, OPA1, and paraplegin all cause different forms of axonal degeneration.


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