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**UNIVERSITY OF CALIFORNIA,
IRVINE**

**Mitochondrial Metabolism and Morphology
in Mitochondrial Disease States**

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

In Biological Sciences

by

Mariella Theresa Simon

Dissertation Committee:

Professor Susanne Rafelski, Chair

Professor Jose Abdenur, Co-Chair

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2016

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DEDICATION

To

those individuals who believe(d) that the suffering of mitochondrial disease patients (young and old) is real and not made up or self-inflicted.

For those who did this long before modern science could explain the underlying mechanisms.

For all their pain that we cannot see that is real.

“Die Elementarkörnchen der Zellen, welche noch heute ihre analogen Vertreter in den primären Mikroorganismen haben und welche seit jenen Perioden in den Zellen existieren, vermögen nicht mehr selbständige Lebewesen zu werden.”

“The elementary granules of cells, which still have their analogs in primary microorganisms and which exist since these time periods inside of cells, are no longer able to exist independently.”

Richard Altman 1890

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LIST OF ABBREVIATIONS

Auditory brainstem response (AABR)
Abnormal carbohydrate deficient transferrin (CDT)
Adenosine triphosphate (ATP)
Adult onset polyglucosan body disease (APBD)
Alanine aminotransferase (ALT)
Alzheimer's disease (AD)
Amyloid beta (A β)
Antibiotic-antimycotic (Anti-Anti)
ATPase associated with diverse cellular activities (AAA)
Basal respiration (BR)
Base pair (bp)
Beta-2 microglobulin (B2M)
Beta-hydroxybutyrate (b-OH-Butyrate)
Biotine-thiamine-responsive basal ganglia disease (BBGD)
Blue native gel analysis (BNG)
Cerebral spinal fluid (CSF)
Charcot Marie Tooth disease (CMT)
Clinical exome sequencing (CES)
Combined respiratory deficiency (COXPD)
Creatine Kinase (CK)
Cybrids (cytoplasmic hybrids)
Cytochrome C (CYCS)
Cytochrome c oxidase subunit IV isoform 1 (COX4-1)
Cytochrome oxidase (COX)
2,6-dichlorophenolindophenol (DCPIP)
Deoxyguanosine kinase (DGUOK)
Detergent compatible (DC)
E. coli lon protease (EcLon)
Echocardiogram (Echo)
Electrocardiogram (EKG)
Electroencephalography (EEG)
Electron microscopy (EM)
Electron transport chain (ETC)
Endoplasmic reticulum (ER)
Endoplasmic reticulum unfolded protein response (UPR)^{ER}
Enzyme carbamoyl phosphate synthetase I (CPS1)
Exome aggregation consortium (ExAC)
Exome variant server (EVS)
Extracellular acidification rate (ECAR)
Fibroblast (f)
Fluid-attenuated inversion recovery (FLAIR)
Free fatty acids (FFA)
Fresh frozen plasma (FFP)

Glutathione peroxidase (GPx)
Glycogen brancher enzymes (GBE)
Glycogen debrancher (GDE)
Glycogen storage disease (GSD)
Growth hormone (GH)
Hearing loss (HL)
Heavy strand promoter (HSP1)
High-mobility group (HMG)
Human gene mutation database (HGMD)
Immunoprecipitates (IP)
Induced pluripotent stem cells (iPSC's)
Institutional review board (IRB)
Intrauterine growth restriction (IUGR)
Leber's hereditary optic neuropathy (LHON)
Leigh syndrome (LS)
Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation in brain (LBSL)
Light strand promoter (LSP)
Liver (l)
Magnetic resonance imaging (MRI)
Magnetic resonance spectroscopy (MRS),
Mitochondrial associated endoplasmic reticulum membranes (MAM)
Maximal respiration (MR)
Methylmalonic acidemia (MMA).
Methylmalonic aciduria cblC type, with homocystinuria gene (MMACHC)
Minor allele frequency (MAF)
Mitochondrial aminoacyl-tRNA synthetases (mt-aaRSs)
Mitochondrial and nuclear DNA (mtDNA, nDNA)
Mitochondrial complex I, II, III, IV, V (COI, COII, COIII, COIV, COV)
Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS)
Mitochondrial respiratory chain (MRC)
Mitochondrial transcription factor alpha (TFAM)
Mitochondrial transfer RNA (mt-tRNA)
Mitochondrial transfer RNA for glutamic acid (mt-tRNA^{Glu})
Mitochondrial unfolded protein response (UPR)^{MT}
Mitofusin 2 (MFN2)
Mn-superoxide dismutase (SOD-1)
MtDNA depletion syndromes (MDS)
MtDNA polymerase gamma gene (POLG)
Muscle (m)
Myoclonic epilepsy and ragged red fibers (MERRF)
N-acetylcysteine (NAC)
Nicotinamide adenine dinucleotide (NADH)
Nonessential aminoacids (NEAA),
Online mendelian inheritance of man (OMIM)
Optic coherence tomography (OCT)

Otoacoustic emissions (OAEs)
Oxidative phosphorylation (OXPHOS),
Oxygen consumption rate (OCR)
Parkinson's disease (PD)
PAS (periodic acid-Schiff)
Pediatric autoimmune neuropsychiatric disorder associated with streptococcus infections (PANDAS)
Peripheral astrocyte processes (PAPs)
Progressive external ophthalmoplegia (PEO)
Protein data bank (PDB)
Quantitative PCR (qPCR)
Reactive oxidative species (ROS),
Reversible infantile respiratory chain deficiency (RIRCD)
Scanning laser polarimeter (GDx)
Short tandem repeat (STR)
Spare respiratory capacity (SRC)
Spinal muscular atrophy (SMA)
Steroidogenic acute regulatory protein (StAR)
Succinate dehydrogenase (SDH)
Superoxide dismutase 1 (SOD1),
Transporter of the inner mitochondrial membrane complex (TIM)
Transporter of the outer mitochondrial membrane (TOM)
Ubiquinol-Cytochrome-C Reductase (CYC1),
Uncoupling protein (UCP)
Variant of uncertain clinical significance (VUS)
Whole exome sequencing (WES)
Whole genome sequencing (WGS)

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When I started the process of writing this dissertation, my advisor Susanne Rafelski told me that I should not underestimate the importance of composing my acknowledgment section. I thought at the time, well, I will just thank everybody and acknowledge them. But looking back now at where I was six years ago, as a “first-year student,” this is a tall order. So many people have touched my life these past six years, have supported me and walked with me along this path, believing that I would eventually reach my goal. I will therefore just simply start at the beginning of my journey of wanting to understand better what underlies the many faces of mitochondrial disease and list the laboratories and people I am grateful for. I am certain I will forget somebody. And if that is the person that is reading these lines right now, please know that you are acknowledged and it is due to the sad forgetfulness of the writer, but not the lack of wanting to acknowledge your contribution and importance.

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field and have patiently answered the many questions I have had that pertained to their specialty.

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Finally, if you have actually read through all of this, here are my final thanks and, of course, it is to the most bestest sons in the world: Liam and Sam the Bam.

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1. **Simon M**, Conley E, Gutman GA, Chandy KG. "Mapping the 5'NCR of mKv1.3 and localization of its promoter within a GC-rich island" Abstract and Poster: Annual Conference for Physiology and Biophysics *Biophys. Jour*, 1994; 66
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ABSTRACT OF THE DISSERTATION

Mitochondrial Metabolism and Morphology
in Mitochondrial Disease States

By

Mariella Theresa Simon

Doctor of Philosophy in Biological Sciences

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Professor Susanne Rafelski, Chair

Mitochondria are the hub of cellular metabolism, respiration and energy production. They are sites of reactive oxidative species (ROS), heme and steroid generation. They buffer cellular calcium flux, regulate redox states, control cell cycle and death and are therefore at the nexus of human health and disease. Syndromes encountered in the mitochondrial disease clinic, associated with mutations in mitochondrial genes, are generally rare and require detailed clinical mitochondrial disease workups, including next generation sequencing technology. The discovery of new genes often presents itself with inconclusive findings, which need to be further pursued and delineated in the research setting. It is therefore of great importance to apply the “bedside to bench and back to bedside” principle in the field of mitochondrial medicine to advance diagnostic and treatment modalities. In this dissertation, I demonstrate bioenergetic findings in three novel mitochondrial disease genes, *NARS2*, *TFAM* and *LonP1*, in a collaborative effort. By doing so, I have helped to delineate the underlying cause of pathologies for families with mitochondrial disease. Presented are also two case studies of patients with

mutations in *POLG* and *GFM1*, with unusual clinical findings, which may point to new, not yet described cellular processes associated with mitochondrial dysfunction. By correlating gene function with patient's disease, taking into consideration clinical parameters observed, I have also shown new metabolic findings in a primary fibroblast tissue culture system from patients with mutations in the mitochondrial matrix protease *LonP1*. The patients have mutations in a domain of the gene, which has never been associated with disease. Findings suggest that the patient phenotype depends on the location of the mutations in *LonP1* and that clinical expression is domain specific. Findings also show increased susceptibility to stress, elicited by the use of antibiotic/antimycotic in the tissue culture system. In the future, I plan to build on the results from this study, to assess the effects of potential treatment modalities on mitochondrial function in fibroblast culture.

CHAPTER 1

Introduction

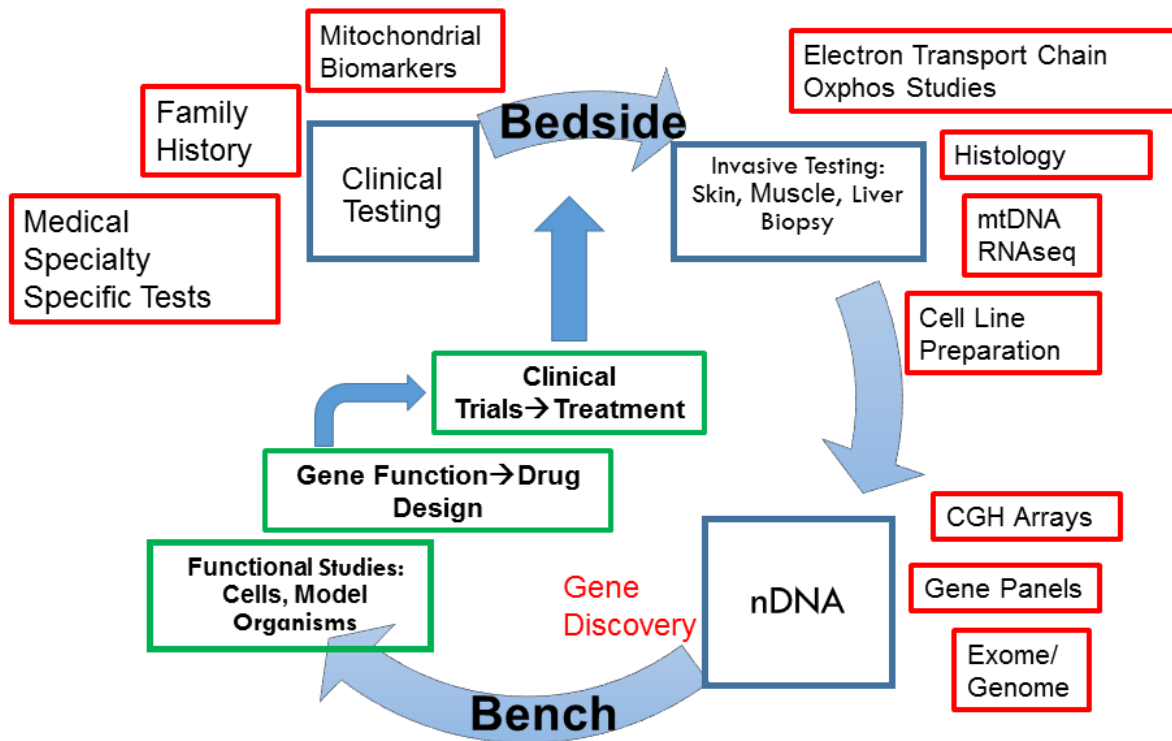
1.1 Principles of mitochondrial medicine

In grammar school we were first introduced to the “Venn Diagram” in a class called “Mengenlehre” which translates into the “study of groups or sets”. My dissertation focuses on the intersection of two groups that are dear to my heart: Medicine and Mitochondria. This Venn diagram holds at its center the patient, an unsuspecting human being who is shortly to find out that everything that makes her or him human depends on the flawless interplay of two organisms that united to form the building block of eukaryotic life, i.e. the cell. There are many beautiful discussions and studies as to whether two specialized prokaryotes eventually formed the eukaryotic cell together (symbiogenesis scenario) or if an “amitochondrial” eukaryote engulfed an alpha protobacterium (archezoan scenario) (Koonin 2010). Ultimately, this debate is of less importance to the human being who has just discovered that he/she can no longer hear or see or walk or talk because something is wrong with their mitochondria. Of greater importance is to explore what underlies the current dilemma and whether it might eventually be fixed. This dissertation is about what happens when things go wrong in the dance between the “energy factory organelle” and its partner, the cell. The central hypothesis of this thesis is that a range of existing and emerging technical advances in molecular genetics, biochemistry and cell biology can be applied to interrogate human patients with mitochondriopathies, thereby identifying the causative mutation and the mechanism underlying the pathophysiology. To test this hypothesis, I participated in studies to identify the causative mutation and understand the underlying mechanism in five such cases. Although each is different from a molecular

view point, they all demonstrate the process of “bedside to bench and back to bedside” (Sadun et al. 2013). Their progression shows how this process has been accelerated in recent years and now delivers true potential to bring the dilemma of the mitopatent to a satisfactory outcome. For each of the cases investigating mutations in the *POLG*, *NARS2*, *GFM1* and *LONP1* genes it took many years to reach a molecular diagnosis. By contrast, our patient with *TFAM* gene mutations was diagnosed within weeks of disease onset due to the availability of clinical next generation sequencing technology. Powerful molecular diagnostic tools now provide the opportunity to diagnose mitochondrial gene mutations before the disease process becomes apparent (Poulsen et al. 2016). This then opens the door to treat “before it is too late”. It is therefore no longer unrealistic to assume that at least some mitochondrial disease pathologies may become manageable much like phenylketonuria which had once been thought of as untreatable (Aldamiz-Echevarria et al. 2016). I will describe several examples from the literature in this chapter, where mitochondrial symptoms have been reverted or successfully treated. In doing so I will also introduce the principles of mitochondrial medicine that are relevant to understanding this dissertation including a discussion on mitochondria and availability of energy substrates. To make successfully treated cases the norm and not the outlier, we have to accelerate our understanding of newly discovered genes, and how their function and cellular mechanisms affects mitochondrial metabolism and mitochondrial disease progression. This seems a daunting task since approximately one third of the 1100 or so genes defining the mitochondrial proteome still lack a clearly defined cellular and biochemical function (Pagliarini, Rutter 2013). This dissertation argues that we can only overcome this hurdle by the close collaboration of the clinician caring for the mitochondrial patient and the

scientist replicating and interrogating the disease process and progression in cell and animal models at the bench (Figure 1.1).

Figure 1.1: The cycle of bedside to bench and back



A clinical workup for mitochondrial patients follows a path from clinical testing protocols (bedside) to a molecular diagnosis. This diagnosis is often novel and causality needs to be explored. The novel findings are translated to the bench in the research lab where gene complementation and other studies establish causality. Results are relayed to the scientific community and reveal basic biology, open doors to investigations in animal models and clinical trials. Results are translated back to the bedside.

1.1.1 The birth of mitochondrial medicine: Historical perspective

Mitochondria have preoccupied scientists for over a century. Richard Altman's 1890 book "Elementar Organismen und ihr Verhaeltnis zu den Zellen" (elementary organisms and their relationship to the cells) is one such early account (Altmann 1890). Ahead of his time, Altman describes and illustrates the intricate dynamics of these shape shifters,

which he names “elementary organisms” (Elementar Organismen) or “bioblasts”. He argues that cells are not the smallest unit of life but rather are themselves comprised of many even smaller organisms. His exhibits depict cells filled with round, seed like structures and their transformed counterparts containing complicated tubular networks. In 1914 Lewis and Lewis again describe the organelle’s dynamic nature but as it is often the case Altman’s and Lewis’s writings and drawings were quickly forgotten, the bioblasts were re-named mitochondria, only to reappear in modern biology books as static ATP factories reduced to bean shaped blobs (Lewis, Lewis 1914). The standard text book depiction though nicely shows that mitochondria have an outer and an inner membrane framing an inter-membrane space and an innermost compartment, the matrix (Alberts et al. 2008). Peter Mitchell proposed in 1961 that this architecture afforded the coupling of the flow of hydrogen ions, pumped across a gradient from the matrix into the inter-membrane space, back into the matrix fueling ATP production (Figure 3) (Mitchell 1961). Very quickly a brilliant Swedish physician and scientist, Rolf Luft, who worked in the group of Bjoern Afzelius, was able to translate this discovery to the bedside and back to the bench (Luft et al. 1962). In their ground breaking article, published in 1962: “A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: a correlated clinical, biochemical, and morphological study” the group describes the case of a 35 years old woman who suffers from excessive heat intolerance and profuse perspiration, general weakness, and what appeared as emaciation. The clinical workup performed by Luft, attempting to delineate the underlying disease pathology should prove to become a blueprint for many mitochondrial patients in the future.

1.1.2 The balance of heat vs ATP production

From the pioneering studies by Peter Mitchell and many other groups defining the process of mitochondrial electron transport we now know that mitochondrial complexes I and II accept electrons from the electron-donors NADH and FADH₂ respectively and transfer them to mitochondrial complex III via the electron carrier ubiquinone. From mitochondrial complex III they are transferred to mitochondrial complex IV via the second electron carrier cytochrome c. From there they are eventually delivered to the ultimate electron acceptor molecular oxygen which is reduced to water. This flow of electrons generates energy that is used to pump hydrogen ions into the mitochondrial intermembrane space establishing the electrochemical gradient and proton motive force (Figure 3) (Tager et al. 1966). As this force increases, protons then flow back into the matrix inducing the rotational movement of the ATP synthase, generating ATP from ADP and phosphate (Reid et al. 1966). Electron flow can also be reversed for example at mitochondrial complex I during states of high membrane potential or inhibited electron transport, secondary to a defect in a downstream process. Electron flow, which is disconnected from ATP generation is called “uncoupled”. During uncoupled electron flow, hydrogen ions leak directly into the matrix through the inner membrane, circumventing the ATPase. Leakage may occur either through specialized "pores" called uncoupling proteins (UCP) generating heat or they may just simply dissipate through an inner membrane, which has changed its properties for example by exposure to free long chain fatty acids (Lombardi et al. 2015). Electrons, which escape their redox carriers mainly at mitochondrial complex I or III, are transferred to oxygen, giving rise to superoxide and peroxide anions. Electron

transport is therefore carefully balanced between the three processes of generation of energy, heat, or free radicals (Drose, Brandt 2012). As introduced in the previous section, Luft correctly postulated that his patients' feeling of constant overheating and insatiable appetite without weight gain was a sign of uncoupled mitochondria. His detailed clinical investigations in conjunction with his knowledge of recent scientific discoveries prevented Luft's patient from being labeled as "Somatoform Disorder" which sadly is still the fate of too many unfortunate mitochondrial disease patients to date (Liberty University).

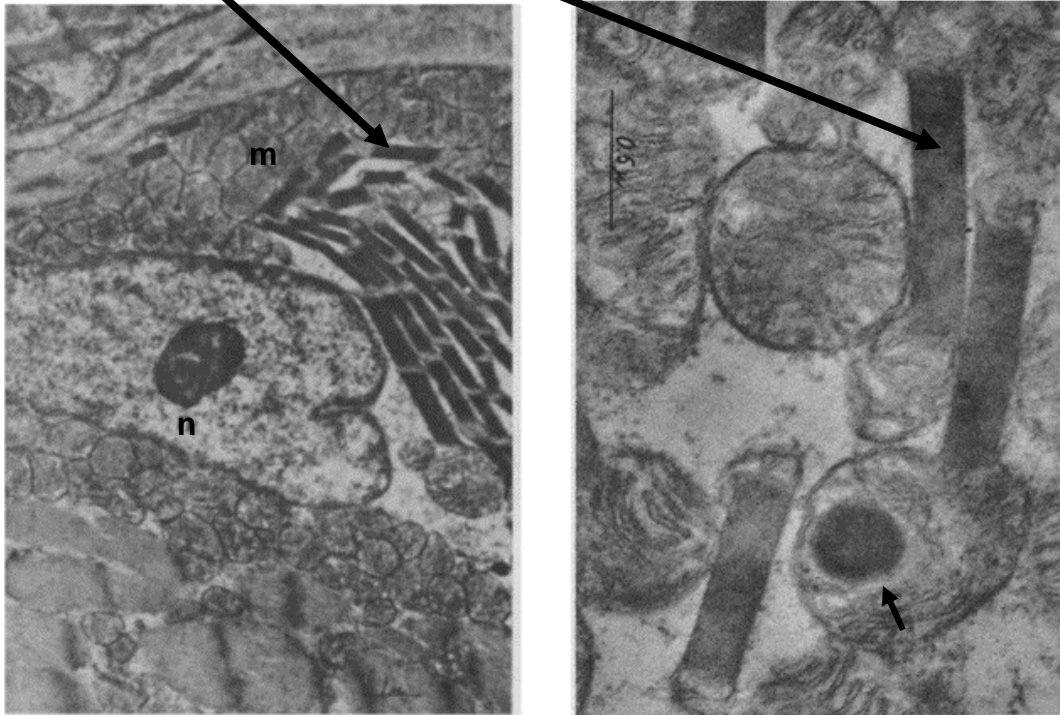
1.1.3 The process of clinical investigation

Luft's paper is an early account of what is now thought of as a standard workup procedure for a patient with suspected mitochondrial disease: (i) An initial consultation allows for gathering of medical and family history including pedigree analysis. (ii) Evaluation of mitochondrial biomarkers in blood, urine and cerebrospinal fluid regularly interrogates Krebs cycle intermediates, amino acid pathways, endocrine function, electrolytes, glucose levels including glucose tolerance, liver function and blood counts. All of these tests were found to be normal in Luft's patient. (iii) Organ specific tests are then performed by the respective specialty like neurology with a focus on magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), cardiology with a focus on electrocardiogram (EKG) and echocardiogram (echo), ophthalmology with a focus on optic nerve and retinal nerve fiber layer exams via optic coherence tomography (OCT) and scanning laser polarimeter (GDx) or audiology with a focus on auditory brainstem response (AABR). In the case of Luft's patient electrophysiological studies interrogating affected organs including electrocardiogram, electromyogram, electroencephalogram as

well as neurological exams, and hearing and vision tests demonstrated normal findings other than the obvious muscle weakness and irregular heartbeat. (iv) Finally, invasive procedures like skin, muscle or liver biopsy are considered for further evaluation. These include sample submission for (v) histology including electron microscopy, (vi) functional mitochondrial studies in live cells or isolated mitochondria, (vii) enzymatic tissue specific testing and (viii) molecular analysis including sequencing of mitochondrial and nuclear DNA (mtDNA, nDNA). Luft's unfortunate patient underwent 4 muscle biopsies, which demonstrated highly uncoupled mitochondrial respiration in isolated mitochondria. Light microscopy revealed signs of degeneration, with 10-50% of fibers demonstrating muscle fibers with a peripheral zone that appeared empty. When stained with the PAS (periodic acid-Schiff) technique, which we now know stains glycogen, the muscle showed PAS positive granules, that had fused in some of the fibers into a large homogeneous intra mitochondrial mass (Raben et al. 2012). Electron micrographs display electron dense particles, which are probably the most beautiful images of formation of mitochondrial paracrystalline inclusions to date (Figure 1.2). These formations have since been shown to be caused by overexpression and eventual crystallization of the mitochondrial creatine kinase and are a phenomenon found in numerous mitochondrial and "non mitochondrial" diseases (O'Gorman et al. 1997; Fernandez et al. 1999). Although the patient's workup closely resembles current clinical testing guidelines, Luft did not yet have DNA based technologies at his disposal. A diagnosis of Luft disease proved to be extremely rare with only one additional case reported. Ironically both remain without a molecular diagnosis (DiMauro, Garone 2010).

Figure 1.2: Electron microscopy of muscle from Luft's patient

Paracrystalline Inclusion



The left panel depicts the nucleus in the center (n) surrounded by regions of mitochondria (m), which are converting to rectangular crystals, “paracrystalline inclusions (crystallized creatine kinase). The right hand higher magnification panel shows dumbbell shaped mitochondria, where creatine kinase crystals are displacing the cristae from the center. The small arrow points to a round electron dense inclusion which may represent glycogen (reproduced and relabeled from Luft et al. 1962 with permission).

1.1.4 Principles of maternal mtDNA transmission

Surely the mtDNA of Luft's patient must have since been sequenced and found to be normal since the first sequences of mammalian mtDNA were published in the 1980s by the groups of David Clayton and Ian Young (Bibb et al. 1981; Anderson et al. 1981). These, and subsequent articles filling in the gaps, showed that human mtDNA contains 13 protein coding genes, 2 mitochondrial ribosomal genes and 22 transfer RNAs (Chomyn et al. 1986). Also during that time Douglas Wallace's group published a

landmark paper, which demonstrated that mtDNA is exclusively inherited through the female germline and hinted that “familial mitochondrial myopathy may be due to alterations in genes located on mtDNA” (Giles et al. 1980). From there a handful of scientists developed what is now known as the field of mitochondrial medicine. In 1988 mtDNA lesions were finally unanimously linked to human disease. Morgan-Hughes’ group demonstrated that deletions in mtDNA are the cause of mitochondrial myopathy and Douglas Wallace’s group associated mtDNA point mutations with Myoclonic Epilepsy and Ragged Red Fibers (MERRF) as well as Leber's Hereditary Optic Neuropathy (LHON) (Holt et al. 1988; Wallace et al. 1988a; Wallace et al. 1988b). This first wave of mitochondrial medicine focused mainly on the mtDNA associated disease process. The terms homoplasmy (all mtDNA molecules are identical at a nucleotide position) and heteroplasmy (mtDNA molecules differ at a nucleotide position) were coined and correlations with disease severity and tissue specificity were made. We now know that maternal exclusivity of mtDNA inheritance is due to elimination of paternal mitochondria via autophagy of sperm mitochondria. This pathway involves ubiquitination of paternal mitochondria, lysosomes and the 26S proteasome in a species dependent manner (Al Rawi et al. 2011; Song et al. 2016).

Although transmission of paternal mtDNA has been demonstrated in other organisms, next generation sequencing technology in samples from buccal cells shows that there is no paternal transmission of mtDNA in samples from healthy individuals (Pyle et al. 2015). Uniparental inheritance is thought to have several benefits. It has been shown in mice that the admixture of two different normal mtDNAs drifts towards homoplasmy. This is most likely a protective selective mechanism since the mice, which retained both

normal mtDNA species, displayed abnormal cognitive development (Sharpley et al. 2012). Investigations into the cellular processes defining these terms are still ongoing to date and their keen understanding is imperative to facilitate prenatal counseling, risk assessment and assisted reproductive technologies for patients with mtDNA related disease (Johnston et al. 2015; Poulton et al. 2010).

1.1.5 The human oocyte and the mitochondrial bottleneck

We now know that a mother who is a carrier for a mtDNA mutation may have several unaffected children and she may never find out that she is a carrier for a pathogenic mtDNA mutation until the mutant load in one of her offspring is high enough to pass the threshold of disease development. This process of unequal inheritance of mtDNA species was first described in dairy cows and was attributed to unequal segregation during cell division of the early embryo (Laipis et al. 1988). Studies on mitochondrial development in the human oocyte predate the modern view of mitochondria as a dynamic network and are restricted to units of mitochondria rather than mitochondrial mass or mtDNA copy number. Primordial oocytes are described as having less than 100 mitochondria. This number increases to about 10,000 during the extended prophase of Meiosis I when the primary oocyte becomes arrested until ovulation. Oocyte growth during maturation is then accompanied by a dramatic 10 - fold increase with 100,000 mitochondria residing in the mature oocyte (Eichenlaub-Ritter et al. 2011; Motta et al. 2000). Founder mutations are thought to occur during the period of mitochondrial contraction also termed “the mitochondrial bottleneck”. These founder mutations, once enriched, were then postulated

to segregate randomly distributing their pathogenic load to various tissues. It has been shown that this is much more complicated than previously anticipated.

1.1.6 Tissue specific segregation of mtDNA variants

To study mtDNA segregation in animals Douglas Wallace's group also developed a mouse model, in which two deleterious homoplasmic mutations were traced through the maternal germ line over several generations. The group found that selections repeatedly occurred against the more severe mutation in the mt-ND1 gene, while the milder mutation in mt-COI remained stable over several generations (Fan et al. 2008). Both animal models have therefore revealed important types of selections: Selection against heteroplasmy and selection against deleterious mutations. Additionally, the animal models uncovered an unexpected phenomenon, namely that the normal mtDNAs segregated in a tissue specific manner revealing that kidney and liver tissues preferred one haplotype of mtDNA over another. This preference most likely points to a tissue specific interaction and preference between mtDNA and nuclear encoded mitochondrial genes involved in mtDNAs replication, transcription and translation (Sharpley et al. 2012). This finding, generated on the bench, is of great importance and may eventually help to explain facets of the intrinsic tissue specificity observed in mitochondrial disease on the bedside. The most striking examples encountered in the clinic are diseases secondary to mutations in the aminoacyl tRNA synthetases. In this dissertation, I describe patients with mutations in the mitochondrial asparaginyl aminoacyl tRNA synthetase *NARS2*. In a group effort we show that depending on severity, mutations in *NARS2* can lead to Leigh syndrome or to hearing loss (Simon et al. 2015). Mutations in other mitochondrial tRNA

synthetases may lead to completely different phenotypes with pathologies restricted to neurological, muscle, liver, heart or reproductive tissues (Schwenzer et al. 2014). This finding is puzzling since the genes are ubiquitously expressed and theoretically mutations should lead to a similar mitochondrial translational defect of mtDNA encoded genes in all tissues. Although aminoacyl tRNA synthetases have been suggested to retain essential cellular functions outside of translation, they may very well interact differently with mtDNA in different tissues depending on variants modulating binding specificity with their nuclear partners (Guo, Schimmel 2013).

1.1.7 From mtDNA to the mitochondrial protein compendium

As the understanding of mtDNA increased, it unfortunately became rapidly apparent that mutations in mtDNA could only explain a small percentage of presumed mitochondrial disease. The realization that in addition to the handful of mtDNA associated genes, another 1500 or so nuclear encoded genes played an important role in mitochondrial biogenesis made molecular diagnosis a daunting task (Lopez et al. 2000). Inheritance of mitochondrial disease is predominantly autosomal recessive but in some instances autosomal dominant or X-linked inheritance patterns are observed. The dogma of maternal inheritance and mitochondrial disease was therefore abolished but is still deeply ingrained in the popular belief. Before the onset of next generation sequencing technology, the gene hunt for these recessive nuclear encoded disease genes was laborious and painfully slow, with only a few new genes reported per year. The second chapter of this dissertation, focused on mutations in *NARS2*, describes my pursuit of such a gene hunt, while serving as an introduction to some important concepts of mitochondrial

biogenesis and function. It also describes the way new sequencing technologies have revolutionized the field. The third chapter, on *POLG*, complements the focus of chapter two on discoveries made at the bench, by stressing the importance of the systematic clinical interrogation of the mitochondrial patient at the bedside. In this chapter the description of the disease process, delineated in a child with Alpers syndrome, leads to a new hypothesis on the importance of glycogen metabolism in the brain, which is revisited in the discussion section of this dissertation. The following two chapters focus on functional studies performed for a patient with mutations in *TFAM* and two patients with mutations in *GFM1*. The cases demonstrate how modern gene hunting techniques can lead to a more rapid diagnosis. Finally, the sixth chapter describes investigations on the bench for two patients with mutations in the gene *LonP1*. While the patients have mutations in the same gene they significantly differ in their phenotypic disease manifestation and the experiments aim to explain some of the cellular pathways underlying the phenotype-genotype differences. All of the works described in this dissertation illustrate that without functional studies, determination of causality of new genes or gene variants is impossible. As more and more genes related to mitochondrial disease are discovered and the mechanism of cellular and organismal pathologies being elucidated, we now are ready to take the next step into the treatment stage. Although mitochondrial diseases are highly heterogeneous, treatment strategies need to be tailored to disease processes rather than individual disease genes. For this we can draw conclusions from a handful of well-studied mitochondrial diseases, which provide insight in the development of pathology during all stages of human development. These diseases are introduced in the next section of this chapter. In addition to the myth that “all

mitochondrial disease comes from the mother” another myth is that all mitochondrial disease is pediatric. Since mitochondrial dysfunction can appear at any age I have picked one representative disease pathology for each stage of human development to give a more complete picture of what the clinician encounters in the mitochondrial clinic.

1.2 Age related mitochondrial disease phenotypes:

1.2.1 Infancy-Leigh syndrome

1.2.1a Leigh syndrome: A nexus for new gene discoveries

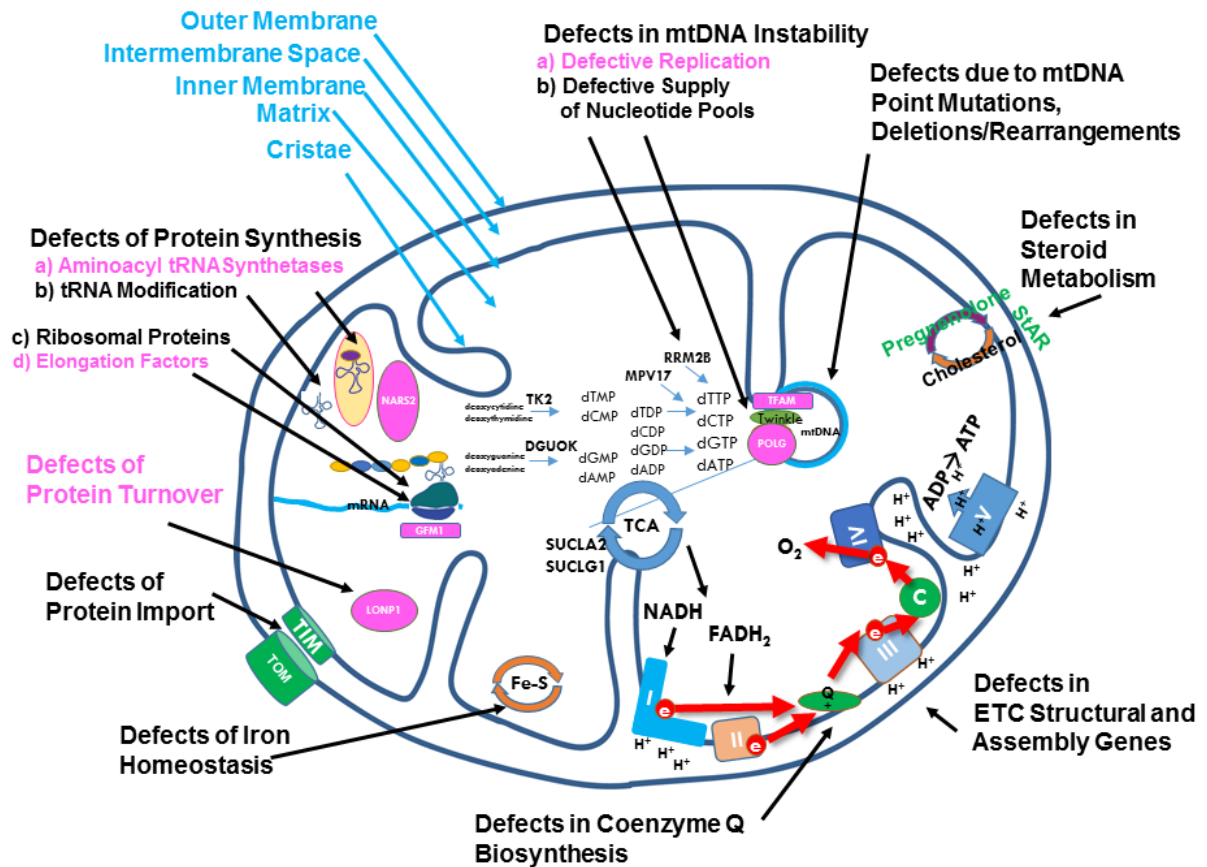
Leigh syndrome (LS; MIM# 256000) is the most common disease pathology of the pediatric mitochondrial clinic. The disease was first described in 1951, with the first genetic lesion being reported in 1991. Since then the number of mtDNA and nDNA disease genes resulting in LS has grown to 75 with 30 of them identified during the last five years (Lake et al. 2016). The diagnosis is based on a set of neurological findings determined by MRI (Baertling et al. 2016). The central diagnostic criteria are bilateral and symmetrical abnormalities in basal ganglia, particularly in the putamen, as well as in the brain stem with or without subthalamic nuclei involvement (Rahman et al. 1996; Bekiesinska-Figatowska et al. 2013). The reason why these structures are preferentially affected in Leigh syndrome is not entirely clear. It has been shown that different brain regions differ significantly in mtDNA encoded gene expression levels which may correspond to different energy needs (Dalwadi, Uht 2013). The onset of symptoms usually begins between three and twelve months of age and present often as non-specific findings such as hypotonia, nystagmus or recurrent vomiting. Associated neurological features include seizures, tremor, dystonia, ataxia and muscle weakness whose onset is

frequently triggered by either a viral infection, prolonged fasting or environmental triggers like surgery (Finsterer 2008). Disease progression is generally rapid with regression of previously acquired skills and death in the first few years of life. Lactic acidosis is a hallmark feature of Leigh's patient but is not a required diagnostic criteria (Ruiter et al. 2007). In some cases individuals with atypical Leigh syndrome may display delayed onset with multiple sclerosis type phenotypes and survival into adulthood (Rahman et al. 1996). The most common causes of Leigh syndrome are dysfunction of the mitochondrial respiratory chain, the pyruvate dehydrogenase complex and rarely coenzyme Q deficiency (Finsterer 2008; Emmanuele et al. 2012). Biochemical defects in the first complex of the mitochondrial electron transport chain represent the most frequent laboratory finding in Leigh syndrome patients (Fassone, Rahman 2012). This large protein complex is comprised of 44 subunits, 7 of which are encoded by mtDNA and 37 by nDNA (Wallace 1999; Rhein et al. 2013). In addition, there are 25 putative assembly factors for complex I and at least 4 of them have been implicated in Leigh syndrome (Fassone, Rahman 2012; Pagliarini et al. 2008). It has been suggested up to 50% of Leigh syndrome cases are still without a molecular diagnosis and that many of them will eventually fall into the category of newly delineated mitochondrial genes (Gerards et al. 2016). Gene products involved in complex I assembly, cardiolipin biosynthesis, coenzyme Q synthesis, processes involving the mitochondria associated endoplasmic reticulum membrane (MAM) as well as calcium and pyruvate carriers and mitochondrial post translational modification have high potential to underlie novel causal pathologies (Pagliarini, Rutter 2013). Also as discussed previously, since mtDNA encodes 13 proteins of the respiratory chain the mitochondrion has retained its own protein synthesis

machinery to translate these 13 proteins within the mitochondrial matrix. All other proteins need to be generated on ribosomes in the cytoplasm and be imported through the outer mitochondrial membrane (Rötig 2011). Mitochondria have developed a complicated complex of transporters to facilitate this process. The transporter of the outer mitochondrial membrane (TOM) and the transporter of the inner mitochondrial membrane complex (TIM) selectively translocate, unfold and refold mitochondrial proteins and help with their shuttling in to the correct mitochondrial compartment (Figure 1.3) (Endo, Yamano 2010). Many genes of the mitochondrial protein synthesis and import machinery have recently been described in Leigh syndrome (Boczonadi, Horvath 2014b; Harbauer et al. 2014; Messmer et al. 2011). Chapter two and three on *NARS2* and *GFM1* describe two gene defects in this category (Figure 1.3). Additionally several cases with Leigh syndrome have recently been associated with gene defects in the Valine degradation pathway. The two major genes responsible for these cases are *ECHS1* and *HIBCH* (Tetreault et al. 2015). Another important recent discovery in the field of Leigh syndrome pathologies is the delineation of the Ugo1 like outer mitochondrial membrane protein SLC25A46 (Janer et al. 2016). It had long been known in yeast that Ugo1 is an essential member of the outer membrane fusion machinery and is involved in maintenance and organization of cristae junctions (Sesaki, Jensen 2001; Jayashankar et al. 2016). A mammalian Ugo1 homolog had remained elusive until the discovery of mutations in *SLC24A46* first in families with Charcot Marie Tooth disease (CMT) and pedigrees with optic atrophy to be quickly followed by the delineation in patients with Leigh syndrome (Abrams et al. 2015; Janer et al. 2016). This example demonstrates that disease discoveries rapidly advance the field of cell biology. Leigh syndrome will therefore not

only be at the center of modern mitochondrial gene hunting but will also help to elicit new gene functions leading to targeted treatment modalities as discussed in the next section.

Figure 1.3: Cartoon of a mitochondrion with its basic structure of outer and inner membrane, intermembrane space and matrix



The folding of the inner membrane creates cristae. Included in the drawing are major mitochondrial gateways to disease. The gene defects discussed in chapter 2-6: *NARS2*, *GFM1*, *TFAM*, *LONP1* and *POLG* are labeled in pink.

1.2.1b Reversible infantile respiratory deficiency: A new hope?

A set of Leigh syndrome related genes termed “reversible infantile respiratory deficiency” have shed light on an interesting cellular process, which offers opportunity for new treatment options. These genes all relate directly or indirectly to mitochondrial protein synthesis defects involving the mitochondrial transfer RNA for glutamic acid (mt-tRNA^{Glu})

(Boczonadi et al.). Infants with a homoplasmic mtDNA mutation at position mt14674T>C/G in mt-tRNA^{Glu} may present as hypotonic at birth with feeding difficulties and abnormal respiratory chain enzymes. Some progress rapidly to severe myopathy and respiratory failure, which is frequently preceded by a viral illness. Interestingly mutation carriers can develop normally as long as they survive the first two years of life (Uusimaa et al. 2011). The nuclear encoded mitochondrial glutamyl tRNA synthetase mt-RS^{Glu} (*EARS2*) is responsible for the charging of mt-tRNA^{Glu} with its cognate amino acid (Bonfond et al. 2005). It was therefore interesting to find that infants with mutations in *EARS2* also present with a labile period during the first one to two years of life with clinical improvement and stabilization of symptoms after the sensitive period in some (Steenweg et al. 2012). The third player in this group of genes is the mitochondrial tRNA modifying enzyme *TRMU*. *TRMU* attaches thiol groups to a modified nucleoside found in the wobble position of mt-tRNA^{Glu}. Thiolation of mt-tRNA^{Glu} stabilizes its structure, assists in aminocylation via interaction with *EARS2* and assists with codon recognition during protein synthesis (Guan et al. 2006). Mutations in *TRMU* cause an infantile hepatopathy between 2 to 4 months of age. Infants who survive the period of liver failure can display spontaneous recovery. The group of Rita Horvath hypothesized that these three gene defects must have a common denominator based in a developmental process of cellular metabolism (Boczonadi et al. 2013). They and others attributed the labile period to disease right after birth to low cytoplasmic pools of reduced cysteine and thiols secondary to low cystathionine gamma lyase activity during the first months of human development (Viscomi et al. 2010). Experiments in patient fibroblasts demonstrated improved mitochondrial respiratory function after supplementation with L-cysteine. Another gene

responsible for infantile partially reversible hypertrophic cardiomyopathy is the mitochondrial tRNA modifier gene *MTO1*. Together with *TRMU*, *MTO1* is responsible for modification of tRNA residues in the wobble position to ensure accurate translation of mtDNA (Ghezzi et al. 2012). Due to these findings discovered on the bench and the fact that N-acetylcysteine is readily available with low or no toxicity CHOC physicians have taken the laboratory findings to the bedside and are currently treating a patient with mutations in *EARS2* with this compound (Boczonadi et al.). Defects in thiamine transport are another group of disorders that are treatable if initiated early in the disease process. A combination treatment of biotin and thiamine has been shown to even revert cortical and subcortical lesions in patients with the disease and stabilize disease progression (Ortigoza-Escobar et al. 2014). The disease categories discussed in this section would therefore present themselves as a logical candidates for newborn screening and it is surprising that there is no mention of N-acetylcysteine or thiamine supplementation in recent reviews on improved therapies for inborn errors of metabolism (Ginocchio, Brunetti-Pierri 2016). Successful treatment options are not limited to the newborn period, as discussed in the next section focused on Leber's Hereditary Optic Neuropathy and also require early detection and intervention.

1.2.2 Puberty- Leber's Hereditary Optic Neuropathy (LHON)

1.2.2a LHON is a disease of incomplete penetrance and skewed sex ratio

Leber's hereditary optic neuropathy is the most well-known mitochondrial disease affecting the eye and the most common mitochondrial disease overall (Chinnery et al. 2000). Inheritance is maternal with most mutations presenting as homoplasmic mtDNA

point mutations in the complex I subunit genes MT-ND1, MT-ND4 and MT-ND6. Initial loss of central vision occurs in both eyes with an average onset between 15 to 35 years (Barboni et al. 2006). The disease provides a window of approximately 6 months before progression to complete loss of vision. Treatment therefore needs to be initiated pre-symptomatically or when symptoms first become apparent. Although all maternal family members in a homoplasmic LHON cohort carry the respective LHON mutation only 50% of all males in the family will develop vision loss (incomplete penetrance). I will address this phenomenon in more depth in the next section. Disease incidence is even lower in females of a LHON family with 10% affected individuals reported (skewed sex ratio) (Yu-Wai-Man et al. 2009). This is thought to be due to the protective role of estrogens, which decrease after menopause, when some female mutation carriers develop a multiple sclerosis-like disease (Pisano et al. 2015). Large pedigrees with LHON mutations have been studied and serve as models for other mitochondrial diseases, which are usually rare, and do not have the same statistical power. The clinically observed incomplete penetrance of LHON is an intensively pursued puzzle of the research lab.

1.2.2b Studies in fibroblasts from unaffected LHON mutation carriers correlate mtDNA copy number with incomplete penetrance

Insights into why individuals with the same homoplasmic mutation segregating in the same family become affected while others do not may hold promise for the design of new treatment options. The study of Valerio Carelli's group sheds some light on this phenomenon by investigating mtDNA copy number in fibroblasts from controls, from unaffected LHON "carriers" and from LHON patients (Giordano et al. 2015). Cells from

individuals with LHON mutations had increased mtDNA copy number compared to controls, indicating a compensatory response to the mutant genotype, i.e. mtDNA proliferation. Significantly, the ability of the cell to counteract depressed respiration by increasing mtDNA levels was more pronounced in cells from unaffected carriers compared to those from LHON patients. Cells from unaffected carriers also displayed an ability to recover from ethidium bromide induced mtDNA depletion while those from affected LHON individuals did not. Interestingly, recovery from induced mtDNA depletion was suggested to be carbon source dependent as demonstrated in a glucose vs galactose culture experiment. In galactose media cells from unaffected carriers and normal controls were able to recover from depletion equally, while fibroblasts from affected individuals were only able to reach 50% of their counterparts mtDNA content (Giordano et al. 2015). However, in glucose, cells from affected patients and carriers were able to increase mtDNA recovery even above the levels in control cells. These results point to a compensatory response of cells with compromised oxidative metabolism to preferably use aerobic glycolysis – a concept explained in depth in the conclusion chapter of this dissertation. Unfortunately, the glucose vs galactose study design of Valerio Carelli's group was potentially flawed and may have had a different outcome if culture conditions were chosen more carefully. The investigators used high carbon plus antibiotic for the glucose media and low carbon without antibiotic in the galactose media. I will demonstrate later in this dissertation that inclusion of antibiotics in culture medium has significant impact on mitochondrial respiration and mtDNA copy number in cells from patients with mutations in *LONP1*. Nevertheless the correlation of mtDNA copy number levels and health is a valuable insight of the Carelli study and warrants further exploration

since mtDNA copy number has for example shown to be inducible by exercise in animal models, as discussed later in this chapter (Safdar et al. 2011).

1.2.2c Inefficient electron transport leads to increased oxidative stress

It has been puzzling why the pathology of homoplasmic mutations in mitochondrial complex I subunits, which should theoretically affect all tissues equally, often presents with a restricted, cell specific manner. Vision loss in LHON patients is further defined by loss of retinal ganglion cells restricted to unmyelinated regions of the optic nerve, which have the highest energy demand. This then leads to degeneration of small-caliber optic nerve fibers and central vision loss while peripheral vision is spared (Maresca et al. 2013). Since all LHON mutations compromise mitochondrial complex I functions it was suggested that pathology is most likely due to increased oxidative stress in affected tissues. Stalled electron transport at mitochondrial complex I as well as complex III results in the formation of superoxide anion $O_2^{\cdot-}$ the precursor to most reactive oxygen species (ROS) (Turrens 2003). ROS can damage proteins, lipids, DNA, RNA and other biomolecules. Mitochondria have several defense mechanisms against oxidative damage specifically the enzymes Mn-superoxide dismutase (SOD-1) catalase and glutathione peroxidase (GPx) (Drose, Brandt 2012). These enzymes may then in theory modulate the expression and severity of pathogenic nuclear and mtDNA encoded genes and provide a potential explanation for incomplete penetrance. Damage in LHON due to ROS has recently been confirmed in a LHON mouse model. This model opened the door to the development of several antioxidant treatment modalities as described in the next section (Lin et al. 2012).

Many other mitochondrial treatment modalities focus on upregulation of the antioxidant defense system or administration of molecules that can serve as electron acceptors for free radicals, as ways to decrease oxidative stress.

1.2.2d Cigarette smoking aggravates the LHON phenotype

Environmental factors that modulate expression of mitochondrial mutations have long been suggested but have been hard to prove (Caito, Aschner 2015). Alfredo Sadun and Valerio Carelli's group studied the effects of exposure to alcohol and cigarette smoke in a large multigenerational LHON family from Brazil, and postulated that smoking triggered the disease in mutation carriers, who might otherwise have never become affected. Their clinical descriptions include the reversal of vision loss in individuals who stopped smoking (Sadun 1998). Having demonstrated previously that upregulation of mtDNA copy number is protective in LHON carriers, a multicenter group built on these findings when interrogating mechanisms of toxicity of cigarette smoking and LHON mutations *in vitro*. The study shows that fibroblasts exposed to cigarette smoke condensate displayed decreased mtDNA copy number but that this was more pronounced in cells with the LHON mutation. The exposure also resulted in significantly decreased ATP levels, a finding restricted to cells from patients with LHON mutations. Cells from unaffected LHON carriers had the highest level of mRNA expression of genes involved in the antioxidant defense system (Giordano et al. 2015). These studies are of utmost importance to the clinician and the mitochondrial patient to encourage lifestyle changes with a measurable positive outcome. They also demonstrate that treatment may even be initiated after loss of vision. Reversal of vision loss has since been observed in clinical trials after the use

of antioxidants, particularly idebenone. The group of Alfredo Sadun reports on treating a 31 year old woman shortly after onset of vision loss with complete recovery over a period of 9 months (Sabet-Peyman et al. 2012). As a preventative treatment strategy Carla Giordano's group also investigated the effect of non-feminizing phyto-estrogen compounds on cybrid cells with a LHON mutation with increased cell viability and decreased ROS levels (Pisano et al. 2015). Recently administration of adenovirus associated gene therapy carrying the ND4 gene has been shown to be safe and to improve vision in patients with the most common LHON mutation (Wan et al. 2016). The LHON experience therefore proved seminal for designing treatment regimens and strategies for other mitochondrial disorders with less conclusive outcome measures (Koene et al. 2016).

1.2.3 Adulthood- Charcot Marie Tooth Disease (CMT)

1.2.3a CMT: From gene function to disease

It is ironic that although Charcot Marie Tooth disease is the most common inherited neurological disorder with 1/2500 people affected, few people have heard of the disease. The central diagnostic criterion is chronic polyneuropathy of the sensory and motor neurons. The disease is highly heterogeneous, with over 70 genes reported to date (Rossor et al. 2013). Two main types of CMT (as well as an intermediate type) are distinguished by nerve conduction velocities: CMT1, which displays chronic demyelination and nerve conduction velocities below 38 m/s, is caused by mutations in genes essential for Schwann cell biogenesis and Schwann cell metabolism. CMT2, which is not demyelinating and defined by nerve conduction velocities above 45 m/s, displays

mainly abnormal axonal development (Harding, Thomas 1980). A number of genes associated with CMT have frank mitochondrial involvement. This is particularly true for CMT2 related genes, which are mainly involved in cytoskeletal organization, as well as in mitochondrial axonal transport. Mutations in the mitochondrial GTPase mitofusin 2 (*MFN2*) represent the most common cause of CMT2 and are responsible for 20% of all CMT2 cases. Symptomatology associated with *MFN2* mutations is therefore further sub classified as CMT2A (Zuchner et al. 2004). CMT2A represents a classical axonal neuropathy with variable age of onset from infancy into the 6th decade but symptoms most commonly start in early adulthood. Due to the fact that CMT2A mainly follows autosomal dominant inheritance but may also display recessive inheritance patterns it is still unclear whether all CMT2A is caused by a gain of function or also a loss of function (Pitt et al. 2003). Onset before 10 years of age may be found in autosomal recessively inherited cases and has shown more severe “CMT2plus” phenotypes with optic atrophy and other CNS involvement (Chung et al. 2006; Genari et al. 2011). CMT2A is a common neurological disorder and it is therefore further surprising that no approved treatment modality is available. In the following section I propose this is mainly due to the fact that the mechanism by which mutations in *Mfn2/MFN2* confer pathology are still being investigated and highly debated, since findings observed in rodents do not corroborate findings observed in human cell lines.

1.2.3b CMT2A displays modulated mitochondrial dynamics in rodent

models but less so in human neurons

As introduced earlier, mitochondria are organelles that are organized as a tubular network and are in constant transition between varying levels of connectedness (fusion) and separation (fission). Strikingly, this behavior of fusion and fission is directly correlated to mitochondrial energy states and health (Cogliati et al. 2013a). The most important proteins facilitating fusion are the optic atrophy related protein Opa1, which is involved in fusion of the inner mitochondrial membrane, and the mitofusins (Mfn1 and Mfn2), which participate in fusion of the outer membrane. Studies in rat dorsal root ganglion cells point to a mitochondrial transport defect with mitochondria clustering in axonal bodies and decreased levels of mitochondria found in distal axonal regions (Baloh et al. 2007). Interestingly, the finding is not correlated with Mfn2's role in mitochondrial fusion but rather has been shown to be due to dysfunctional attachment of mitochondria to microtubules (Misko et al. 2010). This type of defect would then starve axons of much needed energy due to the lack of ATP generating mitochondria at the axon terminals. Before the onset of stem cell technologies, investigations of CMT2A pathology in human cell models had been complicated since primary fibroblasts from Mfn2 patients do not display abnormal mitochondrial dynamics. Since then it has been shown in two independent studies that peripheral nervous system cell types from CMT2A patients derived via iPSC technology do not replicate the findings from rodent models. The group of John Dimos demonstrates normal levels of mitochondrial number and size in iPSC derived spinal chord motor neurons. Surprisingly, although mitochondria moved more slowly along microtubules the studies could not replicate the severe axonal transport

defects observed in rodents (Saporta et al. 2015). The investigators argue that the animal models used to demonstrate the severe axonal transport deficits were due to knockdown or biallelic mutations of *MFN2* while CMT2 patient derived neurons were from heterozygous *MFN2* mutation carriers (Saporta et al. 2015). This explanation seems oversimplified since the group was able to reproduce the axonal mitochondrial transport defects in motor neurons derived from heterozygous NEFL mutation carriers, which is responsible for CMT2E. It has been shown that Mfn2 has several important functionalities related to cellular metabolism, connecting mitochondrial morphology to energy production (Sebastian et al. 2012). A closer look at those studies may aid in understanding the pathophysiology of Mfn2 and help in designing new treatment modalities.

1.2.3c Mfn2 Defects due to abnormal bioenergetics

In addition to the proposed role of Mfn2 in mitochondrial axon transport other groups are investigating its role in bioenergetics (Bach et al. 2003). These studies have involved gain of function (resembling autosomal dominant) as well as loss of function (resembling autosomal recessive) experiments. Although most conclusions have been drawn from experiments in muscle cells, conclusions are also drawn from nervous system abnormalities found in obese mice with *MFN2* defects (Dietrich et al. 2013). These type of investigations are particularly interesting since the neuropathies seen in CMT2A have significant overlap with diabetes induced nerve damage (Lozeron et al. 2002). A recent study by the group of Sergio Lavandero showed that in rat skeletal muscle Mfn2 controls metabolism in at least two ways - through control of mitochondrial morphology, as well as other non-canonical and less well understood mechanisms. The group demonstrated that

silencing of *MFN2* resulted in mitochondrial fragmentation and decreased insulin induced cellular glucose absorption. The observed glucose dysregulation was explained by disrupted mitochondrial Ca^{2+} uptake secondary to the abnormal mitochondrial morphology. The study also showed that the decrease in mitochondrial Ca^{2+} uptake translated into a decrease in activation of the protein kinase AKT, which directly controls the glucose transporter GLUT4 (del Campo et al. 2014). Additionally, since pyruvate, isocitrate and oxoglutarate dehydrogenases are directly controlled by mitochondrial matrix Ca^{2+} level, disrupted Ca^{2+} uptake further exacerbates low energy states via disruption of the TCA cycle (Santo-Domingo, Demaurex 2010). Interestingly, it has also been shown that Insulin itself is involved in preservation of mitochondrial morphology via stimulation of mitochondrial fusion. Therefore, the hormone protects the integrity of its own signaling cascade (Parra et al. 2014). Finally Mfn2 is able to stimulate glucose metabolism by a mechanism unrelated to mitochondrial fusion and Ca^{2+} dysregulation (Sebastian et al. 2012). The group of Antonio Zorzano overexpressed a truncated *MFN2* construct (Δ Mfn2) lacking mitochondrial fusion domains in mouse skeletal muscle cells. The study shows enhanced glucose oxidation and gluconeogenesis secondary to the overexpression of the deletion Mfn2 protein (Segales et al. 2013). Dysregulated glucose homeostasis is one of the hallmarks of a vast number of mitochondrial and metabolic syndromes and this topic winds like a thread through this dissertation. Tissue specific hypoglycemia in particular may eventually be recognized as a trigger point for the degenerative CNS process associated with syndromes of energy deficiency (Amiott et al. 2008; Simon et al. 2013). Regulation of glucose homeostasis in energy-hungry tissues may prove to be a unifying mechanism for mitochondrial treatment modalities.

1.2.4 Aging: Alzheimer's disease

1.2.4a Lessons learned from the aging field

The balance between glucose demand and tissue specific glucose availability plays a major role in age related diseases like Alzheimer's and Parkinson's disease (AD, PD), which have long been known to have a strong mitochondrial component (Wallace 2005). Mitochondrial involvement has been shown to be in part due to oxidative damage, caused by free radicals generated by the organelle, which is known as the "mitochondrial free radical theory of aging" (Harman 1956). In reality many factors play a role in cellular aging but their convergence regularly plays out at a mitochondrial nexus (Loeb et al. 2005). Pathological processes observed during aging and processes observed in frank mitochondrial disease are closely related or essentially the same. The difference is the pace, at which mitochondrial damage accumulates in tissues, and is translated into mitochondrial disease states. For example, mtDNA point mutations and deletions will accumulate rapidly due to a primary mutation in the mitochondrial mtDNA polymerase gamma gene (*POLG*) leading to early onset Leigh syndrome. Mitochondrial DNA mutations continuously accumulate at a slow and steady pace during normal aging in post-mitotic tissues, since the enzyme inherently has a relatively high mutation frequency. Another example is type 2 diabetes with its hallmark findings of insulin resistance and relative insulin deficiency leading to dysfunctional glucose uptake and an intracellular hypoglycemic state. Type 2 diabetes develops slowly in non-mitochondrial mutant patients but shows often young and rapid onset in patients with the mt-tRNA^{Leu} A3243G mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) mutation

(Sue et al. 1999). Type 2 diabetes also has shown to have significant and direct impact on the development of AD. Interestingly, mitochondrial abnormalities have directly linked the two disease pathologies in rodent models (Peila et al. 2002; Carvalho et al. 2012). A study by Paula Moreira's group showed that mice exposed to sucrose sweetened water had increased levels of A β protein and displayed mitochondrial abnormalities in brain reminiscent of that observed in the AD mice (Carvalho et al. 2012). These are just a few examples to demonstrate the direct correlation and lessons that can be drawn from the extensive efforts afforded in the aging field and their translation to the mitochondrial clinic. These deserve a closer, more in depth discussion in this introductory chapter since many concepts and new insights in mitochondrial metabolism directly affect the mitochondrial disease process.

1.2.4b The POLG mutator mouse is a powerful tool to study mitochondrial degeneration and metabolism

One of the best studied models of aging is the mitochondrial POLG "mutator mouse". In this animal model the exonuclease domain of the nuclear encoded *Polg* gene was mutated, resulting in increased mtDNA point mutations and deletion rates leading to respiratory chain deficiency. The mice develop normally until 6 months but then age rapidly with greying hair and premature hair loss, skeletal involvement, including kyphosis and osteoporosis, and anemia. They also appear emaciated, display hearing loss and cardiomyopathy all leading to a reduced life span (Kujoth 2005; Trifunovic et al. 2005). It was, therefore, surprising that increased oxidative stress and ROS production was not a finding associated with this animal model (Zhang et al. 2000). This finding contradicted

established models that supported a role for ROS in aging and instead suggested that defects in electron transport were directly responsible for the aging effect. Additional experiments in a double mutant mouse again point to involvement of use and availability of energy sources and metabolism. Knockdown of the uncoupling protein UCP2 significantly aggravated the phenotype with increased lactic acidosis, decreased life span and worsened cardiomyopathy (Safdar et al. 2011). The study showed that UCP2 enabled increased use of glutaminolysis and utilization of fatty acids, allowing the cell to rely less on glucose, which was abolished by the loss of the uncoupling protein (Kukat et al. 2014). The knockdown of UCP2 therefore further worsened the metabolic defects of the POLG mutator mouse including hypoglycemia in an animal model that heavily relies on glycolysis for energy generation (Saleem et al. 2015). Therefore the POLG mutator mouse is no longer just a model of premature aging but resembles the hypoglycemia and lactic acid states, which we see in primary mitochondrial disease patients with lactic acidosis, and are a useful tool to study energy flux *in vivo* (Simon et al. 2014). There are many more studies, which have successfully used the POLG mutator mouse model to elicit new pathways in mitochondrial metabolism, and disease development, leading to new treatment regimens and clinical trials (Pfeffer et al. 2012). This animal model is therefore one of the most successful examples of "bedside to bench to bedside" and the discussed studies demonstrates the value of creating the laboratory scenario of a "double mutant" or mutant plus environmental impact to study the effect of genetic mitochondrial disease in conjunction with confounding factors.

1.2.4c The POLG mutator mouse reveals metabolic dysfunction in

Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia and has been shown to be associated with faulty cleavage and aggregation of amyloid precursor proteins to form neuritic plaques. The presence of neuritic plaques then induces the formation of intracellular tangles composed of Tau and amyloid beta (A β) protein leading to progressive cortical atrophy and dementia (Coskun et al. 2012). One familial type of AD is due to mutations in the amyloid beta (A β) A4 protein gene APP. In Alzheimer's disease the APP protein has been shown to accumulate in the translocation channels of the outer mitochondrial membrane blocking import of other mitochondrial proteins (Anandatheerthavarada et al. 2003). To further explore the role of APP and mitochondria, Vassar's group crossed the POLG mutator mouse with an AD-APP mouse model. The researchers found that increased plaque formation was not due to an increase in APP production, but rather due to metabolic changes, with double mutant mice having decreased levels of insulin degrading enzyme IDE that is known to clear A β (Kukreja et al. 2014). IDE has since been found to regulate not just insulin levels, but also the levels of the glycogenolysis inducing hormone glucagon. A potent and highly selective IDE inhibitor "6bK" has shown improved glycolytic flux during feeding in a different mouse model of diabetes and obesity by inhibiting the degradation of insulin, glucagon and amylin in a disproportional manner (Maianti et al. 2014). It would therefore be interesting to investigate the effects of 6bK on the APP-POLG mutator mouse to explore feasibility of 6bK as treatment in mitochondrial disease. Another promising diabetes drug in the mitochondrial field is acarbose, which is currently being tested as a treatment for Leigh

syndrome in mice with a Leigh-like disease (Kaeberlein and Bitto unpublished results, UMDF2016). The above findings of dysregulated glucose metabolism in mitochondrial disease are consistent with previously discussed findings for the POLG mutator mouse demonstrating that the ability to efficiently use glucose is at the center of mitochondrial and cellular health. This finding is also consistent with previous discussion addressed in section 1.4.2 on CMT and non-canonical functions of Mfn2 on glucose homeostasis. I will revisit this topic in the dissertation's conclusion chapter with a focus on tissue specific use of glycolysis and oxidative metabolism during brain development. For the future it will be interesting to see whether treatment options designed for AD like the IDE inhibitor as well as for diabetes will find their way in to the mitochondrial clinic.

1.3 Exercise as treatment for mitochondrial disease

Although not applicable to mitochondrial cases that present in infancy, exercise is to date the most effective mitochondrial treatment and preventative of mitochondrial disease, as shown in animal models. Subjecting POLG mutator mice to endurance exercise completely averted mitochondrial abnormalities that would otherwise result in the progeroid phenotype. Mice started treadmill exercise program at 45 minutes, three times per week when they were 3 months old (i.e. equivalent to middle-aged humans). After 5 months of exercise the mice displayed increased mtDNA copy and decreased mtDNA mutations as compared to their sedentary littermates. A reduction in protein levels of subunits of the mitochondrial respiratory chain, observed in the sedentary mice, was also averted in the exercised mice. Additionally, EM of muscle from sedentary POLG mice displayed mitochondria with fragmented cristae and disrupted membranes reminiscent of

the patient with Luft disease, while the exercised mutator mice revealed normal mitochondrial ultrastructure (Safdar et al. 2011). These studies bring our journey through the lifecycle of the mitochondrial disease processes full circle and back to the patient with Luft Disease (Luft et al. 1962). The patient's underlying genetic defect remains unresolved but the fact that her symptoms were due to a mitochondrial dysfunction were clearly shown. In a study by Marianna Sikorska's group a young athlete had volunteered for a study on gender differences in intramuscular lipid. Surprisingly, needle biopsy showed numerous paracrystalline inclusions reminiscent of what had been seen in the patient with Luft disease in this apparently healthy individual (Tarnopolsky et al. 2004). Sequencing of the athletes' mtDNA showed a pathogenic mutation in the mitochondrial complex 3 subunit gene MT-CYTB at position G15497A and revealed maternal family history of seizure disorder. Studies using cybrids ('cytoplasmic hybrids' - a cell where the nucleus has a neutral genetic background but the mitochondria are patient derived) showed highly uncoupled mitochondria with decreased ATP generation and high ROS generation reminiscent of the patient with Luft disease. Interestingly the patient's paracrystalline inclusions found in muscle tissue were reversible after treatment with creatine monohydrate for five weeks (Tarnopolsky et al. 2004). This case without an initial pathogenic indication shows that the disease process starts long before symptoms are observed. More importantly the case shows that intervention with targeted treatment can reverse the pathology as long as we are aware of the underlying defect and are able to deploy intervention early in the disease process.

CHAPTER 2

Mutations of Human *NARS2*, Encoding the Mitochondrial Asparaginyl-tRNA Synthetase, Cause Nonsyndromic Deafness and Leigh Syndrome

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2.1 Introduction

Mitochondrial respiratory chain (MRC) disease represents a large and heterogeneous group of energy deficiency disorders (McCormick et al. 2013) (Molecular genetic testing for mitochondrial disease: from one generation to the next). A significant percentage of MRC disorders is caused by both nuclear and mitochondrial encoded genetic variants that impact molecules of the mitochondrial protein synthesis machinery (Lightowlers et al. 2014). Among these genes, those coding for the mitochondrial aminoacyl-tRNA synthetases (mt-aaRSs) have emerged as being frequently associated with human disease (Schwenzer et al. 2014). The primary function of mt-aaRSs is to charge mitochondrial tRNA (mt-tRNA) molecules with their cognate amino acids (Ibba et al. 2005). Scheper and co-authors first linked mutations in *DARS2*, encoding the mitochondrial aspartyl-tRNA synthetase, to leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation in brain (LBSL; MIM: 611105) (Scheper et al. 2007). Since then, mutations of 14 other mt-aaRSs have been associated with mitochondrial disease, including *NARS2*, which has recently been linked to intellectual disability, epilepsy and myopathy (Vanlander et al. 2014). The significant tissue specificity and phenotypic heterogeneity of mutated mt-aaRSs was unexpected, considering that a deficiency in these ubiquitously expressed enzymes should affect all tissue types (Yao, Fox 2013). The delineation of the underlying cellular mechanisms is subject to intensive investigation for cytoplasmic as well as mitochondrial aaRS (Guo, Schimmel 2013; Tynismaa, Schon 2014).

Mutations of five (mt) aaRS genes cause syndromic forms of deafness, including Perrault syndrome (*LARS2*, *HARS2*), Charcot Marie Tooth disease type 2N (*AARS*)

(McLaughlin et al. 2012) and pontocerebellar hypoplasia type 6 (*RARS2*) (Cassandrini et al. 2013). Homozygous mutations in *KARS*, an aaRS which functions in the cytoplasm as well as the mitochondria, have been shown to cause nonsyndromic hearing impairment (DFNB89) (Santos-Cortez, Regie Lyn P et al. 2013). The gene shows similar pleiotropism as it is demonstrated for *NARS2* in this study since in addition to DFNB89, compound heterozygous *KARS* mutations have been shown to cause Charcot Marie Tooth disease and developmental delay as well as severe infantile disease with microcephaly and white matter abnormalities, seizures and vision loss (McMillan et al. 2014; McLaughlin et al. 2012).

Mutations in *EARS2* and *FARS2* have been shown to cause fatal epileptic mitochondrial encephalopathy and/or Alpers syndrome (Elo et al. 2012; Almalki et al. 2014; Steenweg et al. 2012) and mutations in *IARS2* have previously been associated with Leigh Syndrome or Leigh-like disease. Our report therefore adds *NARS2* to the list of mt-aaRS associated fatal epileptic mitochondrial encephalopathy and represents the second Leigh syndrome associated mt-aaRS. Leigh syndrome is a neurodegenerative disease caused by mitochondrial dysfunction resulting in symmetric, bilateral lesions in the basal ganglia, thalamus, and brain stem (Baertling et al. 2014). Leigh syndrome is the most common clinical finding associated with mitochondrial disease of childhood and displays significant genetic heterogeneity (Baertling et al. 2014; Sofou et al. 2014). To date there are over 60 genes associated with Leigh syndrome, and a large proportion is caused by defects in molecules involved in the mitochondrial translational machinery (Baertling et al. 2014). In two families, we report phenotypic variability associated with

different mutations of the same mt-aarSs, NARS2. One family is segregating nonsyndromic hearing loss (DFNB94) and another with Leigh syndrome.

2.2 Results

2.2.1 Clinical findings

2.2.1.1. Family LS06

Subject II.1, from family LS06, (Figure 2.1A) was born to unrelated healthy Caucasian parents without contributory family history. He was considered normal at birth but then failed the post-natal hearing screen. Follow up testing at 1 month of age showed absent Auditory Brainstem Response (ABR) with preserved cochlear microphonics, diagnostic of bilateral auditory neuropathy. Pure tone otoacoustic emissions (OAEs) testing at 11 weeks showed absent transient evoked emissions. Early developmental milestones were normal. Myoclonic movements started at 3 months of age, rapidly worsened, eventually involving all four extremities and complex partial seizures were recorded. A CT scan and MRI of the head were normal. Extensive laboratory studies for metabolic disease showed abnormal urine organic acids with elevations in multiple TCA cycle metabolites (Table S2.1). CSF lactate was elevated at 3.9 mmol/L (Normal <2.0 mmol/L) and 5.4 mmol/L by 5 months of age, while plasma lactate was normal.

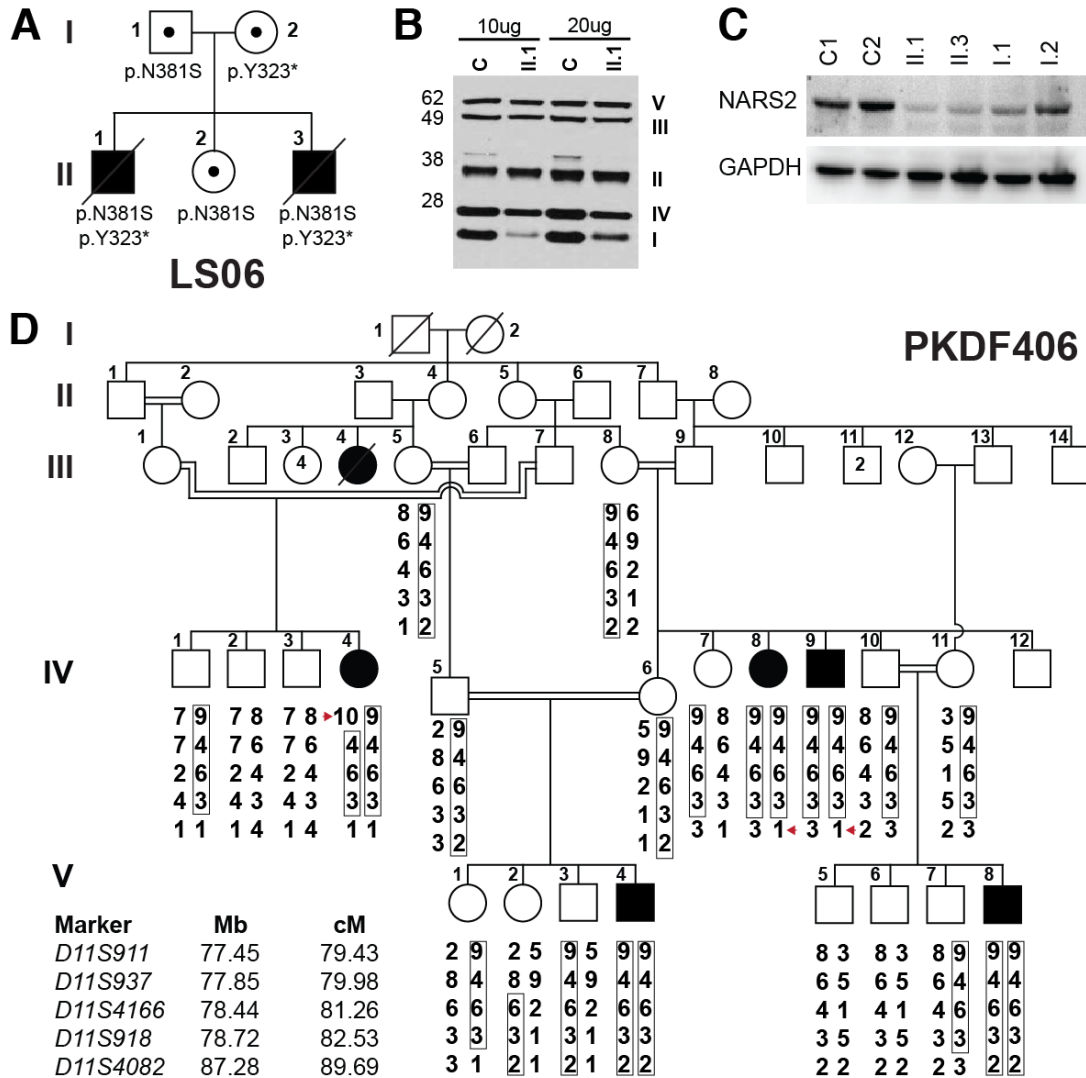
Follow up MRI showed multiple areas of hyperintensive T2-weighted and Fluid-attenuated inversion recovery (FLAIR) signal within periventricular white matter and posterior corona radiata with extension into the posterior limbs of the internal capsule. There was also a hyperintensive signal in the thalami and dentate nuclei. Electroencephalography (EEG) was abnormal, consistent with status epilepticus. Anti-

seizure medications were tried including Topamax®, Dilantin® and Klonopin®, but were not effective. By 10 months of age, he had developed laryngomalacia with pharyngeal hypotonia, his condition progressed and he died of respiratory failure at 15 months of age. Post mortem examination of the brain showed cortical atrophy with laminar necrosis, atrophy of the corpus callosum, significant white matter oligodendroglial loss. Neuronal gray matter loss was widespread with gliosis. Multifocal prominent hypervascularity, as well as, symmetrical lesions in the brainstem and thalamus were characteristic of Leigh syndrome.

Table S2.1: Abnormal levels of urine organic acids of patient II.1.

Organic acids	Patient II.1 (mM/mol creatinine)	Reference Ranges (mM/mol creatinine)
Benzoic acid	44	0-6
Ethylmalonic acid	14	0-11
Malic acid	52	0-13
Pyruvic acid	27	0-22
2-OH-glutaric acid	29	0-22
3-OH-adipic acid	35	0-20
Suberic acid	9	0-7
Citric acid	>1344	120-675
Hippuric acid	>3166	0-784
OH-decanedioic acid	7	0-2
N-acetyltyrosine acid	5	0-2

Figure 2.1: NARS2 mutations identified in two unrelated families



(A) Pedigree of the LS06 family. Filled symbols represent affected individuals and small circles represent carrier individual. The pedigree shows autosomal recessive inheritance of compound heterozygous NARS2 variants [c.969T>A; p.Tyr323*] and [c.1142A>G; p.Asn381Ser]. (B) SDS PAGE and western blot of control and patient II.1 muscle homogenates (10 μ g and 20 μ g of protein), samples were probed for mitochondrial respiratory chain complexes via MitoProfile total OXPHOS human WB antibody cocktail. The result showed significantly decreased amounts of mitochondrial respiratory complex I and IV. (C) SDS PAGE and western blot of fibroblast lysates from both affected probands (II.1, II.3), their parents (I.1, I.2) and controls using anti-NARS2 antibody and anti-GAPDH antibody as loading control. (D) Pedigree of the PKDF406 family. Filled symbols represent affected individuals, and a double horizontal line represents a consanguineous marriage. Alleles forming the risk haplotypes are boxed. The short tandem repeat (STR) markers, their relative map positions (Mb) according to UCSC Genome Bioinformatics build GRCh37 (hg19), and their genetic positions (cM) based on the Marshfield genetic map are shown next to the pedigree. A haplotype analysis revealed a linkage region delimited by a proximal meiotic recombination at marker D11S911 in individual IV:4 (arrowhead) and distal recombination at marker D11S4082 in individuals IV:8 and IV:9 (arrowhead).

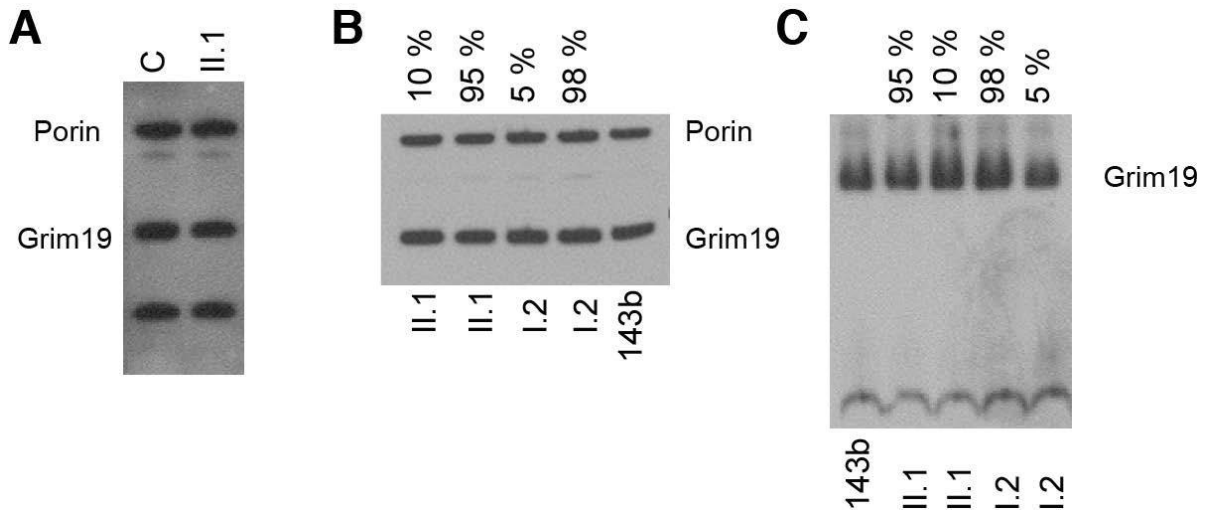
A muscle sample was obtained post-mortem within the acceptable time frame for preservation of mitochondrial enzyme activities. Histology, including mitochondrial stains and electron microscopy, was normal. ETC studies showed absent activity of NADH cytochrome C reductase (COI/III), indicating a block in electron flow from the membrane bound arm of mitochondrial complex I (COI) to mitochondrial complex III (COIII) and a milder reduction in complex IV (COIV) activity. Mitochondrial ferricyanide reductase activity probing the matrix arm of COI, which is thought to contain mainly nuclear encoded subunits was normal (Table S2.2).

Additionally SDS PAGE and western blot for muscle and fibroblast lysates from proband II.1 were performed with a standard protocol (Potluri et al. 2004). Consistent with the ETC results, western blot on muscle lysates showed significantly decreased levels for mitochondrial complex I (NDUFB8-18kD) and moderate decrease for mitochondrial complex IV (MTCOII-22kD) (Figure 2.1B). However, western blot performed with anti-GRIM19 antibody for mitochondrial complex I (GRIM19 corresponds to the mitochondrial complex I subunit NDUFA13) in fibroblast lysates was normal (Figure S2.1A).

Table S2.2: Mitochondrial respiratory chain complex activities of muscle homogenate

Complex	Enzyme analyzed	II.1	II.2
I	NADH-Ferricyanide reductase	94%	84%
I / III	NADH-cytochrome c reductase (rot. sens)	0.01%	12%
II	Succinate dehydrogenase	112%	102%
II / III	Succinate-cytchrome c reductase (antimycin sensitive)	42%	71%
III	Decylubiquinol-cytochrome c reductase	59%	68%
IV	Cytochrome c oxidase	44%	103%

Figure S2.1: Western blot and Blue Native Gel analyses for patient fibroblasts and transmittochondrial cybrids



(A) SDS PAGE followed by western blot of fibroblast cell lysate from patient II.1 does not show lower levels of mitochondrial complex I (COI) subunit NDUFA13 (Grim19). Porin is used as a loading control. (B-C) We generated mitochondrial cybrid cell lines for II.1 and I.2 to delineate mtDNA vs nDNA origins of the complex I defect. For this we fused enucleated patient fibroblasts with a human osteosarcoma (143b) ρ^0 cell line and selected clones displaying 5, 10, 95 and 98% of heteroplasmy respectively for mt-tRNA^{Cys}A5793G. western blot (B) and BNG analyses (C) for COI (NDUFA13-GRIM19) were both normal for II.1 and I.2 irrespective of heteroplasmy levels.

The family's third child, a male who was born after an uneventful pregnancy via spontaneous vaginal delivery (II.3, Figure 2.1A) also failed the new born hearing screen but was otherwise normal. ABR and OAE testing at 1 month of age showed comparable bilateral auditory neuropathy with normal middle ear function. He first presented with myoclonic movements of the right arm at three months of age, accompanied by lethargy and decreased feedings, which necessitated the placement of a gastrostomy tube. The MRI showed restricted diffusion in the left basal ganglia, and external capsule junction as well as the left frontal lobe in cortical distribution. EEG showed continuous left hemispheric focal seizures. Laboratory testing for organic acids revealed mild elevation in TCA cycle metabolites, fumaric, malic, and 2-keto-glutaric acids; suggestive of mitochondrial disease. Plasma amino acids, as well as, lactic acid in blood were within normal range. A muscle biopsy at three months of age was normal for coenzyme Q levels and histology, while ETC studies (CIDEM) showed complex I/III deficiencies similar to his deceased brother. Individual II.3 passed away at 6 months of age. Autopsy also showed severe encephalopathy and prominent basal ganglia involvement consistent with Leigh syndrome. There were fewer vaso-proliferative lesions than in proband II.1. The striate cortex showed severe degeneration, which correlated with the patient's cortical blindness. Microscopically there was widespread gliosis and prominent diffuse metabolic astrocytosis.

2.2.1.2. Family PKDF406

We ascertained family PKDF406 from Punjab province of Pakistan. The pedigree suggested that deafness was segregating in this family as an autosomal recessive trait

(Figure 2.1D). Pure-tone audiometric evaluations of the affected individuals from the PKDF406 family revealed pre-lingual, profound, bilateral sensorineural hearing loss (HL) (Table 2.1 and Figure S2.2). We found no evidence of co-segregation of vestibular dysfunction, retinitis pigmentosa or an obvious cognitive disability with HL in the PKDF406 family (Table 2.1).

Table 2.1: Clinical findings of affected PKDF406 family members

No.	Age years	Sex	Hearing status	Retinitis pigmentosa	Vestibular function ^a	Hypotonia	Seizure history	Brain CT scan	Menstrual history
IV:4	40	F	Profound	No	Normal	No	None	ND	Menopause ^c
IV:8	45	F	Profound	No	Normal	No	None	ND	Menopause ^c
V:8	26	M	Severe to profound	No	Normal	No	None	Right maxillary sinusitis	NA
V:4	30	M	Profound	No	Positive ^b	No	None	Normal	NA

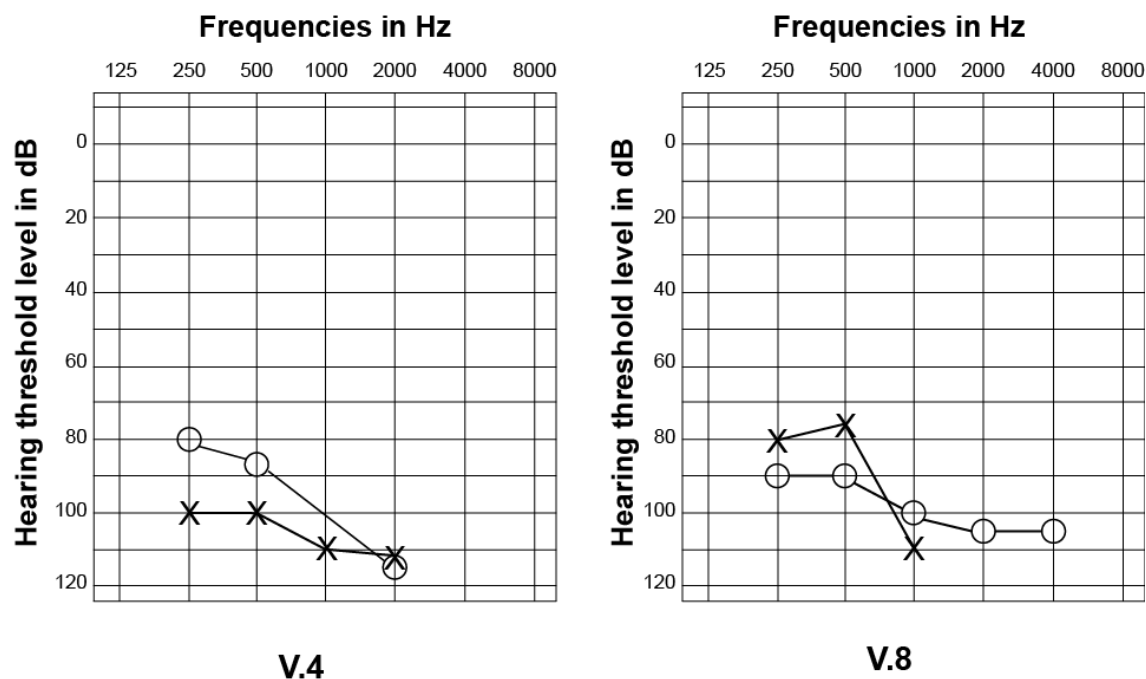
^aEvaluated using Tandem gait and Romberg tests.

^bPositive: fumbled during both tests.

NA: Not applicable; ND: Not determined.

^cHad history of normal menstrual cycles before premature menopause

Figure S2.2: Audiograms of individuals V.4 and V.8 from family PKDF406



Hearing loss in the affected family members was evaluated by pure-tone audiometry, which tested frequencies that ranged from 250 Hz to 8 kHz. It was determined to be severe to profound, sensorineural and bilateral. The symbols 'o' and 'x' denote air conduction pure-tone thresholds in the right and the left ears, respectively.

2.2.2 Leigh syndrome and nonsyndromic hearing loss caused by mutations of NARS2

2.2.2.1 Leigh syndrome caused by mutations of NARS2

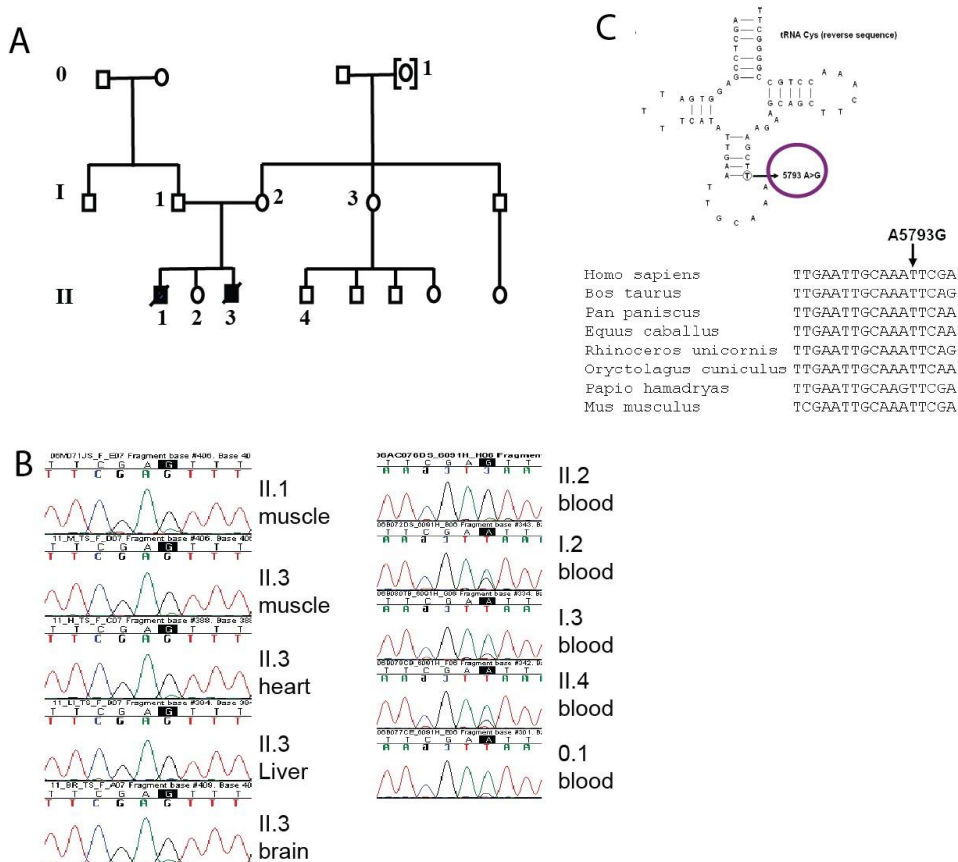
mtDNA studies for patient II.1 from family LS06, identified a heteroplasmic variant in the mitochondrial tRNA cysteine (mt-tRNA^{Cys}) at position A5793G while all other studies were normal (Figure S2.3). Segregation analysis showed the variant to be homoplasmic in the unaffected sister and could therefore not have been the underlying cause of the phenotype (Figures S2.3B). Since the variant could cause suboptimal translation of mtDNA encoded respiratory chain subunits we performed transmitochondrial cybrid studies. The variant did not affect mitochondrial complex I levels when introduced into a

neutral nuclear background (Figure S2.1). Therefore, to identify the disease-causing gene, we performed whole exome sequencing (WES) for probands II.1 and II.3 and their father (I.1). This analysis revealed compound heterozygous variants in the *NARS2* gene (NM_024678, MIM: 612803), encoding mitochondrial asparaginyl-tRNA synthetase (mt-AsnRS or NARS2), at positions [c.969T>A; p.Tyr323*] and [c.1142A>G; p. Asn381Ser] (Figure 2.1A, Figure S2.4). Carriers of these mutations were not found in the 1000 Genome Project or the NHLBI Exome Variant Server (EVS). The *NARS2* variants represent the only candidate gene, which could explain the proband's phenotype (Table S2.3).

Table S2.3: Summary of Exome sequencing analysis for LS06

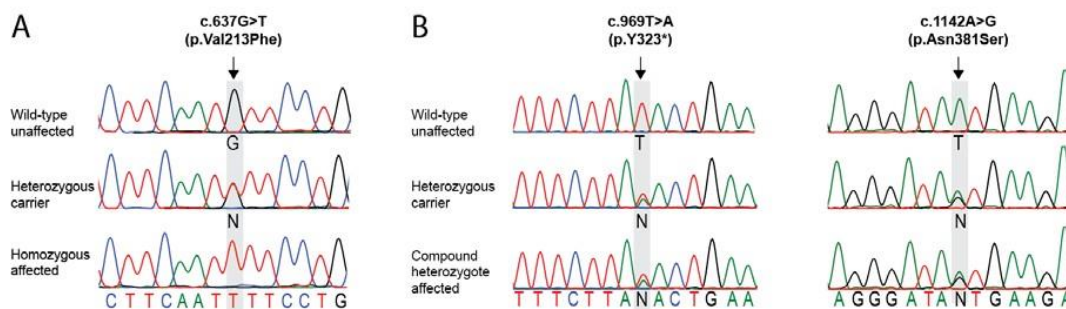
	Exome	Gene
Variants in common for both probands and with same zygosity	65,339	
Coding region and splice variants	12,168	
1000g2012apr_all MAF <0.01 filtering	1,456	
dbSNP138_NonFlagged threshold 0.01	181	
Ensemble annotations popfreq_all threshold 0.01	90	
X-linked genes	3	<i>Gria3, IRS4, MAGEC1</i>
Genes with two or more variants in trans	2	<i>ANKRD36, NARS2</i>
Genes with mitochondrial association	1	<i>NARS2</i>
Mitochondrial clinical variant	1	<i>Mfn2</i>

Figure S2.3: Segregation and Sanger sequencing of mt-tRNA^{Cys} A5793G



(A) Extended pedigree. (B) A novel mt-tRNA^{Cys} variant at position A5793G had been shown in the proband and other maternal relatives via full mtDNA sequencing. The variant displays heteroplasmy (varying levels of variant vs wild-type mtDNA) (C) Position of A5793G mt-tRNA^{Cys} at the base of the acceptor stem. The variant is completely conserved in mammals.

Figure S2.4: Sequencing chromatograms



(A) Nucleotide sequence chromatograms of exon 6 of *NARS2* comparing the wild type sequence, heterozygosity and homozygosity of the c.637G>T mutation. (B) Nucleotide sequence chromatograms of exons 10 and 11 of *NARS2* comparing the wild type sequence, heterozygosity and compound heterozygosity of the c.969T>A and c.1142A>G mutations.

2.2.3 Nonsyndromic hearing loss caused by mutation of *NARS2*

In family PKDF406, linkage analysis was undertaken using short tandem repeat (STR) markers for many of the reported recessive nonsyndromic deafness loci. PKDF406 family was found to be segregating deafness linked to markers for *DFNB2* (Figure 2.1D) on chromosome 11q13.5 (Weil et al.). Previous studies have shown that mutant alleles of *MYO7A* are responsible for the *DFNB2* phenotype in humans (Riazuddin et al. 2008). Using the NGS-based mutation screening test OtoSeq (Shahzad et al. 2013), we sequenced the affected individual IV:8 (Figure 2.1D) from the PKDF406 family and did not find any pathogenic variants in all of the coding and non-coding exons of *MYO7A*. Furthermore, refined mapping and haplotype analyses using additional PKDF406 family members excluded the *MYO7A* gene from the linkage interval on chromosome 11q13.4-q14.1. Therefore, the HUGO nomenclature committee assigned the designation *DFNB94* to the locus defined by the PKDF406 family. A maximum two-point LOD score of 5.10 ($\theta = 0$) was obtained for marker *D11S937*.

Next, genomic DNA from individual IV:8 of family PKDF406 was processed for WES. All the variants found in the WES data are summarized in Table S2.4a. Since there was significant evidence of linkage of deafness segregating in family PKDF406 to STR markers on chromosome 11q13.4-q14.1, we focused only on the variants present in the *DFNB94* linkage interval. We found a c.637G>T transversion variant in *NARS2*. Using Sanger sequencing (Figure S2.4), we confirmed the segregation of the c.637G>T allele with hearing loss in the PKDF406 family. Sanger sequencing of all the coding and non-coding exons of *NARS2* (Table S2.4b) did not reveal any other changes besides c.637G>T in family PKDF406. No carrier of the c.637G>T mutation was found in the 500

ancestry-matched control chromosomes, the 1000 Genome Project or the NHLBI EVS database.

Table S2.4a: Summary of Exome sequencing analysis for PKDF406

	Exome
Total changes	61,250
Changes not in dbSNP135 (MAF < 1%)	2359
Non-synonymous/Splice site/insertions/deletions	352
Homozygous/compound heterozygous changes	31
Changes not found in Pakistani control samples	21
Changes not found in 1000 genome or NHLBI ESP	21
Potential pathogenic changes in known deafness genes	0
Changes present in the <i>DFNB94</i> linkage interval	1
Changes predicted to be pathogenic [#]	1
Changes segregating with hearing loss in family PKDF468	1

[#]Pathogenic predicted by at least two of the four prediction programs: Polyphen-2, SNPs3D, MutationTaster and SIFT.

Table S2.4b: Primer sequences used to amplify and sequence human *NARS2* coding exons

Exon	Forward Primer	Reverse Primer	Product (bp) ^a
1	CTCTGGAGTGCCTTAGAGCAGAG	CGACGCCTACACTTTCTAACTTTTC	399
2	GAAGTTGATTTGTTGAGGCAGGAT	TATTCGGAGTTGATCCTCAAAGCTA	379
3	TCTATGGGAGAACTTGAACCTCGAAC	TTAACTGTGACACACACCACAGAGA	820
4	CGTCCTACCATTCCACTTACCTG	GCACCCAAAGCTAGGTCATGTAG	495
5	AAAATTATTTGTGCCCTGTGG	TGTCACAGCATAAGACTGCTGGT	468
6	CCTTTATTTTCCTGAGAACAACCTG	CATGTTTGGGATTCAAGCAAGA	477
7	AGCAAATATGTGTCAGTCTGTGGAC	AATGGCAGAGCTGGGATATAAACTT	475
8	GAATGCGGTCCTTATTGAGTCCT	CCTGGAATCTGGGGAGTGTCTAT	495
9	TGGTTCTCTCAAATGTCCTGAAG	CCTGGAATCTGGGGAGTGTCTAT	375
10	GTCTTCAGGCAGATCATTCTTTTCAT	AAACGTGGGAGACTCCTTTATAACC	495
11	CCTTAGCAGCTAGCTCCACACAT	GACCTTGTGACAGAAATGAGGAAGT	472
12	ACTACACAAATGGTGCAAGAGAGAG	TTGCTCAAGGACATAGAGAATCTGA	419
13	AGAGGCTTTGTCGTCTACTTCTTCA	CCCTTCCCTGACCTAAGAACAGTTA	286
14	TTCTTCCTTTCCATTGTCTGTCTGT	CATCCTTACGTGAAATACCCTCAAC	500

^aAll PCR products were amplified with 1.5 mM MgCl₂ and 60°C annealing temperature.

2.2.4 *Nars2* expression in the brain and inner ear

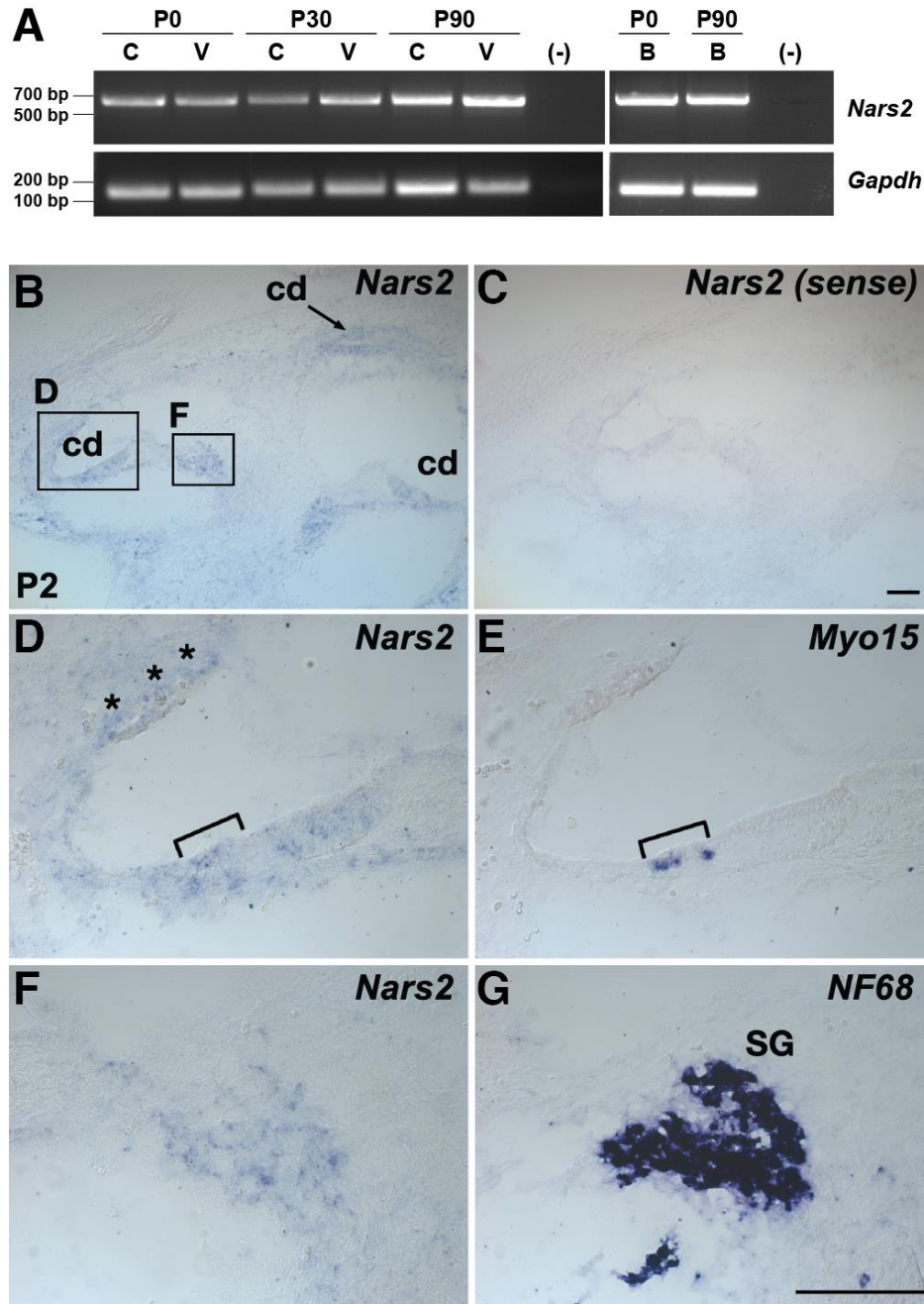
NARS2 is widely expressed in human and mouse tissues (UniGene, see Web Resources), including the brain, cochlear and vestibular systems (Figure 2.2A, Table S2.5). In mouse brain, *Nars2* is also broadly expressed with prominent *in situ* hybridization signals in regions such as the cortex, hippocampus, cerebellum and brain stem (Allen Brain Atlas; see Web Resources). A transcriptome analysis showed a 10-fold

increase in *Nars2* mRNA in mouse cochlear and vestibular spiral ganglion cells at postnatal day 0 (P0) compared with sensory hair cells (SHIELD; see Web Resources). As there is no commercially available antibody for murine *Nars2*, we performed *in situ* hybridization studies to highlight the expression of *Nars2*. They revealed a broad *Nars2* expression pattern in the spiral ganglion (Figure 2.2B, F), the cochlear duct including the organ of Corti (Figure 2.2D, bracket), and some of the mesenchyme surrounding the duct (Figure 2.3D, asterisks), at P2 (Figure 2.2B, D, F). A *Nars2* sense probe was used as a negative control (Figure 2.2C), and sense probes for *Myo15a* and *NF68* (Figure 2.2E, 2.3G) were used as positive controls for labeling sensory hair cells and spiral ganglion, respectively (Morsli et al. 1998).

Table S2.5: Primer sequences used to amplify mouse *Nars2*

	Forward Primer	Reverse Primer	Product (bp)
<i>mNars2</i>	TGCACATTCATACCCCAGTAC	ACCATCTTCATTCTCCCTCATG	626
<i>mGAPDH</i>	TCAACAGCAACTCCCCTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA	115
Riboprobe	TTGTTGCGGATTCAAGCTTCG	CCCTGAGGAACGCTGAGA	1579

Figure 2.2: Expression of *Nars2* in mouse inner ear and brain



(A) RT-PCR analysis of *Nars2* expression in the C57Bl6/J mouse cochlear (C), vestibular (V) tissues and brain (B) at different developmental stages (P0, P30 and P90). *Gapdh* expression was used as an internal control. (B-G) Expression of *Nars2* in the P2 mouse cochlea is shown. Hybridization signals of *Nars2* antisense (B, D, F) and sense (control) probes (C) in mid-modiolar sections of P2 cochlea are shown. Positive signals were detected in the cochlear epithelium, including the region, organ of Corti, (D) as indicated by the Myo15a-positive hair cells (E, bracket). Positive signals were also detected in the cells surrounding the cochlear duct (D, asterisks) and neurofilament-positive spiral ganglion (G, SG). B, C, E and G are 12 μm adjacent sections. Abbreviations: cd, cochlear duct, SG, spiral ganglion. The scale bar in C is 100 μm and applies to B and C. The scale bar in G is 100 μm and applies to D-G.

2.2.5 Mutations located in exons encoding the predicted catalytic domain of NARS2

NARS2 is composed of 14 exons that encode a protein of 477 amino acids (Figure 2.3A). *NARS2* was first described in yeast (Landrieu et al. 1997), and belongs to the class II aminoacyl-tRNA synthetases, a classification based on three consensus sequence motifs in the catalytic subunit (Eriani et al. 1990). Generally, aaRS are comprised of an anticodon binding domain and a catalytic domain. Some aaRSs have an additional domain with editing functions to prevent the insertion of inappropriate amino acids during protein synthesis. However, InterProScan (Zdobnov, Apweiler 2001) and SWISS-MODEL (Arnold et al. 2006) molecular modeling software predicted that *NARS2* does not contain this additional domain (Figure 2.3A). All mutations observed in our families are located in the predicted catalytic domain (Figure 2.3A). The Leigh syndrome associated p.Tyr323* variant results in a premature termination codon and is therefore considered damaging. The stop codon occurs instead of the tyrosine residue which is conserved through yeast (Figure 2.3B) and hypothetically results in a truncated protein of 323 amino acids. The second Leigh syndrome variant p.Asn381Ser substitutes Serine for a highly conserved Asparagine with a GERP score of 4.59 (Figure 2.3B). This variant is predicted highly pathogenic by all ten interrogated pathogenicity prediction programs (Table S2.6). The hearing loss p.Val213Phe variant is moderately conserved with a GERP score of 3.89. It is deemed highly damaging by 5 out of the 10 interrogated pathogenicity algorithms (Chang, Wang 2012).

Table S2.6: Predicted effect of p.Val213Phe and p.Asn381Ser missense mutations on NARS2

<i>In silico</i> pathogenicity prediction tool	p.Val213Phe predicted effect (score)	p.Asn381Ser predicted effect (score)
¹ PolyPhen-2 HDIV	Probably damaging (0.978)	Probably damaging (0.999)
² PolyPhen-2 HVAR	Possibly damaging (0.815)	Probably damaging (0.978)
³ LRT	Deleterious (1)	Deleterious (1)
⁴ Mutation Taster	Disease causing (1)	Disease causing (1)
⁵ SIFT	Deleterious (0)	Deleterious (0.01)
⁶ FATHMM	Tolerated (0.445)	Damaging (0.465)
⁷ METASVM	Tolerated (0.481)	Deleterious (0.582)
⁸ GERP++	3.89	4.59

¹Scores range from 0-0.452 (Benign) 0.453-0.956 (possibly damaging) 0.957 -1 (probably damaging)

²Scores range from 0-0.446 (Benign) 0.447-0.909 (possibly damaging) 0.909 -1 (probably damaging)

³Scores range from 0-1; > score signifies that the codon is more constrained and variant is more likely to be deleterious

⁴Scores denote the certainty of the prediction:1- p value

⁵Scores >0.05 are Tolerated, Scores <0.05 are Deleterious

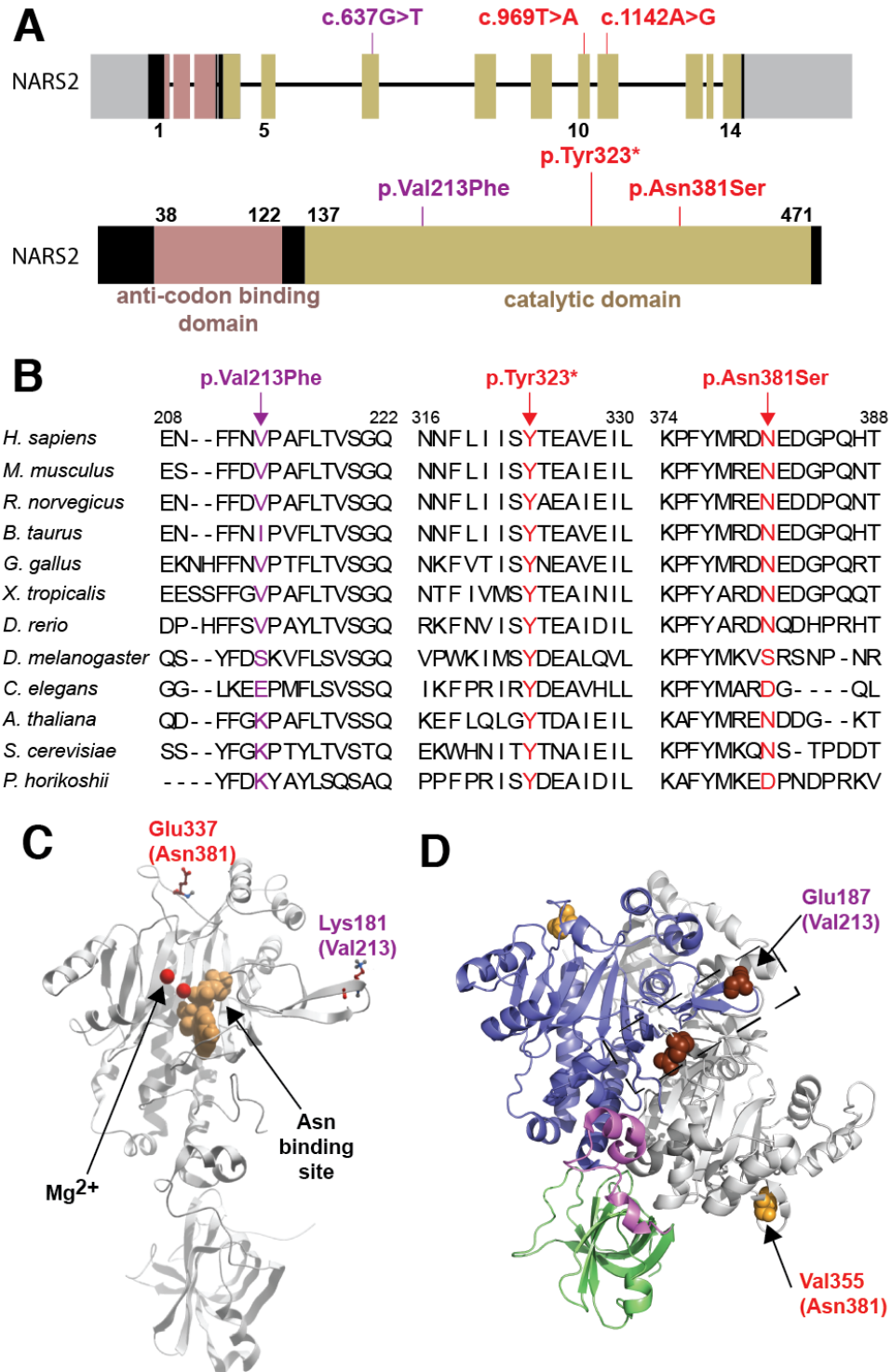
^{6,7}Scores range from 0-1: higher score is more likely deleterious

⁸Scores increase with degree of conservation

The 3D structure of human NARS2 has not been resolved. However, there is 30.5% identity and 50.1% similarity between NARS2 and *Pyrococcus horikoshii* AsnRS, which allowed us to use the crystal structure of *Pyrococcus horikoshii* AsnRS (Iwasaki et al. 2006) to model the effect of the human missense mutations of NARS. Human NARS2 p.Val213 residue corresponds to the p.Lys181 residue in *Pyrococcus horikoshii* (Figure 2.3B-C). Molecular modeling data suggests that the p.Lys181 residue is present on the surface of the molecule (Figure 2.3C). The substitution of this valine residue with phenylalanine in humans is predicted to create a sticky patch on the surface that could

affect the protein-protein interactions of NARS2. Since a dimeric protein form has been described for mt-AsnRS of *Entamoeba histolytica* (Protein Data Bank access # 3M4Q), we used it as backbone for *in silico* modeling. The p.Glu187 and p.Val355 of *Entamoeba histolytica* mt-AsnRS correspond to p.Val231 and p.Asn381 residues in human NARS2 (Figure 2.3D). Molecular modeling data suggest that p.Asn381 is not directly involved in the dimer interphase. However, the variant could affect the dimerization by propagation of a structural perturbation. Conversely, the p.Val213 residue is located within the dimer interphase. We therefore functionally interrogated a potential NARS2 oligomerization defect.

Figure 2.3: NARS2 structure and molecular modeling



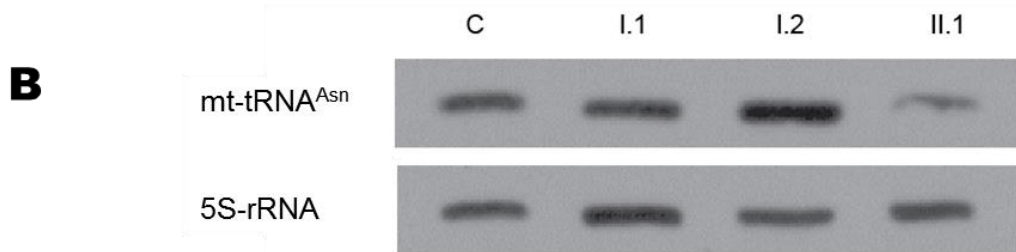
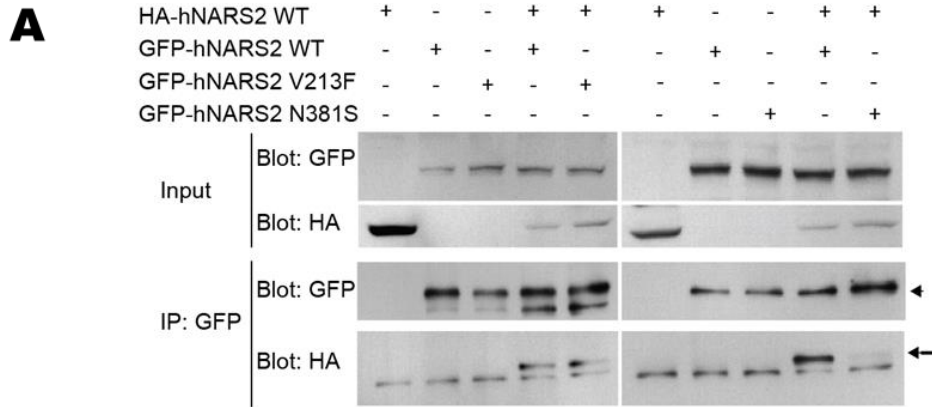
(A) Schematic representation of *NARS2* gene and predicted encoded protein product. Exons are represented with boxes. The anti-codon binding domain is shown in pink, and the catalytic domain is shown in beige. The c.637G>T, c.969T>A, c.1142A>G mutations are located in exons 6, 10 and 11, respectively,

which are all coding for a part of the catalytic domain of the protein. (B) Protein sequence alignments show the evolutionary conservation of the mutated residues (arrows). (C) Mapping of homologs of the p.Val213Phe and p.Asn381Ser mutations in a 3D structure of NARS2. This 3D model of the NARS2 protein is based on the structure of *Pyrococcus horikoshii* AsnRS. The asparagine binding site is represented in beige; Mg²⁺ ions are shown in red. The human mutations p.Val213Phe and p.Asn381Ser were mapped on the *Pyrococcus horikoshii* molecule at position p.Lys181 and p.Glu337, respectively. (D) Dimeric representation of *Entamoeba histolytica* AsnRS (PDB: 3M4Q) using PyMol molecular graphics system. Anticodon binding domain is represented in green, hinge region in pink, catalytic core in blue and second monomer is shown in grey. The *E. histolytica* residue corresponding to human NARS2 p.Asn381 is p.Val355 and shown by an orange sphere. The *E. histolytica* residue corresponding to human p.Val213 is p.Glu197 and is displayed in dark brown.

2.2.6 p.Asn381Ser-NARS2 does not dimerize with wild type NARS2

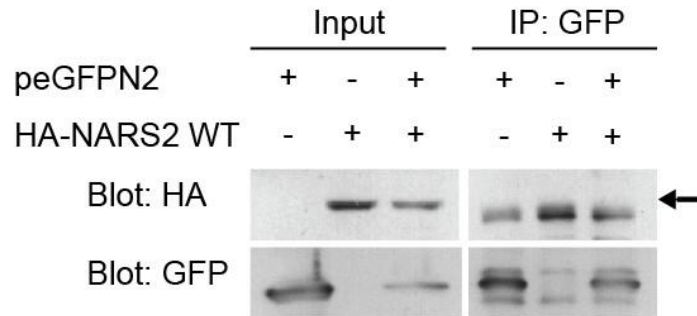
Most class II aminoacyl tRNA synthetases function as homodimers (Eriani et al. 1990; Mudge et al. 1998; Barbour, Turner 2014). Extensive studies in autosomal dominant GARS related Charcot-Marie-Tooth disease type 2D have shown that tRNA charging is dependent on GARS protein dimerization (Mudge et al. 1998; Chihara et al. 2007). We therefore wanted to assess whether NARS2, like its cytosolic counterpart would form homodimers. Additionally we wanted to assess whether the missense mutations would disrupt protein conformation sufficiently to alter dimerization with a wild type monomer. For this, we performed co-immunoprecipitation studies using GFP- and HA- tagged wild type and mutant proteins that were co-expressed in HEK293T cells. These studies revealed that *in vitro* wild type NARS2 can homodimerize (Figures 2.4D, Figure S2.5) and the p.Asn381Ser mutation changes protein structure sufficiently to affect the dimerization with the wild type monomer (Figure 2.4D) while the p.Val213 variant does not.

Figure 2.4: NARS2 homodimerization and RNA level: effect of the p.Val213Phe and p.Asn381Ser mutations



(A) Immunoprecipitates (IP) with anti-GFP antibodies from HEK293T cells transiently transfected with GFP-tagged (arrowhead) and HA-tagged NARS2 (arrow) constructs. Precipitates were immunoblotted with antibodies to the GFP and HA tags. NARS2 homodimerizes, and the p.Val213Phe mutation does not affect the dimerization process. No dimerization was detected with p.Asn381Ser NARS2 construct. (B) Steady state level for mt-tRNA^{Asn} was assessed by northern blot and the results were validated by two independent laboratories. 5S-rRNA probe was used as a loading control on the same membrane. In fibroblasts of patient II.1, from LS06 family, the level of mt-tRNA^{Asn} is decreased compared to his parents and a control sample. Due to high passage number, we could not measure the mt-tRNA^{Asn} levels in the fibroblast of patient II.3.

Figure S2.5: Interaction between HA- and GFP-tags



Immunoprecipitates (IP) with anti-GFP antibodies from HEK293 cells transiently transfected with GFP and HA-tagged NARS2 constructs. Precipitates were immunoblotted with antibodies to the GFP and HA tags. No dimerization was detected between GFP and HA-NARS2 construct (black arrow).

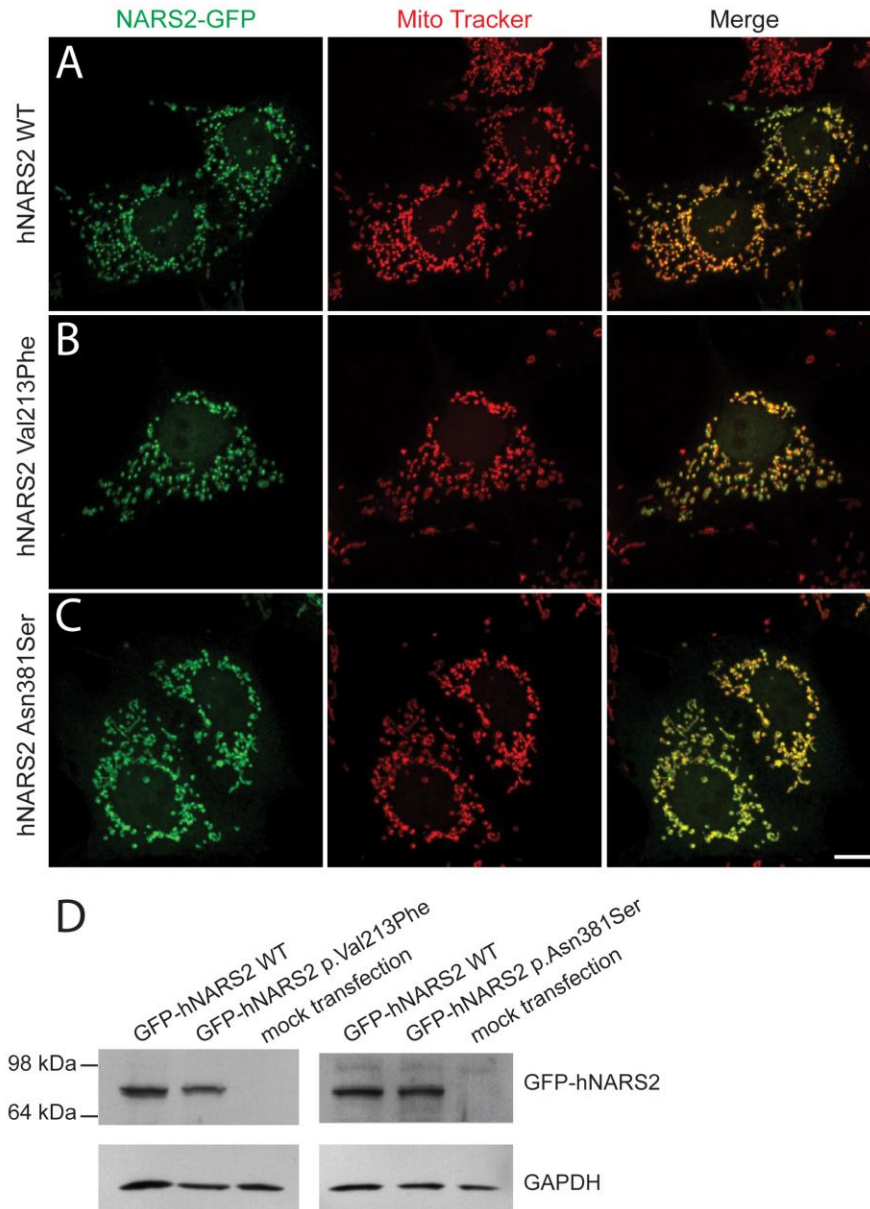
2.2.7 Neither missense mutation affects NARS2 expression, stability or mitochondrial targeting in heterologous cells

To determine the effect of the p.Val213Phe and p.Asn381Ser missense mutations on the expression and stability of NARS2, we transiently transfected HEK293T cells with GFP-tagged cDNA constructs encoding either wild type or mutant NARS2. Western blot analysis and quantification, after normalizing against the GAPDH expression level, revealed no significant difference in the steady state levels of the p.Val213Phe or p.Asn381Ser mutant proteins compared with wild type NARS2 protein (Figure S2.6D and data not shown).

To determine if the pathogenic mutations have an effect on the cellular location of NARS2, we performed immunofluorescence analysis in COS7 cells transiently transfected with GFP-tagged wild type and mutant NARS2 cDNA constructs (Figure S2.6A-C). Confocal imaging of the MitoTracker® Red FM and GFP-tagged protein revealed an overlap of both signals, which demonstrates the mitochondrial localization of NARS2 (Figure S2.6A), which is not affected by the p.Val213Phe and p.Asn381Ser

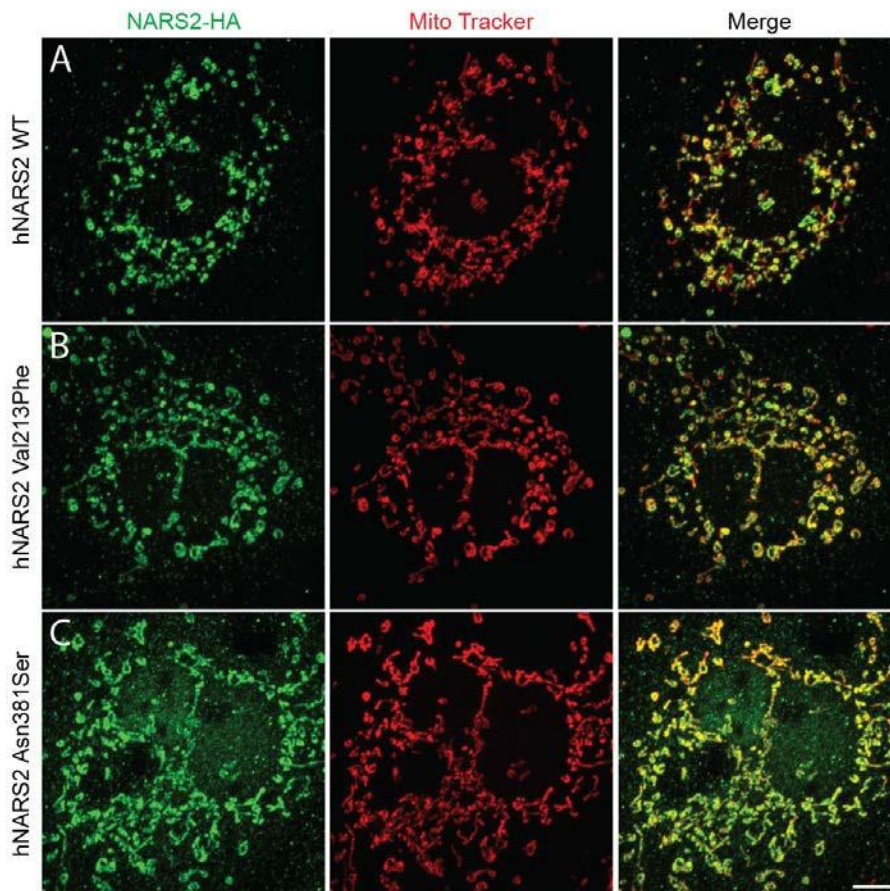
alleles (Figure S2.6B, S2.6C). Identical results were obtained with a C-terminal HA tagged wild type and mutant *NARS2* constructs (Figure S2.7).

Figure S2.6: Impact of the p.Val213Phe and p.Asn381Ser mutations on the NARS2 expression level *in vitro*



(A) Wild type NARS2-GFP (green) was transiently expressed in COS-7 cells, and Mito Tracker Red FM was used to stain mitochondria. Co-localization of the fluorescent signals indicates the mitochondrial targeting of wild type NARS2. (B) p.Val213Phe NARS2-GFP (green) and (C) p.Asn381Ser NARS2-GFP (green) were also targeted to mitochondria, indicating that these disease-causing mutations do not affect NARS2 localization. The scale bar is 5 μ m and applies to all panels. (D) Immunoblot analysis of transfected GFP-tagged NARS2 constructs. HEK293 cells were transiently transfected with the same quantity of wild type or mutant NARS2 constructs. Protein extracts from the cell lysates were analyzed by western blot using an anti-GFP antibody. The expected size of both GFP-fused proteins is 81 kDa. Wild type, p.Val213Phe and p.Asn381Ser mutant NARS2 appear to be equally expressed in the transfected cells. A GAPDH antibody was used as a loading control.

Figure S2.7: Effect of the p.Val213Phe and p.Asn381Ser mutations on NARS2 localization



(A-C) The localization of HA-tagged wild type and mutant NARS2 in COS-7 cells. (A) Wild type, (B) p.Val213Phe NARS2-HA construct and (C) p.Asn381Ser NARS2-HA construct were transiently expressed in COS-7 cells. Mito Tracker® Red FM (Invitrogen) was used to stain mitochondria. NARS2 was labeled using a monoclonal HA antibody (Millipore, green). The two signals co-localized for wild type and mutant NARS2, suggesting that both mutations do not affect NARS2 targeting to the mitochondria. The scale bar is 5 μ m and applies to all panels.

2.2.8 NARS2 levels are decreased in fibroblasts from family LS06

In order to assess *in vivo* protein stability due to *NARS2* variants in the LS06 family, we performed SDS PAGE and western blot analysis on whole fibroblast cell lysates from both parents and the probands (Figure 2.1C). A truncated NARS2 protein product (Δ 154aa) stemming from the p.Tyr323* allele was not observed (Figure 2.1C, black arrow). Both

probands had significantly reduced NARS2 levels when compared to controls (C1 and C2; Figure 2.1C). Since the probands' full-length NARS2 bands represented translation solely from the paternal allele, western blot results deemed the p.Asn381Ser NARS2 variant as unstable at the RNA or protein level. This is further demonstrated by a 50% reduction in NARS2 levels for the paternal sample (Figure 2.1C).

2.2.9 Compound heterozygous mutations (p.Tyr323* and p.Asn381Ser)

decrease steady state levels of mt-tRNA^{Asn}

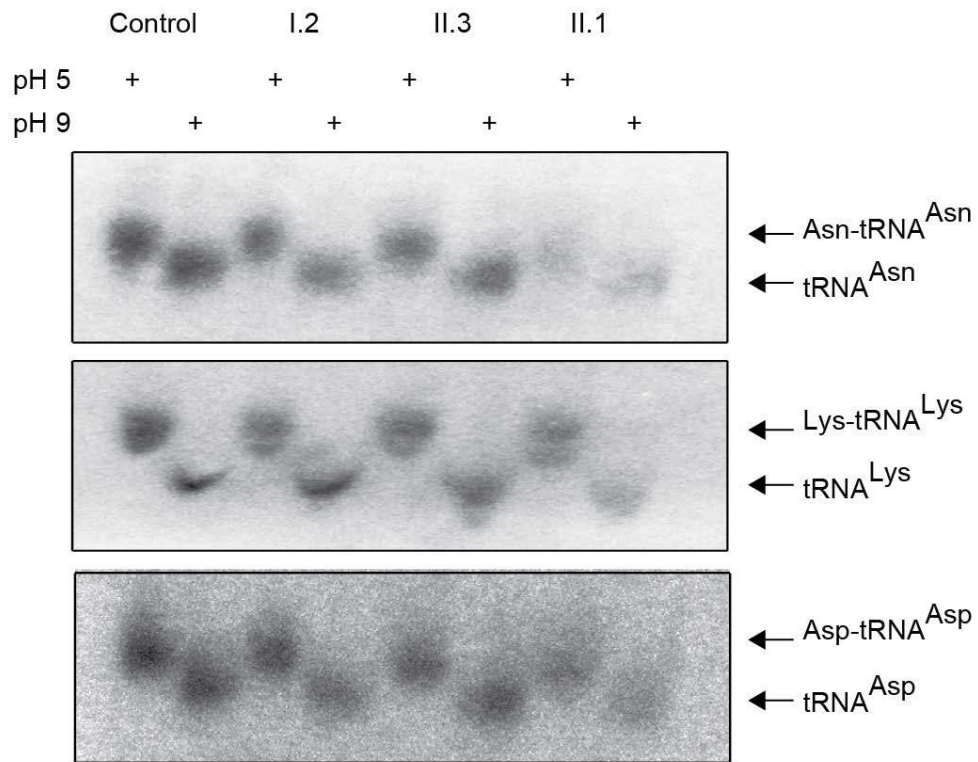
We next examined whether the aminoacylation and steady state levels of mt-tRNA^{Asn} were affected in patient fibroblasts. For this we first analyzed the aminoacylation level of tRNAs in total RNA extract isolated from fibroblast cells obtained from probands II.1 and II.3 from family LS06 as well as their mother (I.2) (Figure S2.8). NARS2 capacity of aminoacylation was measured by evaluation of the ratio between charged and uncharged mt-tRNA^{Asn}. Our results showed aminoacylation activity of NARS2 to be normal in patient fibroblasts (Figure S2.8). Quantification of overall levels of the interrogated mt-tRNAs was suggestive of decreased levels when compared to control (data not shown). We also measured steady state mt-tRNA^{Asn} levels for proband II.1, his parents and a control. When normalized against the loading control (5S-rRNA), reduced steady state level for mt-tRNA^{Asn} for patient II.1 was observed (Figure 2.4E).

2.2.10 Defective MRC is associated with mutations in NARS2

To test whether the NARS2 mutations in family LS06 are directly correlated with MRC (Mitochondrial Respiratory Chain) dysfunction, we reasoned that the reintroduction of wild

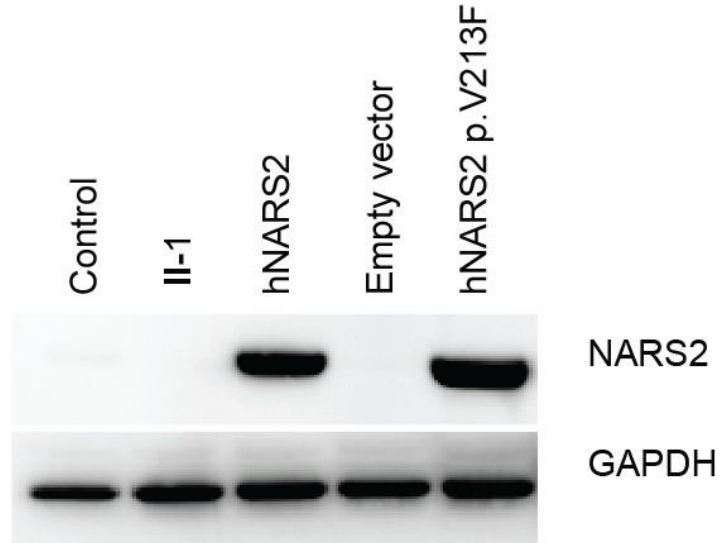
type *NARS2* would correct a hypothetical defect in mtDNA encoded mitochondrial protein translation. Therefore, we overexpressed wild type *NARS2* in patient fibroblasts. We also reasoned if p.Val213Phe is a pathogenic mutation associated with nonsyndromic hearing loss (DFNB94), it would not rescue the MRC defects observed in family LS06. To test these hypotheses, stable *NARS2* over-expression cell lines were generated by lentiviral transduction of patient II.1 fibroblasts with cDNA constructs encoding human wild type or p.Val213Phe mutant *NARS2* cloned into a pLVX-IRES-tdTomato lentiviral plasmid. Transduced cells were sorted by flow cytometry, and tdTomato-positive cells were selected for *NARS2* overexpression. The overexpression of *NARS2* proteins was confirmed by SDS PAGE and western blot (Figure S2.9).

Figure S2.8: Aminoacylation assays for mitochondrial tRNA^{Asn}



The aminoacylated tRNAs were separated from nonaminoacylated tRNA species on acidic denaturing polyacrylamide-urea gels and then electro-blotted and hybridized with specific probes for mt-tRNA^{Asn}, mt-tRNA^{Lys} and mt-tRNA^{Asp}. Samples of mitochondrial tRNA were deacylated by being treated at pH 9. The blot shows normal aminoacylation for mt-tRNA^{Asn} in II-1. Northern Blotting for mt-tRNA^{Asn} levels was performed 3x for RNA isolated from mitochondrial fractions from I.2, II.1 and II.3 as well as a control sample (data not shown).

Figure S2.9: SDS-PAGE and western blot analysis of lentiviral transduction and expression of NARS2

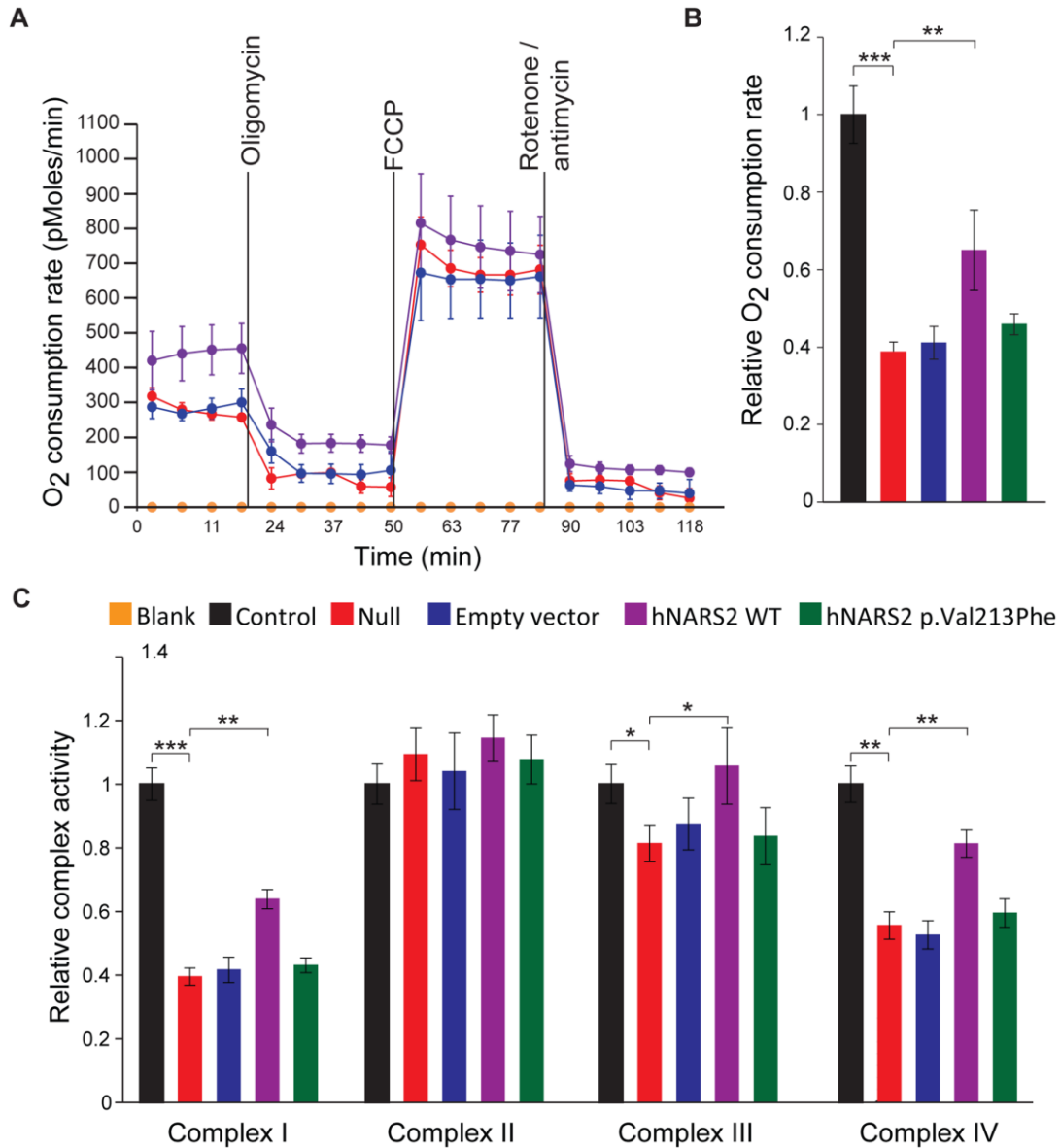


NARS2 lentiviral constructs were made by cloning human *NARS2* cDNA into pLVX-IRES- tdTomato vector and then packaged into pseudoviral particles. *NARS2* expression was assessed with western blot in transduced patient cells to monitor the transduction efficiency, using an anti-*NARS2* antibody. GAPDH antibody was used as a loading control.

To examine if overexpression of *NARS2* rescued the respiratory deficiencies caused by the *NARS2* mutations (p.Tyr323*, p.Asn381Ser), O₂ consumption of transfectants and their parental cell lines II.1 were measured by using a Seahorse XF24 Analyzer (Figure 2.5A-B) and the manufacturer supplied “Mitostress” kit. The kit uses sequential addition of substrates and inhibitors of the mitochondrial respiratory chain. After measuring basal respiration, oligomycin is added to block the mitochondrial ATP synthase. The reduction in oxygen consumption reflects the ATP need of the cell, while the remaining respiration reflects the proton leak. Next the mitochondria are uncoupled with FCCP which causes an increase in electron flux, as the mitochondria attempt to restore the loss of the proton gradient. After addition of FCCP, rotenone and antimycin A are added to inhibit complex I and III, which then stops all mitochondrial respiration. This

allows calculation of respiration due to non mitochondrial oxygen consumption. The oxygen consumption rate (OCR) was significantly higher in the cell line expressing wild type *NARS2* although rescue was not complete (Figure 2.5B). These data suggest that the overexpression of *NARS2* can enhance the rate of respiration in the patient cell line carrying the *NARS2* mutations. Furthermore, the transduction with mutant *NARS2* did not rescue the OCR function, which supports the pathogenic nature of the p.Val213Phe allele (Figure 2.5B).

Figure 2.5: Analysis of the impact of the *NARS2* mutations on mitochondrial functions



(A) Oxygen consumption in intact patient cells with *NARS2* overexpression. Oxygen consumption in *NARS2* overexpression cells was analyzed using the Seahorse XF24 analyzer. 2 μ M oligomycin, 4 μ M FCCP and 2 μ M rotenone/antimycin were added at the indicated points. (B) Oxygen consumption rates from fibroblast mitochondria of the indicated genotypes. “Null” corresponds to fibroblasts from patient II.1 of LS06 family. Overexpression of the p.Val213Phe *NARS2* construct failed to improve the oxygen consumption rate, but the wild type *NARS2* construct significantly rescued it (wild type *NARS2*: OCR ratio = 0.650 ± 0.103 , p.Val213Phe *NARS2*: OCR ratio = 0.459 ± 0.027 , $n=3$, $p=0.003$). (C) Enzymatic activity of the individual respiratory chain complexes from fibroblast mitochondria of the indicated genotypes. Complex II activity was unaffected in all genotypes. Overexpression of the wild type *NARS2* construct significantly improved the activities of complexes I, III and IV ($p=0.002$, $p=0.032$ and $p=0.004$, respectively), and expression of the p.Val213Phe *NARS2* construct had no effect. Students t-test have been performed for statistical analysis. Data are represented as the mean \pm SEM.

Next, we measured the activity of mitochondrial complexes I to IV. The null cell line derived from patient II.1 has decreased activity in complexes I, III and IV. No significant difference was observed in the activity of complex II, which does not have mtDNA encoded subunits. The overexpression of wild type and p.Val213Phe mutant NARS2 did not affect the activity of complex II (Figure 2.5C), but the overexpression of wild type NARS2 significantly rescued the activity of complexes I, III and IV in the null fibroblasts (Figure 2.5C). Rescue was not complete for mitochondrial complex I and IV. Contrary to WT construct, the over-expression of the p.Val213Phe mutant NARS2 in null fibroblasts did not rescue the activity of complexes I, II and IV (Figure 2.5C). Together with the OCR measurements, the results from these functional studies revealed that Leigh syndrome and DFNB94 associated alleles impair the mitochondrial function of NARS2.

2.3 Discussion

Two brothers diagnosed with Leigh syndrome defined by symmetrical lesions in the brain stem and thalamus, had similar clinical courses including congenital auditory neuropathy followed by myoclonus, intractable seizures and early demise. CSF lactic acid was elevated, suggestive of mitochondrial dysfunction. ETC studies confirmed this suspicion, revealing abnormal complex I/III activity. WES uncovered compound heterozygous mutations in the *NARS2* gene. Compound heterozygous mutations resulted in decreased levels of the tRNA^{Asn}, impaired the activities of mitochondrial complexes I, III and IV and negatively affected the oxygen consumption rate in patient fibroblasts. Electron transport through mitochondrial complex II was not enhanced, which is consistent with the fact that the architecture of mitochondrial complex II is completely nuclear encoded. Introduction

of wild type NARS2 protein significantly improved OCR and activity of mitochondrial complexes I, III and IV but rescue was incomplete. There could be many reasons for incomplete rescue, including the altered spatiotemporal expression of NARS2, tissue specificity, non-repairable damage to mitochondrial proteins due to endogenous mutant NARS2, cellular health, and genetic modifiers. We have identified a heteroplasmic mt-tRNA^{Cys} variant at position A5793G, located in the tRNA's acceptor stem. This variant was found once before in a family with epileptic seizures and visual disturbances (personal communication with Dr T. Dorn), however, unaffected maternal relatives in that family also carried the variant. We cannot rule out the possibility that mt-tRNA^{Cys} variant might add to the translational defect and hence resulted in incomplete rescue by overexpression of wild type NARS2.

In the Pakistani family PKDF406, we identified a novel homozygous missense mutation in *NARS2*, which co-segregates with nonsyndromic congenital hearing loss DFNB94. In contrast to wild type NARS2, over-expression of p.Val213Phe NARS2 did not restore OCR or mitochondrial electron transport chain function in fibroblast cells derived from family LS06. Recently, mutations in mt-aaRS (*ARS2*) and cytoplasmic aaRS (*ARS*) have been associated with human diseases such as Charcot-Marie-Tooth disease, Perrault syndrome (*LARS2*, *HARS2*) (Pierce et al. 2011; Pierce et al. 2013), and pontocerebellar hypoplasia, all of which include sensorineural hearing impairment (Table 2.2). The available clinical data indicate that the hearing impairment in the PKDF406 family is nonsyndromic (Table 2.1). Clinical studies ruled out Charcot-Marie-Tooth disease and pontocerebellar hypoplasia. However, definitively ruling out Perrault syndrome, a disorder of hearing impairment and premature ovarian failure, in affected

individuals from family PKDF06 is clinically challenging. Only two females in our family were homozygous for the p.Val213Phe missense mutation and had hearing loss (IV:4 and IV:8; Figure 2.1A). At the time of examination, family members IV:4 and IV:8 were 40 and 45 years old, respectively (Table 2.2), were both post-menopausal, and had no children. The average age for menopause in Pakistan is estimated to be 49.3 years (Nisar et al. 2012), suggesting that these two affected females may have experienced early menopause. The degree of ovarian insufficiency in Perrault syndrome is highly variable and early menopause would be consistent with a diagnosis of Perrault syndrome. We also Sanger-sequenced *NARS2* in 14 unrelated Perrault syndrome probands to assess whether mutations in *NARS2* are a common finding in this patient population, but did not find any pathogenic variants.

Pleiotropism is a common phenomenon in ARS associated disease. In our study, comparative mutation and resulting functional deficit analyses for both families may explain the syndromic versus nonsyndromic phenotype. The nonsense Leigh syndrome mutation did not result in a truncated *NARS2* protein product in patient fibroblast, which is indicative of nonsense mediated *NARS2* mRNA decay (Baker, Parker 2004). The remaining *NARS2* protein product, expressed from the missense allele, showed additional instability. We were unable to assess stability of p.Val213Phe mutant protein *in vivo* since no patient tissue is available, but the protein was stable *in vitro*.

Table 2.2: Summary of the known aminoacyl tRNA-synthetase genes associated with deafness

Gene	Localization	Phenotype	Mutations	References	OMIM#
<i>LARS2</i>	Mitochondria	Perrault syndrome	p.Thr522Asn p.Ile360Phefs*15 p.Thr629Met	(Pierce et al. 2013)	604544
<i>HARS2</i>	Mitochondria	Perrault syndrome	p.Leu200Val p.Val368Leu c.del200-211	(Pierce et al. 2011)	600783
<i>AARS</i>	Cytoplasm	Charcot Marie Tooth type 2N	p.Arg329His	(McLaughlin et al. 2012)	613287
<i>RARS2</i>	Mitochondria	Pontocerebellar hypoplasia type 6	p.Met404Lys c.del471-474	(Cassandrini et al. 2013)	611523
<i>KARS</i>	Cytoplasm Mitochondria	Nonsyndromic hearing impairment DFNB89	p.Asp377Asn p.Tyr173His	(Santos-Cortez, Regie Lyn P et al. 2013)	613916
<i>NARS2</i>	Mitochondria	mild intellectual disability, epilepsy , severe myopathy	p.Gln274His	(Vanlander et al. 2014)	612803
		Leigh Syndrome	p.Tyr323*; p.Asn381Ser	This study	
		Nonsyndromic hearing loss	p.Val213Phe	This study	

Furthermore the secondary structure of *NARS2* harbouring p.Asn381Ser must be significantly affected since the *NARS2* p.Asn381Ser protein product has reduced dimerization with wild type *NARS2* protein. In contrast, the DFNB94 allele (p.Val213Phe) did not alter *NARS2* protein stability, or the ability to dimerize with wild type protein *in vitro*.

During the course of our work, two siblings with variable clinical manifestation and c.822G>C (p.Gln274His) mutation in *NARS2* were reported by Vanlander and coauthors

(Vanlander et al. 2014). One affected individual displays mild intellectual disability and epilepsy, while another sibling has severe myopathy, fatigability and ptosis (Vanlander et al. 2014). Neither sibling has a hearing loss. The .822G<C allele also results in abnormal splicing of intron 7 in patient lymphoblastoid cell lines. In the patient cell lines, NARS2 protein level is somewhat decreased, but is completely absent in muscle from both affected siblings suggesting additional instability of the abnormal splice product in that tissue. To explain the mild phenotype, particularly of the sibling without myopathy, the authors suggest the presence of normally spliced NARS2 protein product below the detection level in muscle and a low threshold for essential NARS2 enzymatic activity. An ETC assay, performed in skeletal muscle homogenate as well as isolated mitochondria shows significantly reduced COI activity. The different methodology makes a direct comparison with our study difficult, but absence of COI/COIII activity in muscle homogenate of the Leigh syndrome patients is striking and suggests a more severe COI defect than what was observed by Vanlander and co-authors. We also observed decreased mt-tRNA^{Asn} levels in our patient fibroblasts, which may have further increased severity of the mitochondrial dysfunction, particularly in CNS tissue. The decreased levels of mt-tRNA^{Asn} may be suggestive either of a regulatory role of NARS2 in the expression of its cognate tRNA or of a decrease in stability of the tRNA by poor interaction with the mutant NARS2 (Belostotsky et al. 2011). Fluctuations in mitochondrial tRNA levels have significant influence on disease expression. The best example is reversible infantile respiratory chain deficiency (RIRCD) due to a homoplasmic mutation in the mt-tRNA^{Glu}. The mutation may cause lethality in infancy and is associated with low levels of mt-tRNA^{Glu}. However, as mt-tRNA^{Glu} levels increase surviving infants spontaneously

recover, with good prognosis (Horvath et al. 2009; Boczonadi, Horvath 2014b). Decreased mt-tRNAs levels secondary to defects in a mt-tRNA's cognate *ARS2*, have been observed in several other *ARS2*-related diseases (Cassandrini et al. 2013; Isohanni et al. 2010). Intriguingly, recent studies have shown that overexpression of cognate and non-cognate *ARS2* can rescue mitochondrial dysfunction secondary to mt-tRNA mutations (Hornig-Do et al. 2014), further reinforcing the importance of the interaction between *ARS2* and their cognate mt-tRNAs.

The absence of hearing deficit in the family studied by Vanlander and co-authors, while the two families described in our study exhibit syndromic or non syndromic hearing loss, is an additional example of the pleiotropism of *ARS2* associated disease. Interestingly mutations in *KARS* show phenotypic variability to an extent comparable to our findings associated with *NARS2* defects. In three independent studies, different mutations in *KARS* lead to Charcot Marie Tooth disease (McLaughlin et al. 2012), non syndromic hearing impairment DFNB89 (Santos-Cortez, Regie Lyn P et al. 2013) and more recently visual impairment and progressive microencephaly (McMillan et al. 2014). The link between *ARS* mutations and phenotypes still need to be unraveled and require a better understanding of the symptoms as well as the involved molecular mechanisms. The phenotypic diversity can also be explained by tissue specificity. Consistent with defects in other mitochondrial disorders, and more particularly with other *ARS2* disorders (for review (Antonicka et al. 2006)), our patients display significant tissue specificity with prominent central nervous system and/or inner ear pathogenicity, despite an ubiquitous expression. Molecular mechanisms underlying this tissue specificity are poorly characterized. Mitochondrial translation might be sufficiently supported by residual *ARS2*

activity in most of the tissues but may have reached threshold in affected tissue(s) and may depend on the availability of mitochondrial chaperone proteins, in a tissue specific manner (Konovalova, Tynismaa 2013). Differential expression of other molecules of the mitochondrial protein synthesis machinery in response to the altered steady state levels of the mutated protein was also shown to be tissue dependent (Antonicka et al. 2006). Several studies have, recently, highlighted the influence of modifying factors and more particularly factors that would modulate the mitochondrial translation. Additional function(s) of ARS2 proteins, yet to be described, may be restricted to specific cell types or to specific developmental stages. The fact that in many mt-aaRS related disorders even severe mutations do not display an aminoacylation defect has prompted further studies which have highlighted the importance of the aminoacylation process (Scheper et al. 2007; Cassandrini et al. 2013; Edvardson et al. 2007), protein folding (Huang et al. 2002; Wilcox et al. 2005), and refolding (Banerjee et al. 2011). Therefore, the NARS2 mutations described in this study may disrupt protein refolding in the mitochondrial matrix in a tissue-specific manner.

In conclusion, our findings implicate mutant alleles of *NARS2* as another cause of Leigh syndrome as well as DFNB94 hearing loss in humans. Animal models are needed to elucidate the crucial functional roles that *NARS2* play in the inner ear and central nervous system as well as in the mitochondrial respiratory chain. Future studies will explore the mechanistic differences in tissue-specific phenotypic expression of *NARS2* mutations causing Leigh syndrome, hearing loss, epilepsy and intellectual disability.

2.4 Materials and Methods

2.4.1 Linkage analysis

For family PKDF406, screening for linkage to the reported recessive deafness loci was performed using at least three short tandem repeat (STR) markers each for these loci. Data was analyzed using GeneMapper software (Applied Biosystems). LOD scores were calculated using a recessive model of inheritance assuming a fully penetrant disorder and a disease allele frequency of 0.001.

2.4.2 Whole exome sequencing

For family LS06, genomic DNA was extracted from fibroblast cell lines using the Gentra-Puregen kit (Qiagen) according to the manufacturer's instructions. Exon capture and enrichment was performed (Illumina TruSeq Exome) in solution and libraries were sequenced as 100 bp paired end reads on an Illumina HiSeq 2000 instrument. Mapping to the human reference sequence hg19 and variant calling was performed using CLC bio genomics workbench 6.5.1 employing the "default stand-alone mapping" algorithm. Variant calling parameters were minimally modified with a neighborhood radius of 9 bp, minimum allele frequency of 35% and minimum coverage of 4 bidirectional reads (Mapping statistics are summarized in Table S2.7). Coding region and splice junction variants (6bp) common to probands II.1 and II.3 were filtered using the Annovar "filtered annotation" functions tool (Chang, Wang 2012). We discarded variants with Minor Allele Frequency (MAF) ≥ 0.01 present in the "1000 genome project April 2012", "dbSNP138-non flagged" and "ensembl annotations popfreq all" databases. We then selectively

analyzed variants compatible with X-linked or autosomal recessive inheritance (Table S2.3).

Table S2.7: Summary of sequencing statistic for LS06

Sample	Total reads	Mapped reads	% mapped	% reads in pair	Average read	Average length	Cov 1x (%)	Cov 5x (%)	Cov 10x (%)	Cov 20x (%)
I.1	246,260,344	242,668,234	98.5	95.9	105	221	96.3	94.6	93.1	90.6
II.1	192,208,754	163,146,325	84.9	71.9	105	262	96.4	93.8	91.6	87.7
II.3	247,022,204	243,329,645	98.5	95.6	105	230	96.8	94.6	93	90.4

For family PKDF406 whole exome sequencing analysis used a genomic DNA sample from one affected individual (NimbleGen SeqCap EZ Exome Library v2.0, Roche) and 100bp paired-end sequencing was performed on an Illumina HiSeq2000 instrument. Sequencing data were analyzed following the guidelines that are outlined in the Broad Institute's Genome Analysis Toolkit (DePristo et al. 2011; Li, Durbin 2009). The raw data were mapped using the Burrows Wheeler Aligner (Li, Durbin 2009) the variants were called using the Unified Genotyper, and the data underwent further processing and quality control (Li, Durbin 2009; DePristo et al. 2011). Low-quality reads (less than 10x coverage) were removed, and the remaining variants were filtered against the dbSNP133 database and all of the known variants in the NHLBI 6500 Exome Variant database that had a minor allele frequency (MAF) of greater than 0.05% (Table S2.3). We used Sanger sequencing to analyze the segregation of alleles in the other family members. Primers were designed with Primer3 software (Rozen, Skaletsky 2000) to amplify exons as well as flanking introns and untranslated regions (Table S2.4). PCR amplified products were purified with

exonuclease and alkaline phosphatase (Fermentas) treatments. Purified products were then sequenced with BigDye v3.1 (Applied Biosystems) and run on an Applied Biosystems 3730xl DNA Analyzer.

2.4.3 Animals

Post-natal day 0 (P0), P30 and P90 C57BL6/J mice were used. The mice were obtained from Jackson Laboratories and bred in Cincinnati Children's Hospital Medical Center (CCHMC) animal facility.

2.4.4 Molecular modeling

Two homology models of NARS2 were constructed, using PYMOL (see Web resources). The templates, used as backbone, were the crystal structure of *Pyrococcus horikoshii* AsnRS (Iwasaki et al.) (Protein Data Bank access # 1X55) and the dimeric protein form of mt-AsnRS of *Entamoeba histolytica* (Protein Data Bank access # 3M4Q).

2.4.5 Nars2 expression analysis

Mouse inner ear tissues were harvested from three to five mice at P0, P30, and P90. The vestibular system and the cochlea were separately dissected and immediately frozen in TriReagent (Ambion). RNA were isolated with RiboPure kit (Ambion) and used to synthesize cDNA (SuperScriptII Reverse Transcriptase, Life Technologies). Inter-exonic primers (Table S2.5) were designed with Primer3 software and PCR amplifications were performed with EconoTaq (Lucigen). Primers that amplify *Gapdh* cDNA were used as an

internal control (Table S2.5). Amplimers were size-separated on a 2% agarose gel and stained with ethidium bromide.

In situ hybridization was performed on mouse cochlear cryosections at post-natal day 2 (P2). *Nars2 in situ* hybridization probes were subcloned from C57BL/6J inner ear cDNA and ligated into pCRII-TOPO vector (Invitrogen) for *in vitro* transcription. *Nars2* was detected with a ~1.5 kb probe transcribed from NM_153591, nt501-2078 (Table S2.5). *In situ* hybridization positive control probes for *Myo15a* and *NF68* were used as described previously (Anderson et al. 2000, Morsli et al. 1998). RNA hybridization was performed in on 12 µm cryo-sections according to standard methods using digoxigenin-labeled probes in weakly acidic hybridization buffer (pH 4.5), anti-digoxigenin-AP Fab fragments (Roche) in TBST buffer, and the NBT/BCIP colorimetric substrate reaction in AP buffer at pH 9.5

2.4.6 Cell culture

HEK293T and COS7 cells were grown in DMEM that was supplemented with 10% FBS, 2 mM glutamine, and penicillin/streptomycin (50 U/ml) (Life Technologies) and were maintained at 37°C in 5% CO₂. Human Fibroblast cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum at 37°C and with 5% CO₂.

2.4.7 Generation and analysis of cybrids cell lines

Fibroblast cells were grown in DMEM medium supplemented with glutamax (446 mg/l), 10% fetal calf serum, 50µg/ml uridine and 1mM sodium pyruvate under standard conditions. Cells were fused with 143B rho0 cells as previously described (Potluri et al. 2009). Several clones were isolated and the resulting cybrid cells were subsequently

expanded. Biochemical assays were performed on isolated mitochondria and/or permeabilized cells. Western blot analysis of a patient's muscle biopsy sample and Blue Native gel analysis (BNG) of a patient's fibroblast were performed as previously described (Potluri et al. 2009). We used "MitoProfile" antibody mix (Total OXPHOS human WB antibody cocktail, Abcam) for muscle lysates. For fibroblast lysates we used antibody GRIM19 (Abcam), which corresponds to the mitochondrial complex I subunit NDUFA13.

2.4.8 Generation of NARS2 stable over-expression cell

The insert of *NARS2* cDNA was cloned into pLVX-IRES-tdTomato lentiviral expression vector using In-Fusion Cloning (Clontech). The construct was packaged into VSV-G pseudotyped viral particles by transfection of HEK293T cells with packaging plasmids and the expression vector for *NARS2*. Lentiviruses were concentrated by ultracentrifugation (CCHMC Vector Core). Transduction of patient fibroblast cell line II.1 was performed according to established methods (Li et al.). Briefly, fibroblast cells from patient were seeded in six-well plates and transduced 22 hours later with 25 μ l of concentrated lentivirus (MOI=6). Polybrene at 4 μ g/ml was added to increase the transduction efficiency. Transduced cell were sorted by flow cytometry with standard FITC filter sets, and tdTomato positive cells were selected for *NARS2* over-expression. Cell sorting was performed at CCHMC Research Flow Cytometry Core with a BD FACSAria II (BD Biosciences).

2.4.9 Immunofluorescence studies

Wild type (WT) human *NARS2* cDNA (clone #Z7860) was obtained from GeneCopoeia and subcloned into pEGFP-N2 vector (Clontech) using InFusion cloning (Clontech). Stratagene QuikChange Lightning mutagenesis (Roche) was used to introduce the c.637G>T transversion and c.1142A>G transition into WT *NARS2* sequence. All constructs were then sub-cloned in pcDNA3.1(+) (Invitrogen) vector and sequence verified. A HA tag was added at the C-terminal part of *NARS2* cDNA using InFusion cloning.

Constructs were expressed in COS7 cells after transfection with PEI (Polysciences) using a 1:5 ratio (1µg cDNA/5µg PEI). Twenty-four hours post transfection, cells were incubated with 100nM Mito Tracker® Red FM (Invitrogen) for 30 minutes, followed by fixation with 4% paraformaldehyde. Fixed cells were mounted with Fluorogel Mounting Medium (EMS) and imaged with a Zeiss LSM700 confocal microscope.

2.4.10 Immunoprecipitation and western blot

GFP- and HA-tagged *NARS2* constructs were co-expressed in HEK293T cells, after transfection using PEI reagent (Polysciences). Forty-eight hours after transfection, cells were harvested and homogenized with sonication in lysis buffer (50mM Tris HCl pH7.4, 100mM NaCl, 1% NP-40, 2mM Na₃VO₄) containing a protease inhibitor mixture (#P8340, Sigma). Immunoprecipitation was performed with an anti-GFP antibody as described previously (Riazuddin et al. 2012). The cell lysates and the immunoprecipitates were

processed for western blot analysis (Jaworek et al. 2013). NARS2 (Abcam), GAPDH (Ambion), GFP (Life Technologies) and HA antibody (Millipore) were used for immunoprecipitation and western blot analyses.

2.4.11 Mitochondrial and nuclear DNA analyses

Genomic and mitochondrial DNA was extracted from skeletal muscle, whole blood and primary fibroblasts using Genra (Quiagen) blood and tissue kits according to the manufacturer guidelines. RNA was extracted from primary fibroblast using Trizol reagent (Life Technologies). The 16 Kb mtDNA genome was PCR amplified in 8 overlapping fragments. Nuclear DNA encoded complex I genes were PCR-amplified from patient cDNA. cDNA was synthesized from RNA from patient fibroblast cells using the iScript cDNA synthesis kit (Biorad). Twenty-nine nuclear complex I subunit genes were amplified. PCR products were purified by ExoSAP-IT® (Amersham) and directly sequenced using the PRISMTM Ready Reaction Sequencing Kit (PE Applied Biosystems) on an automatic sequencer (ABI 3130, PE Applied Biosystems). Sequence data were analyzed using Sequencher (version 4.0.5, Genecode Corp.) software.

2.4.12 Mitochondrial tRNA analysis

Total mitochondrial RNA preparations were obtained from mitochondria isolated from fibroblast cell lines (4.0×10^7 cells). 2µg of total mitochondrial RNA was electrophoresed through a 10% polyacrylamide-7 M urea gel and then electroblotted onto a positively charged nylon membrane (Roche) for hybridization analyses with specific oligodeoxynucleotide probes (Gong et al. 2014). Nonradioactive DIG-labeled (Roche)

oligodeoxynucleotides (mt-tRNA^{Asn} :5'-CTAGACCAATGGGACTTAAA-3' and 5S-rRNA: 5'-GGGTGGTATGGCGGTAGAC-3') were used on the same membrane after stripping in 50% formamide, 5% SDS, 50 mM Tris-HCl, pH 7.5 and extensive washes in 2x SSC (Gong et al. 2014).

2.4.13 Mitochondrial tRNA aminoacylation analysis

Total RNA was isolated under acidic conditions. 2µg of total RNA was electrophoresed at 4°C through an acid (pH 5.2) 10% polyacrylamide-7 M urea gel to separate the charged from the uncharged tRNA as detailed elsewhere (Kohrer, Rajbhandary 2008). The gels were then electroblotted onto a positively charged nylon membrane (Roche) for hybridization analysis with oligodeoxynucleotide probes for mt-tRNA^{Asn} and 5S-rRNA.

2.4.14 Electron transport chain studies

Muscle and skin samples were obtained during autopsy for the first proband, subject II.1, and from a biopsy at 3 months of age from the second proband, subject II.3, from Caucasian family LS06 with parental consents. Spectrophotometric analysis of the respiratory chain complexes was performed in muscle homogenates through the Center for Inherited Disorders of Energy Metabolism (CIDEM), according to established protocols (Case Western Reserve University, Cleveland, OH).

2.4.15 Measurements of oxygen consumption rate by Seahorse

Oxygen consumption rate (OCR) measurements were performed by using a Seahorse Bioscience XF-24 instrument (Seahorse Biosciences). Cells were seeded in XF24-well

microplates in growth medium and the following day growth medium was replaced with assay medium as described (Mullen et al. 2011).

2.4.16 Mitochondrial complex I–IV activity assay

Complex I activity was assessed by following the decrease of NADH absorbance at 340 nm, using decylubiquinone as an electron acceptor. The activity of complex II was measured by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) with the decrease of the absorbance at 600 nm of the oxidized DCPIP. Complex III activity was determined by measuring the reduction of cytochrome c by an increase of absorbance at 550 nm. Complex IV activity was measured by monitoring the oxidation of reduced cytochrome c as a decrease of absorbance at 550 nm (Cary 300 UV-Vis Spectrophotometer Agilent, CA.). Complex I–IV activities were normalized by citrate synthase activity and then used in the analysis (Trounce et al. 1996).

2.4.17 Study approval

2.4.17.1 Human and animal studies

Approval for this study was obtained from the following institutional review boards (IRBs): University of California Irvine (2002-2608), University of Maryland, School of Medicine, Baltimore, Maryland, USA (HP-00059851), Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio, USA (2010-0291 and 2013-7868), National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan, and the Combined Neuroscience Institutional Review Board protocol (OH93-DC-0016) at the National

Institutes of Health, Bethesda, Maryland, USA. Written informed consent was obtained from all the participating family members.

All experiments and procedures were approved by the Institutional Animal Care and Use Committees of CCHMC and the University of Maryland, School of Medicine.

2.5 Acknowledgements

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2.6 Contribution

Mariella Simon consented the family, provided genetic counseling, performed mtDNA sequencing, analyzed the Exome sequencing, and delineated the variants, and co-wrote the paper.

CHAPTER 3

Abnormalities in Glycogen Metabolism in a Patient With Alpers' Syndrome Presenting with Hypoglycemia

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3.1 Introduction

The integrity of physiological regulation of blood glucose levels is of utmost importance to brain metabolism, with prolonged states of hypoglycemia ultimately leading to encephalopathy and death. Therefore, blood glucose levels are tightly controlled to remain within a range of 2.5 and 7.5 mmol/L (45-135 mg/dL) (Lonlay et al. 2004). Several metabolic pathways work in unison to avoid the hypoglycemic state. Mutations in genes involved in glycolysis and gluconeogenesis, glycogen synthesis and degradation, as well as fatty acid oxidation pathways and their hormonal regulators may all lead to primary hypoglycemia syndromes (Haymond 1989). To identify the defective pathway and ultimately the specific enzymatic block requires methodical analysis of various metabolic markers and tissues, which can be costly and time consuming (Mochel et al. 2005). However when hypoglycemia is a secondary finding of a known metabolic disorder these detailed investigations are not usually performed. Mitochondrial disorders are prime candidates for secondary hypoglycemic syndromes, since their underlying energy debt may deem them unable to supply pathways of glucose metabolism with sufficient ATP. The Stanford Mitophenome Database is a tool generated to assist clinicians in phenotype genotype correlations for mitochondrial disease patients (<http://www.mitophenome.org/>). Close to one third of the 174 genes in the database list hypoglycemia as a phenotypic feature (Scharfe et al. 2009). Over a decade after the initial discovery of the disease correlation between progressive external ophthalmoplegia (PEO) and mutations in the mitochondrial DNA polymerase gamma, the *POLG* gene has emerged as one of the most common causes of mitochondrial disease (van Goethem et al. 2002; Nguyen et al. 2005; Wong et al. 2008). With close to 200 pathogenic mutations described to date, the gene is

associated with a wide spectrum of phenotypes and may follow autosomal dominant as well as autosomal recessive patterns of inheritance (Stumpf et al. 2013; Tang et al. 2011). As the sole polymerase responsible for mtDNA replication and repair, abnormalities in *POLG* function lead to increased mtDNA mutability and mtDNA depletion (Del Bo et al. 2003; Naviaux, Nguyen 2004). Severity of mutations correlates with the actual disease presentation, and Alpers' disease is representative of the severe end of the phenotypic spectrum (Stumpf et al. 2013). Unlike most infants with Alpers' disease, which initially present with intractable seizures and eventually progress to liver failure, our patient was ascertained due to isolated intermittent hypoglycemia. Aggressive early investigations into the underlying etiology allowed insights into the disease progression of this *POLG* patient and led us to the finding of abnormal glycogen storage in the liver, with respective absent and severely decreased activities of glycogen debrancher (GDE) and brancher enzymes (GBE) prior to the development of frank liver disease. To our knowledge absent GDE activity has not been previously described in Alpers' patients.

3.2 Results

3.2.1 Case Report

The patient was delivered by cesarean section due to breech presentation; postnatal course was only significant for transient neonatal jaundice. At 4 month of age, she had two episodes of difficult arousal in the morning following uneventful nights, resulting in emergency room visits. Review of outside medical records showed that isolated glucosuria was present, without documented abnormal blood glucose values. This raised the suspicion of a renal tubular defect but repeated urine glucose and biochemical studies

did not confirm this possibility. Neurological consultation, including EEG was unrevealing. At 10 months of age, fasting morning blood glucose was 2.4 mmol/L while the patient was asymptomatic. This prompted admission to our hospital for further studies. Physical exam, including developmental milestones, was normal and there was no clinical or laboratory evidence of muscle or liver involvement. A fasting study was performed at night, after the patient had been feeding regularly during the day. After 8h of fast, a finger stick blood glucose level was 2.7 mmol/L and venous blood samples for critical labs were obtained (Table 3.1).

Table 3.1: Fasting study #1

	Glucose (3.6-6.1 mmol/L)	CO2 (15-28 mmol/L)	ALT (10- 32 U/L)	Cortisol (77.3- 634.6 nmol/L)	GH (2-10 ng/ml)	Insulin (0-90.3 pmol/L)	Lactic Acid (0.5- 2.2 mmo/L)	B-OH- Butyrate (0.05- 0.19 mmol/L)	Alanine (246- 486 µmol/L)	CK (24- 206 U/L)
Results	1.5 mmol/L	21 mmol/L	151 U/L	331.08 nmol/L	29 ng/ml	<6.95 pmol/L	1.6 mmol/L	1.32 mmol/L	175 µmol/L	36 U/L

All reference laboratory ranges are in parentheses. All out of range values are in bold. Reference laboratory ranges were developed for the normal population with normal blood glucose values. Abbreviations: ALT (alanine aminotransferase), GH (growth hormone), CK (creatine kinase).

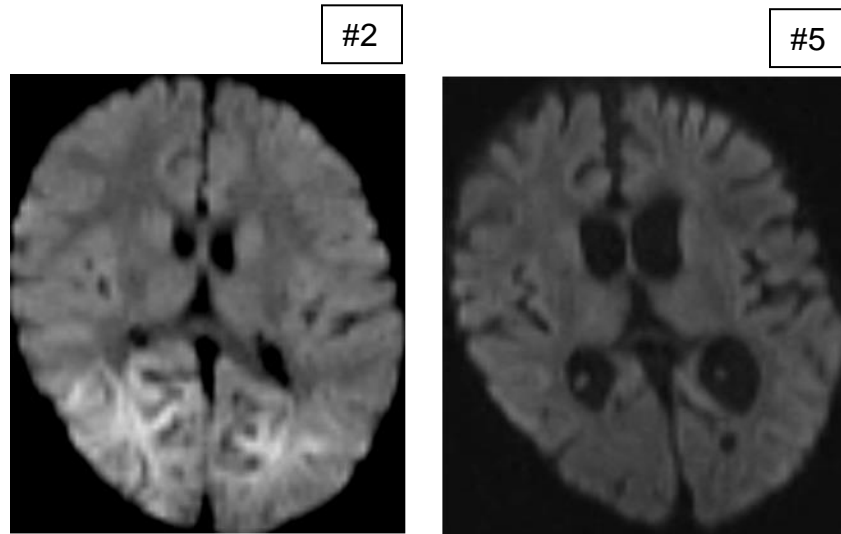
Age 10 months: post 8 hour fast. Organic Acid: significant ketosis, mild increase in dicarboxylic acids. Acylcarnitine: slight elevations of C2 and several other acylcarnitines from C12:1 to C18:1 with no diagnostic pattern

Results were consistent with ketotic hypoglycemia. The normal lactic acid and low alanine suggested intact gluconeogenesis. The response of growth hormone and insulin at the time of hypoglycemia were appropriate. Although serum cortisol level was within the normal reference range it was considered inappropriately low for a child with hypoglycemia. The initial diagnosis of hypocortisolism was made and patient was

discharged on treatment with hydrocortisone and frequent feeds. MRI of the brain and pituitary gland was normal. Two days before her first birthday, the patient had early morning lethargy, with a blood sugar of 1.7 mmol/L. She had missed her 2am feed which resulted in an 11hr fast. The patient fully recovered after formula and orange juice were given. On the day of her birthday, she was asymptomatic and medications and meals were given as scheduled. However in the middle of the night, the patient had a seizure. Blood sugar was normal at 96 mg/dL and she was taken to the emergency room where repeat blood sugar was 3.7 mmol/L. She was admitted to intensive care unit due to lethargy, seizures and apnea requiring intubation and mechanical ventilation. Sepsis work-up and ammonia were normal, cortisol (after stress dose in ER) was high at 5904 nmol/L (214 µg/dL) and comprehensive metabolic panel was normal except for elevated AST and ALT of 173 and 141 U/L respectively.

Due to hypoglycemia while on steroids and mildly elevated LFT's, repeat metabolic testing was obtained. Organic acids once more showed ketosis, whereas lactic acid, acylcarnitines and CK were normal and alanine was again low at 163 umol/L. MRI of brain (2nd) showed bilateral symmetric diffusion abnormalities in both occipital lobes (Figure 3.1 #2) and subtle T2 prolongation in the same areas, which, in view of the normal MRI two weeks prior, was interpreted to be secondary to the recent hypoglycemic episode. A new fasting study was done while on hydrocortisone, after the patient was on frequent feedings for a few days (Table 3.2).

Figure 3.1: Diffusion-weighted images (MRI #2 and #5)



MRI #2 shows bilateral symmetric diffusion abnormalities in both occipital lobes. MRI #5 shows brain atrophy with periventricular and subcortical white matter signal abnormality and full resolution of occipital lesions

Table 3.2: Fasting study #2

Fasting Time	4 h	5 h	6 h	7 h	8 h	8.5 h	9 h	9.5 h	1 h*	2 h*	3 h*
Glucose (3.6-6.1 mmol/L)	5.2	5.8	4.2	3.3	3.4	4.9	3.9	3.1	8.9	6.8	6.7
Alanine (246-486 µmol/L)								281			
Lactate (0.5-2.2 mmol/L)								2	3.3	4.3	4.3
β-OH-Butyrate (0.05-0.19 mmol/L)								0.64	0.26	0.18	0.21
Cortisol** (77.3-634.6 nmol/L)			129.7								
FFA (0.5-0.9 mmol/L)								1.2			

All reference laboratory ranges are in parentheses. All out of range values are in bold. Reference laboratory ranges were developed for the normal population with normal blood glucose values.

Abbreviations: *FFA* (free fatty acids), *β-OH-Butyrate* (beta-hydroxybutyrate).

**Although cortisol value is within normal range it is considered low for a child with hypoglycemia.

*Post Prandial Studies; all specimens are from central line.

Age 24 months: during hydrocortisone treatment + after frequent feedings for +2 days.

The patient developed again hypoglycemia and increased ketone body production, however post prandial samples revealed high blood sugar and lactic acid. Glycogen Storage Disease (GSD) type 0 was suspected but no mutation was found after full sequencing of *GYS2* (Prevention Genetics, Marshfield, WI). The patient did not have further hypoglycemia or seizures while on scheduled feeds and steroid therapy. Repeat MRI of brain (3rd, 18 days following 2nd MRI) showed resolution of previous diffusion abnormalities but persistent abnormal T2 prolongation in the medial aspects of both occipital lobes interpreted as evolution of prior insult. By the time of discharge (20 days stay) the patient had returned to baseline except for cortical visual impairment with expected resolution given improved MRI findings and normal visual evoked potentials. Two months later the patient had new seizures and developed respiratory failure despite being compliant with every 4hr feeding regimen and hydrocortisone treatment. Blood sugar was normal at home as well as in the emergency room. MRI of the brain (4th) showed further improvement of T2 prolongation in occipital lobes without new lesions. Patient recovered fully within a few days and was playful and interactive. No hypoglycemia was documented during this admission, but lactic acid and ALT were persistently elevated and were as high as 17.7 mmol/L (0.5-2.2mmol/L) and 103U/L (10-32 U/L) respectively. A muscle biopsy was ordered and skin and liver biopsies were obtained at 15 months of age. Muscle biopsy was studied on cryostat sections by H&E, modified trichrome stain, ATPase, NADH-TR, PAS, PAS diastase, Oil red 0, phosphorylase A, B kinase, cytochrome oxidase and acid phosphatase (Bonilla et al. 1992). Results showed < 1% COX negative fibers, but were otherwise normal (no ragged red fibers). Muscle electron microscopy also showed normal results (Long Beach

Memorial Medical Center). Electron transport chain studies performed on muscle were at the low end of the control range for NADH cytochrome C reductase (complex I/III) and cytochrome c oxidase (complex IV) (CIDEM, Cleveland, OH). An in vitro fatty acid oxidation probe assay performed in skin fibroblasts showed normal results (Mayo Clinic, Rochester MN) (Blau et al. 2008).

Liver pathology showed relatively uniform nodules divided by fibrous bands with ductular proliferation. The majority of the hepatocytes exhibited cytoplasmic fine vacuolization containing abundant glycogen (confirmed by PAS with and without diastase). The overall histology was reported as consistent with a glycogen storage disease. Liver enzyme activities and glycogen content were measured on frozen liver biopsy tissue using standard spectrophotometric methods that have already been published (Brown, Brown 1989; Wapnir 1985). The residual enzyme activity was measured indirectly by measuring the amount of glucose or phosphate released using glucose or phosphate reagent. Liver glycogen content was borderline elevated at 7.1 % (normal 3.3 ± 1.7), glucose-6-phosphatase, glycogen phosphorylase and glycogen phosphorylase kinase activities were all repeatedly normal with a G-1P/Glu ratio of 50% indicating normal glycogen structure. Branching enzyme activity was low at 20 um/min/g of tissue (normal 85 ± 31) and glycogen debrancher enzyme (GDE) activity assessed in liver tissue was undetectable (performed twice at Duke University Medical Center).

Sequence of the debrancher (*AGL*) gene was normal (Prevention Genetics, Marshfield, WI).

Two weeks after discharge from her third hospitalization, the patient presented with refractory myoclonic seizures. MRI of brain (5th, 1 month apart from 4th) revealed,

for the first time, mild brain atrophy with periventricular and subcortical white matter signal abnormality and full resolution of the occipital lesions (Figure 3.1 #5). The patient progressed rapidly to encephalopathy, lactic acidosis and hepatic failure. *POLG* sequencing revealed compound heterozygous mutations c.1399G>A, p.[A467T] + c.2542G>A, p.[G848S], as underlying cause of the disease.

3.3 Discussion

Since the initial discovery of the causal relationship between *POLG* mutations and Alpers' syndrome, an enormous body of literature elucidating this disease mechanism has accumulated. Large studies exploring phenotype/genotype correlations are mostly focused on the primary clinical features involving liver-pathology, encephalopathy, neuropathy and dysphagia (Horvath et al. 2006; Wong et al. 2008; Tang et al. 2011). Although multiple reports have associated hypoglycemia with Alpers' disease, the underlying etiologies have not been delineated, or remain speculative (Naviaux et al. 1999; Ferrari et al. 2005; Nguyen et al. 2005; Bortot et al. 2009). In a recent article by Scalais et al. an Alpers' patient with the same p.[A467T] + p.[G848S] genotype presented with hypoglycemia and hyperlactatemia as the prominent initial findings. In contrast to our patient who initially presented with isolated ketotic hypoglycemia, normal lactic acid level and normal physical exam at 10 months of age, this infant had already developed severe liver dysfunction at 3.5 months of age with jaundice, ascites and hepatomegaly. A liver biopsy at ~9 months of age showed micronodular cirrhosis and bile ductular proliferation (Scalais et al. 2012). At 18 months a second liver biopsy showed Kupffer cells with PAS positive large granules and glycogen rosettes, a finding usually considered secondary to

glycogen storage (Roels et al. 2009). Enzymatic studies for glucose 6-phosphatase activities were normal in both biopsy samples and fructose 1 phosphate aldolase / fructose 1,6 bisphosphate activities were normal in the first biopsy. Unfortunately, there is no mention whether liver glycogen content or glycogen brancher and debrancher enzyme activities were measured. Interestingly the authors describe that their patient did not respond to glucagon, a response that could be consistent with GDE deficiency depending on the fasting time. Due to the many similarities between our patient and the one previously published (Roels et al. 2009; Scalais et al. 2012), one may speculate whether our findings are genotype specific. A search of the Human DNA Polymerase Gamma Mutation Database (<http://tools.niehs.nih.gov/pol/g>) for p.[A467T] + p.[G848S] revealed 18 additional published cases. Of these, only two publications mention hypoglycemia and neither of them address glycogen metabolism (Ferrari et al. 2005; Nguyen et al. 2005). The relatively high numbers of this particular genotype and the lack of prior pursuit of glycogen studies may encourage retrospective chart review and enzymatic analyses on stored liver samples for this patient population.

To further broaden our search for derangement in glycogen metabolism in mitochondrial disease we took a close look at other mtDNA depletion syndromes which can be associated with hypoglycemia. Insights derived from studies on *MPV17*, a gene of unknown function leading to progressive liver failure in infancy, prove particularly interesting (Karadimas et al. 2006). Patients with mutations in *MPV17* have shown an exceptionally high rate of secondary hypoglycemia. In a recent publication on *MPV17* deficiency Uusimaa reports on 12 different families with *MPV17* related mtDNA depletion syndrome and 50% of them had hypoglycemia (Uusimaa et al. 2013). Furthermore,

detailed metabolic studies on 2 *MPV17* patients revealed early fasting intolerance (3-4 hours postprandially) with physiological parameters resembling glycogen synthesis and glycogenolysis defects. Unfortunately enzymatic tests in liver elucidating the precise etiology of disrupted glycogen metabolism were not done in these patients (Parini et al. 2009). Interestingly a study exploring the potential function of *MPV17* via the yeast homologue Sym1 showed that Sym1 deficiency induces disturbed glycogen metabolism. The authors speculate that this finding may likely be secondary to abnormal levels of mitochondria derived TCA cycle intermediates (Dallabona et al. 2010). Patients with mutations in another mtDNA depletion syndrome associated with hypoglycemia, deoxyguanosine kinase (*DGUOK*), also showed increased glycogen in liver tissues in multiple cases (Dimmock et al. 2008). A lack of enzymatic glycogen metabolism studies in this publication again did not allow further insights into the exact mechanisms of hypoglycemia secondary to liver failure.

The complete absence of GDE activity in our patient's liver sample was confirmed by repeat assay. Additionally, control enzymes run in parallel were normal, ruling out the possibility of technical problems. Since the *AGL* gene had been sequenced previously with normal result, it is possible that the absence of GDE activity was secondary to decreased mRNA and protein levels or due to decreased enzyme activity secondary to a damaged, misfolded protein. We were not able to address this question directly due to the lack of remaining liver sample. Changes in gene expression secondary to *POLG* mutations have been observed previously in fibroblast cell lines derived from *POLG* patients (Voets et al. 2012) as well as in the *POLG* mutator mouse skeletal muscle (Hiona et al. 2010). We accessed the mRNA data deposited in the gene expression omnibus

repository (GEO <http://www.ncbi.nlm.nih.gov/geo/>) (GSE21189) and did not find evidence of altered mRNA levels for the glycogen debrancher enzyme in mouse skeletal muscle (Hiona et al. 2010; Barrett et al. 2012; Edgar 2002). Since the POLG mutator mouse is a model for premature ageing rather than for Alpers' disease we also reviewed another recently submitted GEO dataset by Falk et al: "Transcriptome profiling in primary human mitochondrial respiratory disease" (GSE 42986). Here we compared mRNA data collected on human skeletal muscle from three mtDNA depletion syndrome patients (*POLG*, *MPV17*, *RRM2B*) to three control patients with abnormalities in the pyruvate dehydrogenase complex and normal mitochondrial electron transport. The comparison suggested robust and unaltered expression levels for the *AGL* gene, in human skeletal muscle (data not shown) which may not be representative of expression levels in the liver.

AGL is a large protein comprised of 1532 amino acids (NP_000019.2). It is unusual insofar that it is a monomer with two active sites, catalyzing two different enzymatic reactions (4-alpha-glucotransferase and amylo-1,6-glucosidase) (Nakayama et al. 2001). It's large size and structure may deem the protein more vulnerable to the effects of free radical damage and low levels of ATP both of which have been observed in *POLG* related phenotypes (Voets et al. 2012; Vries et al. 2007). Potential increased production of free radicals in our patient may have been further compounded by conditions of low glucose and hypoxia which may add to the unfolded protein response and free radical damage (Elanchezhian et al. 2012). Abnormalities in glycogen metabolism secondary to *POLG* mutations may also translate to other organs involved in glycogen metabolism. Particularly a closer look at astrocytes may prove to be interesting since it has recently been shown that polyglucosan polar bodies confined to this tissue are able to cause a

mitochondrial disease phenocopy with leukodystrophy and diaphragmatic failure (Dainese et al. 2013) and that cultured astrocytes derived from mice deficient in glutathione activity and increased free radicals have altered glycogen metabolism (Lavoie et al. 2011).

Ultimately hypoglycemia associated with mitochondrial DNA depletion syndromes may have different etiologies depending on the function of the gene involved, the patient's specific genotype, mutations in modifier genes as well as disease stage, progression and degree of liver involvement.

To our knowledge this is the first report on a child with Alpers' syndrome in which the underlying cause for intermittent hypoglycemia has been linked to abnormal glycogen metabolism. This may not only carry significance for Alpers' patients or patients with other *POLG* related syndromes but may hold interesting information for mitochondrial disease in general.

3.4 Acknowledgements

MS was partially supported by grants from Haley's Wish Foundation. We thank Karen Leydiker and Leighann Sremba for editing the manuscript.

3.5 Contributions

Mariella Simon performed the publicly available database search using the gene expression omnibus repository and co-wrote the paper.

CHAPTER 4

Mutations in TFAM, Encoding Mitochondrial Transcription Factor A, Cause Neonatal Liver Failure Associated with mtDNA Depletion

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4.1 Introduction

Mitochondrial diseases are a genetically and phenotypically heterogeneous entity representing one of the most common inborn errors of metabolism with an estimated incidence of ~1 in 5000 live births (Debray et al. 2008; Thorburn 2004). Mitochondria are involved in multiple cellular processes including oxidative phosphorylation (OXPHOS), apoptosis and iron homeostasis (Koopman et al. 2012). As adenosine triphosphate (ATP) is primarily generated by mitochondria, mitochondrial disorders are characterized by impaired energy metabolism and can result from mutations in nuclear encoded DNA (nDNA) or mitochondrial DNA (mtDNA) (Haas et al. 2008). While 13 proteins are encoded by the mitochondrial genome, it is estimated that 1,500 proteins, encoded by nDNA are involved in maintaining mitochondrial structure and function (El-Hattab, Scaglia 2016; Wong 2010).

MtDNA depletion syndromes (MDS) are autosomal recessive disorders caused by molecular defects in nuclear genes that encode proteins involved in mtDNA replication or nucleotide synthesis. Depletion syndromes are characterized by a reduction of mtDNA copy number to less than 30% of normal in the affected tissues (Rotig, Poulton 2009; El-Hattab, Scaglia 2013), which ultimately leads to an inadequate supply of mtDNA gene products resulting in impaired energy production (El-Hattab, Scaglia 2013; Nobre et al. 2012). MDS are phenotypically heterogeneous and may affect different organs or systems which often includes the brain, muscle, gastrointestinal tract, liver and kidney (Nogueira et al. 2014). Depending upon the genetic defect and organ(s) involved, depletion syndromes have been classified as encephalomyopathic, myopathic, hepatocerebral or neurogastrointestinal (El-Hattab, Scaglia 2013).

Hepatocerebral mtDNA depletion syndromes have been reported as caused by mutations in deoxyguanosine kinase (*DGUOK*) (El-Hattab, Scaglia 2013; Nobre et al. 2012; Sezer et al. 2015; Pronicka et al. 2011; Freisinger et al. 2006), *MPV17* (El-Hattab, Scaglia 2013; El-Hattab et al. 2010; Spinazzola et al. 2006; Wong 2007b), polymerase gamma 1 (*POLG1*) (El-Hattab, Scaglia 2013; Nogueira et al. 2014; Cohen, Naviaux 2010; Wong et al. 2008; Muller-Hocker et al. 2011; Ferrari et al. 2005), or *C10orf2* (*Twinkle*) (El-Hattab, Scaglia 2013; Young, Copeland 2016; Suomalainen, Isohanni 2010; Sarzi et al. 2007; Prasad et al. 2013) (Table 4.1).

Here we report the finding of a homozygous variant in *TFAM*, which encodes mitochondrial transcription factor A, identified in two affected siblings from a consanguineous family. Major manifestations were neonatal-onset liver failure progressing to death and mtDNA depletion in liver and skeletal muscle. *TFAM* is essential for the transcription, replication and packaging of mtDNA into nucleoids and *Tfam* knockout mice display embryonic lethality secondary to severe mtDNA depletion (Kukat et al. 2015). We provide evidence that *TFAM* deficiency in humans results in a novel mtDNA depletion syndrome resulting in fatal liver failure.

Table 4.1. Clinical Manifestations of Hepatocerebral Mitochondrial DNA Depletion Syndromes

Gene	Clinical Findings					Mitochondria Impairment				Other Organ Involvement			
	Age of Onset	Liver Failure / Dysfunction	Cirrhosis	Failure to Thrive	IUGR	Lactic Acidemia	Abnorm al NBS	Respiratory Chain Defect in Muscle	mtDNA Depletion in Liver	mtDNA Depletion in Muscle	CNS	Heart	Kidney
TFAM	Neonatal	+	+	+	+	-	+	Normal	+	+	-	-	-
DGUOK	Neonatal	+	+	+	+	+	+	Combined, Complexes I, III, IV	+	+	+	-	+
MPV17	Infantile-childhood	+	+	+	-	+	+	Combined	+	+	+	-	+
POLG1 (Alpers' Phenotype)	Early childhood	+	+	-	-	+	NR	Combined	+	+	+	-	+
C10orf2	Neonatal-infancy	+	+	+	+	+	+	Combined, Complexes I, III, IV	+	-	+	-	+

4.2 Materials and Methods

4.2.1 Human Subjects

Patients 1 and 2 are similarly affected siblings of Colombian-Basque descent (Figure 4.1A). The family self-reports consanguinity as parents being first cousins, in addition, the paternal grandparents are also first cousins. Female sibling (IV-2) died of spinal muscular atrophy (SMA) and prenatal testing for SMA was performed on subsequent pregnancies. Informed consent was obtained for all subjects under approved Institutional Review Boards #130990 at CHOC Children's.

4.2.2 Molecular Analysis

Individual IV-4 underwent trio-clinical exome sequencing (CES) at the Clinical Genomics Center at UCLA. CES was performed using standard protocols from genomic DNA extraction to data analysis (Lee et al. 2014; Rehm et al. 2013) in a CLIA-certified laboratory. Briefly, exome capture was performed using Agilent SureSelect XT Clinical Research Exome, sequencing was performed on HiSeq2500 as 100bp paired end run and variant annotation was performed using GoldenHelix SNP & Variation Suite.

Extracted DNA was obtained from Patient 1 (IV-1) for segregation analysis. Sanger sequencing was performed with ABI BigDye Terminator v3.1 Cycle Sequencing kits on an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems Inc) To evaluate the presence of the *TFAM* variant in family members. The oligonucleotides used to confirm the variant identified in *TFAM* were the following: 5'-GTT GTG GTG GAA GCA TGC AG-3' and 5'-ACA GAA AAG CCT CCA GAA GAA-3' (Integrated DNA Technologies).

All genetic alleles studied were submitted to ClinVar (SCV000262581) <http://www.ncbi.nlm.nih.gov/clinvar/> and were annotated in reference to TFAM NM_003201.2 for cDNA and NP_003192.1 for protein.

4.2.3 In-silico Mutagenesis of Pro178Leu

Mutation modeling for TFAM protein (UNIPROT Q00059-1) was conducted using the crystal structure of TFAM PDB entry 4NNU and Pymol (www.pymol.org) (Ngo et al. 2014).

4.2.4 Pathology/Histological Analysis

The muscle biopsy was divided into three portions for freezing (-80° C), paraffin embedding, and resin embedding, and processed according to standard methods for light and electron microscopy. Cryostat enzyme histochemistry, including H&E, modified Gomori trichrome, succinate dehydrogenase (SDH), nicotinamide adenine dinucleotide (NADH), cytochrome oxidase (COX) and immunohistochemistry were performed on frozen tissue (Dubowitz et al. 2013). A portion of the frozen muscle and liver was sent to Baylor Miraca Genetics Laboratories (Houston, TX) for mitochondrial DNA (mtDNA) content analysis by real-time quantitative PCR (qPCR) and mitochondrial respiratory chain enzyme analysis.

4.2.5 Cell Culture

Primary human skin fibroblasts were established from 3 mm punch biopsy. Tissue was grown on plates coated with fibronectin (10 µg/ml of phosphate-buffered saline (PBS)) in alpha MEM with 1x non-essential amino acids (NEAA), Primocin and 15% FBS (Hyclone).

Established cultures were maintained in growth medium (alpha MEM or DMEM, 1x NEAA, 1x Antibiotic/Antimycotic (Life Technologies) and 15% FBS (Hyclone) at 37°C in 5% CO₂.

4.2.6 Immunoblotting

Cells were lysed on ice in RIPA Buffer (Pierce Biotechnology) supplemented with protease inhibitor cocktail 50 µl/ml (Sigma) for 30 minutes at 4°C and cleared at 16,000 rpm for 20 minutes. Protein concentration was determined in triplicate via detergent compatible (DC) protein assay (Bio-Rad). Lysates were stored in Nupage LDS sample buffer at -20°C (Life Technologies). SDS-PAGE was performed using NuPage Bis-Tris Gels, NuPage MOPS or MES running buffer respectively (Life Technologies). Proteins were blotted onto PVDF membrane (Life Technologies) and blocked for 30 minutes at room temperature with Fast Blocking Buffer (Pierce Biotechnology). Membranes were incubated with rabbit anti-Tfam antibody (Abcam) at 1:10,000 in 0.5% BSA/TBST and 1:100,000 rabbit anti-beta Actin (Abcam) at 4°C overnight, washed briefly with 1x TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at room temperature for 1 hour. Membranes were then washed 3 times for 5 minutes with 1x TBST at room temperature and proteins were detected with SuperSignal Chemiluminescent Substrate (Thermo Fisher Scientific) and imaged on CL-Xposure Film.

4.2.7 Quantification of TFAM Expression

RNA was extracted from patient and control fibroblasts using the Qiagen RNeasy kit according to the manufacturer protocol, and the samples were treated with DNase to produce pure RNA. Quantitative PCR for TFAM was performed in triplicate on CFX

Connect Real-Time PCR Detection System using iTaq Universal SYBR Green One-Step Kit (Bio-Rad). TFAM Primers (5' CAG AAC CCA GAT GCA AAA ACT AC-3' and 5'-GCT GAA CGA GGT CTT TTT GGT-3') were designed against NM_003201.2 using Primer Blast (Ye et al. 2012). We used primers amplifying the housekeeping genes GAPDH, ACTB and HPRT for normalization.

4.2.8 mtDNA Copy Number Determination in Fibroblasts

Genomic DNA was extracted using Puregene DNA extraction according to the manufacturer protocol. Quantitative PCR assay was performed in triplicate on a CFX Connect Real-Time PCR Detection System using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). For this the ratio of mtDNA amplification for the ND1 gene vs the nuclear housekeeping gene beta-2 microglobulin (B2M) was calculated for the patient and an age matched control. Primers were used as previously described (Bonnen et al. 2013a).

4.2.9 Measurements of oxygen consumption rate by Seahorse

Basal and maximal oxygen consumption rate (OCR) measurements were performed on a Seahorse Bioscience XF^e-24 bioanalyzer using the Seahorse XF Cell Mito Stress Test. Cells were seeded in XF24-well microplates in growth medium at 4×10^4 cells/well. The following day growth medium was replaced with unbuffered assay medium supplemented with glucose, pyruvate and L-glutamine. Cells were equilibrated prior to Mitostress testing with unbuffered medium for 45 minutes at 37°C in a CO₂-free incubator. Data was analyzed using XF Cell Mito Stress Test Report Generator. Upon assay completion cells

were rinsed in ice cold PBS and lysed with RIPA buffer supplemented with protease inhibitor cocktail. Protein levels were determined for each well in triplicate using DC protein assay. Values were used for normalization of OCR as per $\mu\text{g}/\mu\text{l}$ of protein.

4.2.10 Immunostaining Studies in Fibroblasts

4.2.10.1 Nucleoids

Primary fibroblasts were plated and fixed with 4% paraformaldehyde, permeabilized and blocked with PBS + 0.1% Triton-X100 and 3% normal donkey serum. Anti-DNA (Millipore, clone AC-30-10) was applied to stain the nucleoids and detected using Alex Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch).

4.2.10.2 Mitochondrial Morphology

Control and patient fibroblasts were lifted with 0.05% Trypsin and plated at a density of 500cells/cm². The following day, Mitotracker-Deep Red dye was centrifuged for 20 minutes at 14,000 rpm, and then diluted in cell culture medium at a concentration of 25 nM. Cells were then incubated with the diluted dye for 15 minutes at 37°C. The cultures were rinsed with PBS and fed with fresh medium, and then allowed to recover for 24 hours before fluorescent imaging. Images were taken at identical exposures for each cell type with an Olympus IX70 microscope.

4.2.10.3 Confocal Microscopy

Confocal images were taken on an inverted stage Nikon Eclipse Ti chassis with a Yokogawa CSUX spinning-disk head and a 60 objective. Z stacks were acquired with

MicroManager ImageAcquisition (v1.4.16) and processed with Image J.

4.3 Results

4.3.1 Clinical Report

Patient 1 (IV-1), a 46XY male, was born at 41 weeks gestational age by normal spontaneous vaginal delivery. The patient's clinical course was nearly identical to that of his sister's (IV-4). He presented with severe IUGR, hypoglycemia, hyperbilirubinemia, cholestasis and ascites in the neonatal period. His NBS, obtained at 27 hours of life, was positive for elevated methionine of 106 $\mu\text{mol/L}$ (cut off <100) with a tyrosine of 483 $\mu\text{mol/L}$, below the cut off for the NBS program (<700). Confirmatory testing revealed elevated tyrosine and methionine in plasma and 4-hydroxy-phenyl derivatives and N-acetyl-tyrosine in urine. He had persistent cholestasis and abnormal liver enzymes with worsening liver synthetic function and ascites with subsequent abdominal distention resulting in respiratory distress requiring ventilation. Echocardiogram, neurological exam and MRI of the brain were normal. Patient rapidly deteriorated and died at 2 months. Shortly before his demise he developed renal and cardiac failure, which appeared to be secondary to complications of his liver failure. Of note patient 1 was born and cared for at another facility and information was obtained through the available medical records.

Patient 1 (IV-4), a 46XX female, was the result of a term pregnancy born via spontaneous vaginal delivery to a G9 P3 TAB2 SAB3 female receiving regular prenatal care. Prenatal microarray revealed 6% genomic homozygosity. Growth lag was noted at 31 weeks gestational age and the pregnancy was complicated by polyhydramnios. She was born at 39 weeks, with a birth weight of 2,085 grams APGAR scores were 9/9 and

she was noted to have asymmetric intrauterine growth restriction (IUGR) with length and weight less than the 10th percentile and head circumference at the 20th percentile. She was transferred to the neonatal intensive care unit for observation and upon admission was noted to have low blood glucose (30 mg/dl), which corrected easily with intravenous fluids. Initial newborn screen obtained at 4 days was positive for an elevated tyrosine of 1,106 µmol/L (cut off <850) with normal methionine and succinylacetone levels. Confirmatory testing was obtained at 7 days of age and patient was discharged at age 13 days in stable condition on breast feedings and formula supplementation. She was first evaluated at the metabolic clinic at 18 days. Her weight was 2,250 gr and physical exam was normal. Review of the NBS confirmatory testing revealed abnormal plasma amino acids with elevated tyrosine (1,236 µmol/L, reference range: 33-160) and methionine (129 µmol/L, reference range: 13-45) and urine organic acids with elevations in 4-hydroxy-phenylacetate, 4-hydroxy-phenylpyruvate, 4-hydroxy-phenyllactate and N-acetyltyrosine. Labs obtained on the day of the visit showed borderline elevations of AST (92 units/L, reference range: <71), ALT (71 units/L, reference range: <32), alkaline phosphatase (807 units/L, reference range: <365) and prothrombin time (16.7 seconds, reference range: <14.6) with normal APTT (37.6 seconds, reference range: 23.1-38.3) and direct bilirubin (0.0, reference range: 0.0-0.6). Additional testing for metabolic and non-metabolic causes of liver disease was initiated and patient was also referred to gastroenterology for evaluation.

At 2 months of age she presented in the emergency department due to decreased feedings and lethargy. Laboratory testing revealed hypoglycemia, hypoalbuminemia, increased liver enzymes, alkaline phosphatase and direct bilirubin and coagulopathy.

Physical exam was positive for jaundice and ascites. A liver ultrasound revealed increased echotexture, concerning for cirrhosis. Patient was treated with IV glucose, albumin, diuretics, fresh frozen plasma (FFP) and cryoprecipitates. An open liver biopsy, skin and muscle biopsies were obtained (see results section, below). The patient's ascites, jaundice and coagulopathy worsened over time and she was unable to maintain glucose levels off IV fluids. Due to poor weight gain and muscle involvement she was not considered a good candidate for liver transplant. Supportive therapy continued until 4 months of age when she died of complications of liver failure. Her cardiac and neurological examinations remained normal until the end, including normal echocardiograms and brain MRI.

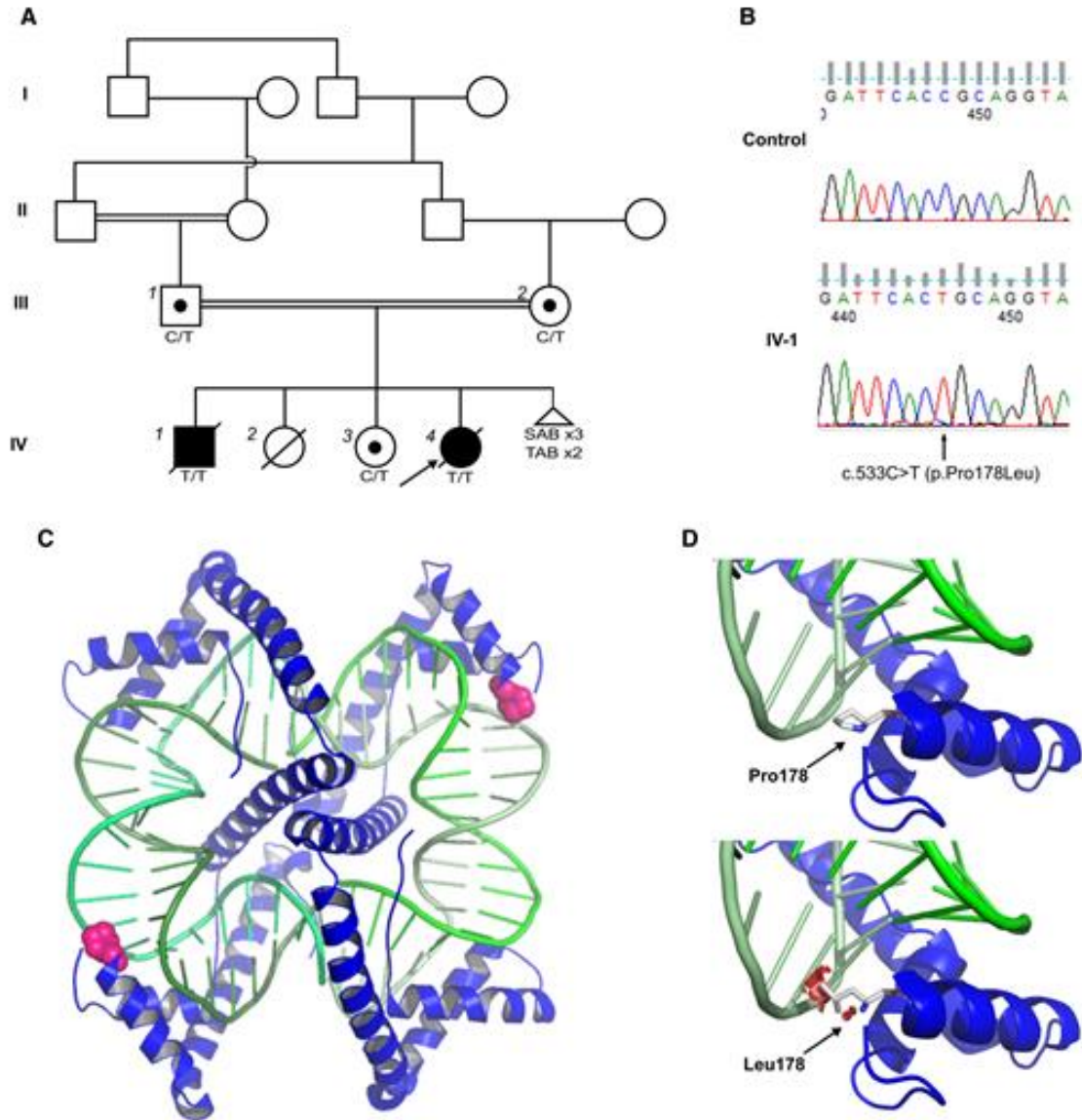
4.3.2 Clinical Exome Sequencing Uncovers a Homozygous Variant in TFAM

In total, 22,884 DNA variants were identified within the coding exons +/- 2bp in the proband with multiple regions of homozygosity (>5Mb) observed, encompassing approximately 5.56% of the genome. A primary genelist from the Human Gene Mutation Database (HGMD) and Online Mendelian Inheritance of Man (OMIM) was generated based on the clinical presentation and phenotype of Patient 2 to prioritize the variants. No pathogenic or likely pathogenic variants were identified within any known disease genes. In total, 25 homozygous and 307 heterozygous rare heterozygous protein-altering variants of uncertain clinical significance (VUS) were identified across 314 genes which included one likely pathogenic variant within a 20Mb region of homozygosity on chromosome 10 (c.533C>T; p.Pro178Leu) in *TFAM* (NM_003201.2). This variant was not observed in the Human Gene Mutation Database (HGMD) or ClinVar and was observed

in 2 out of 118,504 chromosomes in the Exome Aggregation Consortium (ExAC) Cohort (Exome Aggregation Consortium (ExAC) cohort, Cambridge, MA, <http://exac.broadinstitute.org>, accessed April 26, 2016). This variant showed complete segregation with the disease phenotype in all clinically affected family members (Figure 4.1A: IV-1 and IV-4) and both parents and the unaffected sibling were heterozygous carriers (Figure 4.1A: III-1, III-2, and IV-3).

The p.Pro178Leu mutation is located in the high-mobility group (HMG) box B domain of the protein (Figure 4.1C) and the substitution of leucine for the proline at this residue is predicted to cause a steric clash upon binding of mtDNA (Figure 4.1D).

Figure 4.1: Molecular analysis confirms segregation with disease of recessive *TFAM* p.Pro178Leu mutation

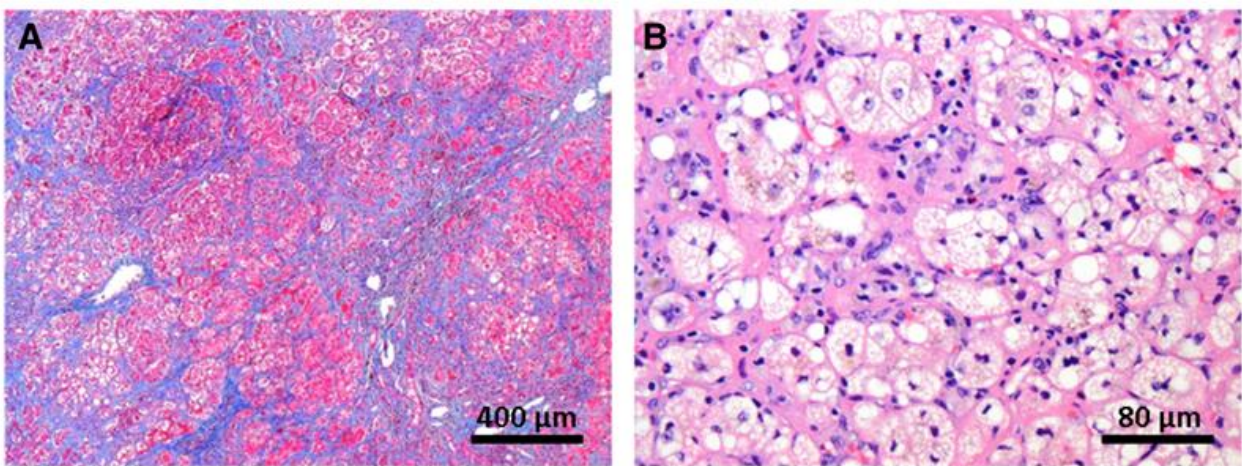


(A) Family pedigree with *TFAM* deficiency. The arrow indicates the proband (IV-4). Filled in symbols represent similarly affected individuals (pedigrees are drawn to accepted standards) (Bennett et al. 1995). T/T or C/T indicates genotype at c.533. (B) An electropherogram obtained by targeted sequencing confirms the presence of the c.533C>T variant in IV-1. (C) Three-dimensional structural modeling of *TFAM*. The molecule is depicted as a dimer with non-specific mtDNA from the ATP6 gene. p.Pro178 is depicted in pink. (D) *In-silico* mutagenesis of p.Pro178Leu. *TFAM* is depicted in blue and mtDNA in green/light green.

4.3.3 Liver and Skeletal Muscle Show Severe mtDNA Depletion and Abnormal Mitochondrial Morphology

A wedge liver biopsy revealed cirrhosis with microvesicular steatosis, cholestasis and mitochondrial pleomorphism (Figure 4.2). In light of the clinical presentation and the results of the liver biopsy, a mitochondrial DNA depletion syndrome was strongly considered. Subsequent mtDNA quantitation in the liver showed a value of 353 which is 11% of the mean value of age and tissue matched controls (3134 ± 733).

Figure 4.2: Liver biopsy reveals cirrhosis and cholestasis

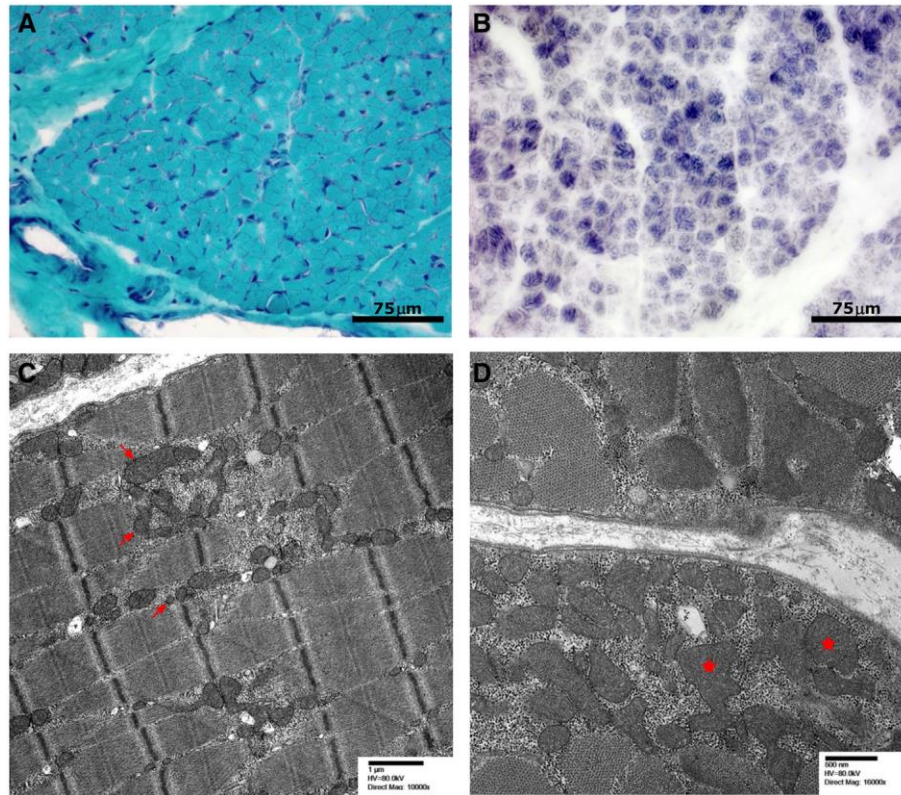


(A) Cirrhotic liver showing nodular regeneration with delicate fibrous septa and perisinusoidal collagen deposition highlighted by trichrome stain. (B) Hepatocytes demonstrate diffuse microvesicular steatosis with rare multinucleated hepatocytes and cholestasis.

Muscle biopsy of the right thigh was performed to study possible manifestations of mitochondrial DNA depletion in muscle. Morphologic evaluation on H&E stain demonstrated well developed and well preserved fascicles of skeletal muscle. Myofibers exhibited age related immaturity consisting of occasional mononucleated fibers, highlighted on modified Gomori trichrome stain (Figure 4.3A). Classic ragged fibers were

not seen, however, sarcoplasmic irregularities were noted throughout the biopsy. No other morphologic abnormalities were noted. Mitochondrial enzyme histochemistry (SDH, NADH, and COX) revealed a mosaic fiber pattern and further suggested sarcoplasmic mitochondrial mal-distribution, especially on SDH (Figure 4.3B). Electron microscopy showed mitochondrial hyperplasia with a haphazard sarcoplasmic distribution (Figure 4.3C) and excessive subsarcolemmal aggregates as well as abnormal mitochondrial morphology with pleoconia and megaconial forms (Figure 4.3D). Furthermore, mtDNA content in the muscle was reduced compared to age and tissue matched controls to a value of 461 (21% of the mean value of age and tissue matched controls (2162 ± 209)).

Figure 4.3: Skeletal muscle reveals findings consistent with mitochondrial myopathy

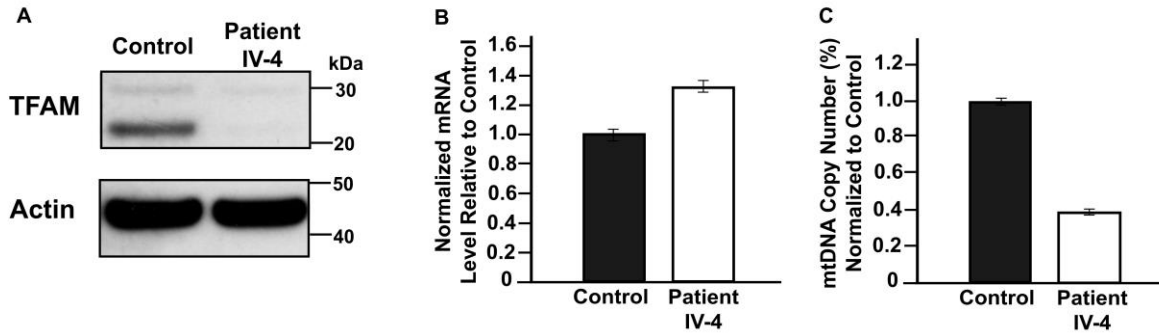


(A) Modified Gomori-trichrome shows myofiber detail and irregular sarcoplasmic pattern. (B) Succinate dehydrogenase (SDH) profile revealed a mosaic fiber pattern and further suggested sarcoplasmic mitochondrial mal-distribution. (C) Electron micrograph shows haphazard mitochondrial sarcoplasmic distribution (D) Higher magnification reveals subsarcolemmal mitochondrial aggregates exhibiting pleoconia and megaconia in the absence of definite internal structural abnormalities

4.3.4 TFAM Expression Studies Reveal Decreased Protein with Increased mRNA Levels

Protein levels in primary fibroblasts were decreased as assessed by immunoblotting (Figure 4.4A) while mRNA levels were upregulated compared to control (Figure 4.4B) as judged by quantitative PCR.

Figure 4.4: Molecular and biochemical studies in primary fibroblasts



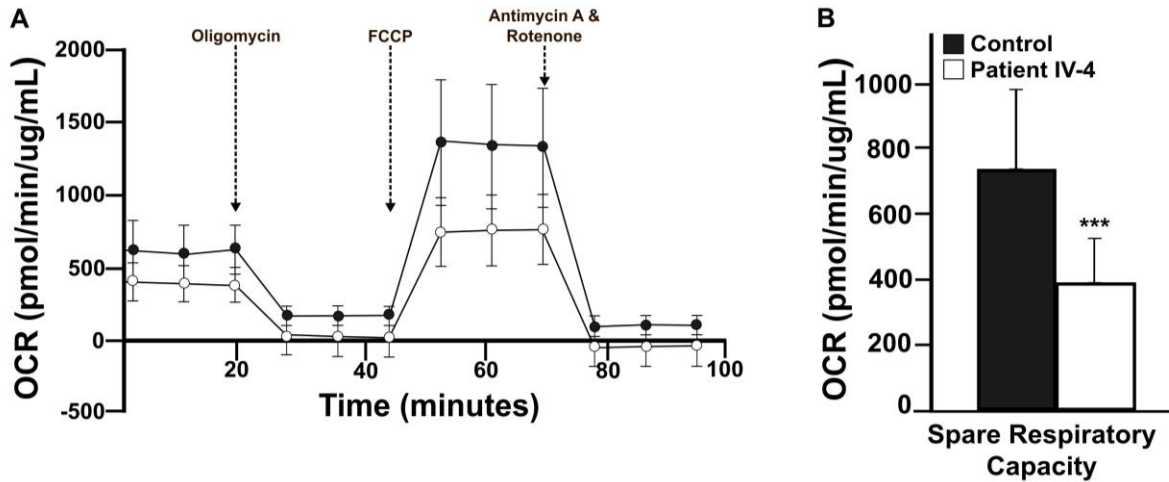
(A) Immunoblot analysis with anti-TFAM was completed to determine abundance compared to control. Beta-actin was used as a loading control. (B) The missense mutation, p.Pro178Leu, was demonstrated to be upregulated as compared to control fibroblasts. (C) Severe depletion of mtDNA was determined in fibroblasts from IV-4 when measured in comparison to age-matched control fibroblasts. For all data error bars indicate standard deviation.

4.3.5 mtDNA Copy Number, Basal Respiration and Spare Respiratory

Capacity is Decreased in Patient Fibroblasts

mtDNA copy number was reduced in fibroblasts to 40% of the normal control (Figure 4.4C). Oxygen consumption rate was assessed in primary fibroblasts (Figure 4.5A) demonstrating that mitochondrial basal respiration and the spare respiratory capacity were significantly reduced (Figure 4.5B).

Figure 4.5: Characterization of respiratory chain function in fibroblasts



(A) Microscale oxygraphy analysis of live fibroblasts demonstrated a respiratory deficiency in cells from IV-4 compared to the control cell line. (B) The ability of fibroblasts from IV-4 to respond to stress, as measured by the spare respiratory capacity (maximal OCR minus basal OCR), is significantly reduced in comparison to the control cell line. For all data error bars indicate standard deviation. Statistical analysis was performed with GraphPad software using Welch's unequal variances t-test.

4.3.6 Mitochondrial Respiratory Chain Enzyme Analysis Showed Evidence of Mitochondrial Proliferation

Mitochondrial respiratory chain enzyme analysis in skeletal muscle demonstrated increased citrate synthase activity, suggesting mitochondrial proliferation. Several respiratory chain complex activities were decreased after correction for increased citrate synthase activity (Table 4.2), but none was sufficiently reduced to satisfy a minor criterion of the modified Walker criteria for the diagnosis of a respiratory chain disorder (Bernier et al. 2002).

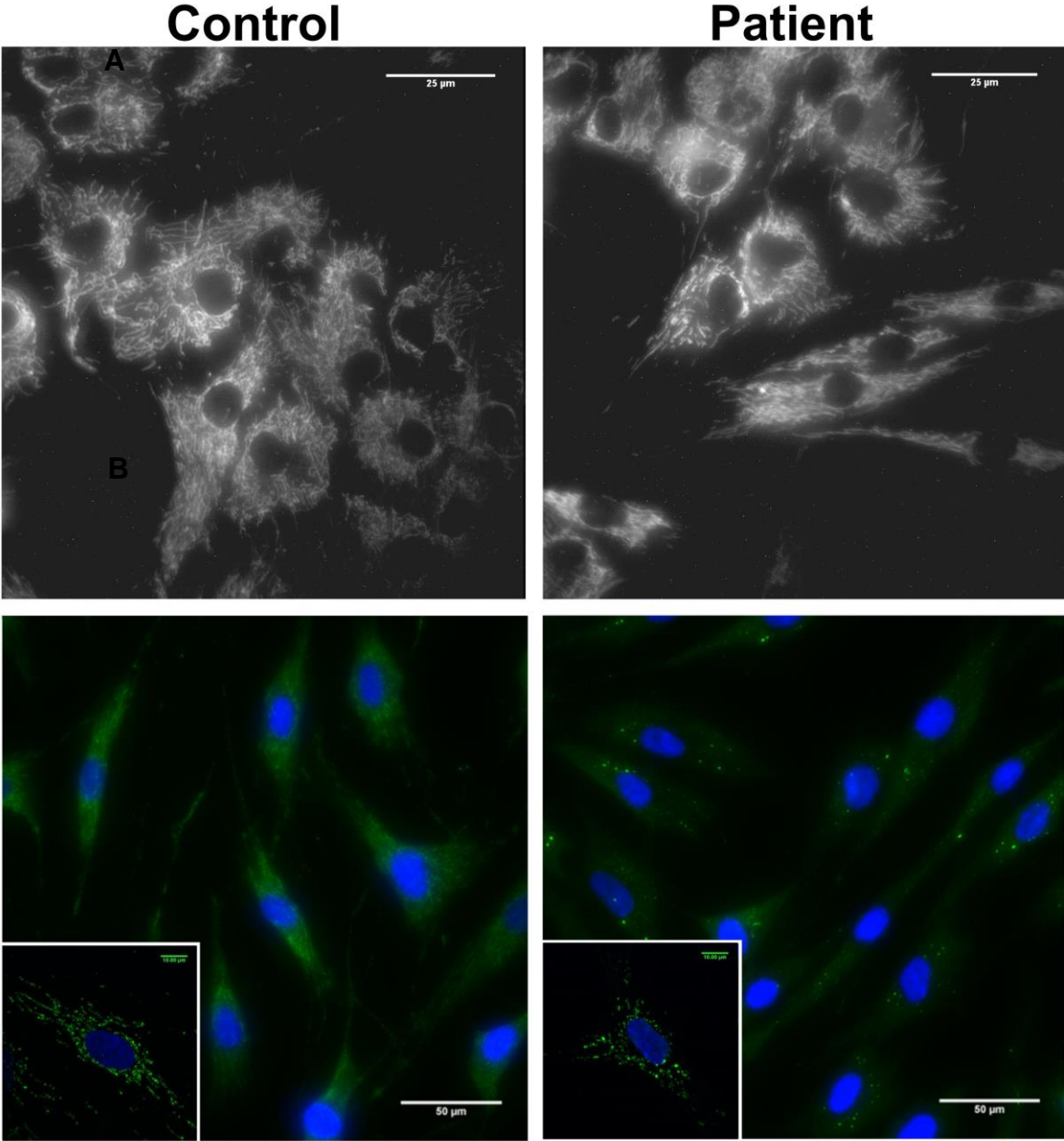
Table 4.2: Mitochondrial respiratory chain enzyme analysis (ETC) - skeletal muscle

ETC Activities	ETC Complexes	Patient	Compared to the Mean	Compared to Citrate Synthase
			Percentage	
NADH:ferricyanide dehydrogenase	I	292	81	44
NADH:cytochrome c reductase	I+III			
Total		24.4	86	47
Rotenone sensitive		9.24	106	58
Succinate dehydrogenase	II	8.61	90	49
Succinate:cytochrome c reductase	II+III	3.67	87	47
Cytochrome c oxidase	IV	28.3	70	38
Citrate Synthase		541	184	100

4.3.7 Immunohistochemistry Reveals Decreased, Aggregated Nucleoids

While there was no apparent difference in mitochondrial morphology and content in primary fibroblasts as compared to controls (Figure 4.6A), mitochondrial nucleoprotein complexes were decreased and revealed large aggregated nucleoids in the patient's cell line (Figure 4.6B).

Figure 4.6: Nucleoid morphology is disrupted



(A) upper panels show representative images of mitochondrial morphology of control (left) and IV-4 (right) in fibroblasts stained with MitoTracker. The lower panels show immunohistochemistry with anti-DNA antibody detected using Alex Fluor 488-conjugated secondary antibody revealing decreased mitochondrial nucleoprotein complexes and large, aggregated nucleoids with perinuclear clustering in cells from IV-4.

4.4 Discussion

We present two siblings with a novel hepatocerebral mtDNA depletion syndrome caused by TFAM deficiency. The disease is characterized by neonatal-onset liver failure progressing to death. Hepatic pathology revealed cirrhosis, steatosis and cholestasis and abnormal mitochondrial morphology was observed in skeletal muscle. We show that mtDNA is depleted in liver and skeletal muscle and provide evidence that this mutation results in decreased TFAM protein expression, reduced mitochondrial function and nucleoid formation.

The clinical presentation of our patients is consistent with that of previously described patients with hepatocerebral mtDNA depletion syndrome. Hepatic involvement in association with mtDNA depletion has been described in patients carrying mutations in nuclear encoded genes involved in mtDNA replication or nucleotide synthesis, *DGUOK*, *MPV17*, *POLG1* and *C10orf2*. These disorders usually present with neonatal-, infantile- or early childhood-onset liver disease and failure to thrive with rapid progression to liver failure resulting in death. Neurological involvement, when present, includes hypotonia, nystagmus and encephalopathy (Nobre et al. 2012). Our patients presented with IUGR, neonatal-onset hypoglycemia and evidence of liver dysfunction. There was a rapid clinical course with progression to liver failure with no evidence of neurological impairment or other organ involvement. The abnormalities found in the muscle biopsy suggest that at this tissue was incipiently affected. However the lack of overt involvement in other organs is surprising as TFAM is ubiquitously expressed (Picca, Lezza 2015). It is possible that involvement in other organs would have become evident had our patient survived longer.

Biochemical investigations reported in hepatocerebral MDS include increased lactic acid, and abnormal plasma amino acids with elevated tyrosine/methionine along with non-specific markers of liver disease which includes hypoglycemia, increased AST and ALT, total and direct bilirubin, coagulopathy, and decreased albumin, which were all found in our patients. Both siblings had an abnormal NBS and confirmatory testing revealed elevations of metabolites in urine and plasma consistent with liver failure with no evidence of mitochondrial dysfunction. The NBS result in patient 1 was flagged for an extremely elevated tyrosine (>1,000 umol/L) and while the NBS in patient 2 was deemed positive for an elevated methionine, her tyrosine levels were >450 umol/L, a value that, although it falls below the cut off for the NBS program, is clearly elevated. Consistent with our findings, abnormal NBS for elevated tyrosine and methionine, has been reported in other MDS syndromes (Table 4.1) (El-Hattab, Scaglia 2013; Pronicka et al. 2011; Prasad et al. 2013).

As expected by the above mentioned biochemical abnormalities, liver pathology in our patient was abnormal showing micro- and macro-vesicular steatosis, cholestasis, and cirrhosis, all findings that have been reported in patients with hepatocerebral mtDNA depletion syndromes (Sezer et al. 2015).

Multiple respiratory chain deficiency and mtDNA depletion in affected tissues are hallmarks of MDS (El Hattab, Scaglia 2014). Evaluation of the respiratory chain in muscle in our patient did not satisfy the requirements for any given respiratory chain complex deficiency, but did show evidence of mitochondria proliferation, an early sign of mitochondrial dysfunction. It is very likely that ETC deficiencies would have been detected in liver, or could have become evident over time in muscle. As could be expected, mtDNA

was depleted in liver and skeletal muscle at levels similar to that of previously reported patients with proven mtDNA depletion syndromes (Dimmock et al. 2010).

It is clear that hypoglycemia, elevated serum transaminases, conjugated bilirubin and lactic acidosis are common signs of mitochondrial hepatopathy and together with a clinical presentation of a neonate with intrauterine growth restriction (IUGR) and histological findings of cirrhosis should prompt evaluation of mitochondrial disease (Fellman, Kotarsky 2011). However, mitochondrial diseases are not always considered early in the differential diagnosis of neonatal-onset liver failure, and confirming a specific diagnosis for a mitochondrial disease remains challenging due to the genetic and clinical heterogeneity (Wong 2007a). Timely diagnosis is important to consider different treatment options including liver transplantation. Clinical exome sequencing (CES), employed as a trio, has been shown to have a higher diagnostic yield than proband-CES or traditional molecular diagnostic methods and can expedite the results in these situations.

CES results were available within a week and revealed a homozygous variant (c.533C>T; p.Pro178Leu) in the mitochondrial transcription factor A gene, *TFAM*. No other gene known to be involved in any MDS was found to have any abnormality and no variants inside or outside of the primary gene list were identified that could explain the phenotype.

Human *TFAM* is a nuclear-encoded protein that is essential for mitochondrial biogenesis (Picca, Lezza 2015) and a member of the high-mobility-group (HMG) family (Gangelhoff et al. 2009). The ~25kDa protein consists of two (HMG)-box domains (HMG-box A, HMG-box B) and a carboxy terminus abundant in basic amino acid residues (Kasashima, Endo 2015; Parisi, Clayton 1991). In the absence of mtDNA, *TFAM* exists

as a monomer and forms a dimer upon binding to the heavy and light strand promoters of mtDNA to activate transcription (Tiranti et al. 1995). TFAM regulates mtDNA copy number indirectly by producing the necessary RNA primer for mtDNA replication. In addition, TFAM dimers have been shown to play a key role in mtDNA maintenance and distribution during cell division through compaction and organization of mtDNA in the nucleoid (Parisi, Clayton 1991; Tiranti et al. 1995; Kang et al. 2007). The p.Pro178Leu variant in our patients occurs in the HMG-box B domain which is involved in mtDNA binding and compaction (Gangelhoff et al. 2009).

The crystal structure of TFAM protein bound to the light strand promoter (LSP), the heavy strand promoter (HSP1) as well as nonspecific mtDNA has been resolved (Ngo et al. 2014). The proline at residue 178 is one of five TFAM residues which intercalates into DNA minor groove and forms a direct contact with LSP, HSP1 and nonspecific mtDNA. The residue is therefore crucial for mtDNA transcription, replication and compaction. *In-silico* mutation of the proline to leucine at residue 178 results in steric hindrance (Figure 4.1D). We speculate that the increased repulsion energy at this location decreases the binding capabilities of TFAM to the mtDNA and subsequently results in decreased mtDNA transcripts, mtDNA content and dysfunction of packaging of mtDNA into nucleoids. Unbound TFAM is subject to turnover by the mitochondrial protease LonP1 which would explain the significant decrease in TFAM protein in patient fibroblasts (Lu et al. 2013).

TFAM is involved in many cellular functions including mtDNA transcription, maintenance, repair, and replication and its activity is vital in the maintenance of mtDNA copy number (Picca, Lezza 2015). The disruption of *Tfam* in mouse results in mtDNA depletion, abolished oxidative phosphorylation and abnormal mitochondria morphology

(Larsson et al. 1998) highlighting that the regulation of TFAM expression and turnover is essential for mtDNA biogenesis. Fibroblast studies in our patient demonstrated decreased mtDNA copy number and TFAM protein levels, with concomitant increase in mRNA expression, which could be expected as a compensatory mechanism. The decreased basal respiration and spare respiratory capacity were consistent with the above results, further sustaining the deleterious effect of the mutation.

As expected by the important role of TFAM in nucleoid formation (Picca, Lezza 2015), immunohistochemistry showed decreased number of nucleoids and large nucleoid aggregates compared to control cells. Differences in the mitochondrial network assessed with mitotracker dye were less evident. Quantitative studies of these cellular abnormalities could be done in the future to further characterize these findings.

Although our patients had a severe clinical course and early death, overall our findings are consistent with a hypomorphic phenotype expected in TFAM deficiency caused by a missense mutation, as it is likely that null mutations are embryonic lethal (Larsson et al. 1998).

In summary we report a novel mtDNA depletion syndrome, characterized by early onset and fatal liver disease, adding TFAM deficiency to the list of mitochondrial diseases.

4.5 Acknowledgements

We thank the family for their steadfast commitment to participation in this study and the generous support of the Fry Family Foundation to CHOC Children's Metabolic Program.

4.6 Contributions

Mariella Simon designed the experiments; performed the protein modeling and created the Figure; performed the following studies: TFAM expression studies, mtDNA copy number studies, Seahorse studies, SDS PAGE for TFAM levels; performed the statistical analysis, created the applicable figures.

CHAPTER 5

**Activation of a Cryptic Splice Site in the Mitochondrial Elongation Factor GFM1
Causes Combined OXPHOS Deficiency in Two Brothers with Microcephaly,
Developmental Delay and Seizure Disorder**

Accepted in *Mitochondrion* for publication (November 2016)

5.1 Introduction

Mitochondria are the hub of cellular metabolism, respiration, energy production as well as sites of reactive oxidative species (ROS), heme and steroid generation. Furthermore they buffer cellular calcium flux, regulate redox states, control cell cycle and cell death. The mitochondrial network is the only cellular organelle of the animal kingdom carrying its own dedicated DNA (mtDNA), which in humans codes for thirteen essential mitochondrial proteins (Wallace 2013). Their translation requires a dedicated protein synthesis machinery, independent from its cytoplasmic counterpart (Smits et al. 2010b). Approximately 1500 nuclear genes have been attributed to mitochondrial biogenesis and metabolism (Pagliarini et al. 2008), of which about 10% are thought to play a role in mitochondrial translation (Rötig 2011). Since mitochondria retain a dedicated mitochondrial protein synthesis machinery to synthesize mtDNA-encoded proteins, mutations in mitochondrial elongation factor genes cause mitochondrial translational defects. Three mitochondrial elongation factors have been described to date: EF-Tu (*TUFM*; MIM#602389), EF-Ts (*TSFM*; MIM#604723), and EF-G1 (*GFM1*; MIM#606639). The mitochondrial elongation factor EF-Tu delivers cognate aminoacylated mitochondrial tRNAs to the ribosomal binding site in a GTP-dependent manner. Once the amino acid has been delivered, GDP bound EF-Tu is released and subsequently recharged with GTP via the elongation factor Ts (EF-Ts). EF-G1, also a GTPase, enforces the elongation-dependent movement of mtDNA encoded tRNAs through the mitochondrial ribosomes by removing the deacylated tRNA and replacing it with the peptidyl-tRNA (Christian, Spremulli 2012). Mitochondria generate ATP via oxidative phosphorylation (OXPHOS), a process which depends on the concerted action of five mitochondrial protein complexes

(complex I through V). Complex I, III, IV and V all contain at least one mtDNA encoded subunit while complex II is encoded completely by nuclear DNA (Wallace 2013). A defect in any of the elongation factors is therefore predicted to result in abnormalities of multiple OXPHOS related complexes, a type of mitochondrial dysfunction since termed “combined oxidative phosphorylation deficiency,” or COXPD (COXPD4 (MIM#610678) for *TUFM*, COXPD3 (MIM#610505) for *TFSM*; COXPD1 (MIM#609060) for *GFM1*). Mutations in all three mitochondrial elongation factors have been associated with severe mitochondrial disease phenotypes (Boczonadi, Horvath 2014a).

Herein we describe clinical, laboratory and molecular findings for two brothers with mutations in *GFM1*. We highlight the survival and stable clinical course in one of the siblings at seven years of age and the unusual findings of repeated transferrin glycoforms suggesting a type I congenital disorder of glycosylation (CDG). We therefore propose to consider mitochondrial disease in the differential diagnosis following abnormal transferrin testing. We also describe a novel, intronic *GFM1* mutation, which results in the predominant use of a noncanonical *GFM1* isoform and secondary *GFM1* protein instability. Our findings demonstrate the importance of choosing exome capture kits and data annotation, which not only cover the canonical isoform of a gene but all potential transcripts (Chilamakuri et al. 2014).

5.2 Materials and Methods

5.2.1 Exome Sequencing

One microgram of genomic DNA was subjected to a series of shotgun library construction steps, including fragmentation through acoustic sonication (Covaris), end-polishing (NEBNext End Repair kit), A-tailing (NEBNext dA Tailing kit), and ligation of 8bp barcoded

sequencing adaptors (Enzymatics Ultrapure T4 Ligase). Libraries were automatically size selected for fragments 350-550bp in length with the automated PippinPrep cartridge system. Prior to sequencing, the library was amplified via PCR (BioRad iProof).

One microgram of barcoded shotgun library was hybridized to capture probes targeting 64 Mb of coding exons and miRNA (Roche Nimblegen SeqCap EZ Human Exome Library v3.0) with custom blockers complimentary to the full length of the flanking adaptor and barcodes. Enriched libraries were amplified via PCR before sequencing (BioRad iProof). Library quality was determined by examining molecular weight distribution and sample concentration (Agilent Bioanalyzer). Pooled, barcoded libraries were sequenced via paired-end 50bp reads with an 8bp barcode read on Illumina HiSeq sequencers.

Demultiplexed BAM files were aligned to a human reference (hg19) using the Burrows-Wheeler Aligner. Read data from a flow-cell lane were treated independently for alignment and QC purposes in instances where the merging of data from multiple lanes was required. All aligned read data were subjected to: (1) removal of duplicate reads (Picard) (2) indel realignment using the GATK IndelRealigner; and (3) base qualities recalibration using GATK TableRecalibration. Variant detection and genotyping were performed using the UnifiedGenotyper (UG) tool from GATK. Variant data for each sample were formatted (variant call format [VCF]) as “raw” calls that contained individual genotype data for one (GATK 1.0) or multiple samples (GATK 1.6-11), and flagged using the filtration walker (GATK) to mark sites that were of lower quality and potential false positives (e.g. quality scores (≤ 50), allelic imbalance (≥ 0.75), long homopolymer runs (>3), and/or low quality by depth ($QD < 5$)).

The single sample VCF files were filtered for 1000 Genomes and NHLBI GO Exome Sequencing Project variants with a frequency of more than 5% cutoff, a minimum quality value (QUAL) of 30, and a minimum coverage (DP) of 8. All remaining variants were further filtered for the overlap with segmental duplications (UCSC genomicSuperDups) and were required a mapability of 1 (UCSC wgEncodeDukeMapabilityUniqueness20bp). Variants were annotated using the Ensembl Variant Effect Predictor.

5.2.2 Cell Culture

Primary human skin fibroblasts were established from 3mm punch biopsy. Tissue was grown on plates coated with fibronectin (10ug/ml of PBS) in alpha MEM with 1x nonessential aminoacids (NEAA), Primocin and 15% FBS (Hyclone). Established cultures were maintained in DMEM, 1x NEAA, 1x Antibiotic/Antimycotic and 15% FBS (Hyclone) at 37C in 5% CO₂.

5.2.3 Immunoblotting

Cells were lysed on ice in RIPA Buffer (Pierce) supplemented with Protease inhibitor cocktail 50ul/ml (Sigma) for 30min at 4C and cleared at 16,000 rpm for 20min. Protein concentration was determined in triplicate via DC protein assay (Biorad). Lysates were stored in Nupage LSD sample buffer at -20C (Life Sciences). SDS PAGE was performed using NuPage Bis-Tris Gels, NuPage MOPS running buffer (Life). Proteins were blotted onto PVDF membrane (Life) and blocked for 30min at RT with Fast Blocking Buffer (Pierce). Membranes were incubated with 1:1000 anti-GFM1 (Abcam, ab176786) and 1:100,000 beta actin at 4C overnight, washed briefly with 1xTBS-T and incubated with

HRP-conjugated goat anti-rabbit IgG at RT for 1hr. Membranes were then washed 3x with 1x TBS-T at RT and Proteins were detected with SuperSignal Chemiluminescent Substrate (Therma) and imaged on CL-Xposure Film.

5.2.4 BN-PAGE with in-gel activity staining assay:

Mitochondria were isolated from cultured fibroblasts following the protocol of Van Coster et al., 2001 (van Coster et al. 2001). Following solubilization of the mitochondria, the isolated OXPHOS membrane protein complexes were analyzed by blue native polyacrylamide gel electrophoresis (BN-PAGE) with in-gel activity stain as described (Smet et al. 2006; van Coster et al. 2001; Chatfield et al. 2015). This assay allows for both the evaluation of the activity and assembly of the OXPHOS complexes. For example, the presence of additional bands of lower molecular weight of complex V due to incomplete assembly can be observed in defects of complex V assembly as well as defects in mitochondrial transcription or translation (Smet et al. 2009).

5.3 Results

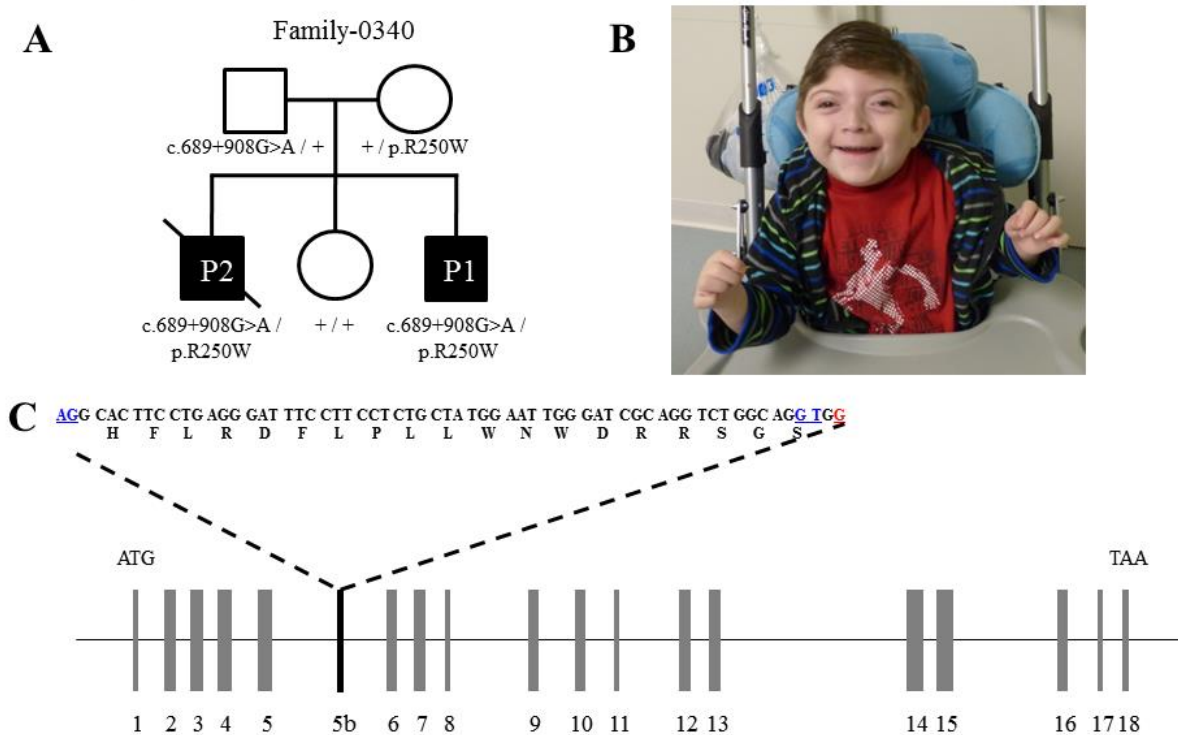
5.3.1 Case report patient 1

Patient 1 (P1) was born after a pregnancy complicated by early 1st trimester bleeding at 37 weeks gestation with normal birth weight and uncomplicated postnatal course. At 3 months of age he was hospitalized due to an RSV infection. Developmental delay, microcephaly, hypertonicity, and visual inattention were noticed and he was referred to our center for evaluation. Family history was significant for an affected, deceased male sibling [patient 2 (P2)], who was born 4 years prior to P1 (Figure 5.1A). Evaluation of P1

was positive for multiple dysmorphic features (Figure 5.1B). Ophthalmological exam, MRI of the brain, and echocardiogram performed at 5 months of age were normal.

Initial biochemical evaluations were significant for abnormal carbohydrate deficient transferrin (CDT) testing performed by affinity chromatography, mass spectrometry (Mayo Clinic) (high mono:di-oligo ratio of 0.176; reference range < 0.100, with normal a-oligo:di-oligo ratio of 0.019; reference range <0.050 , persistent lactic acidemia (2.4-11.1 mmol/L; reference range, <2.2) and increased alanine (595 uMol/L; reference range 143-439). Other routine metabolic screening labs were unremarkable, including normal ammonia, acylcarnitine profile, homocysteine, urine organic acids, urine guanidinoacetate, and 7-dehydrocholesterol level. Repeat CDT testing with the same methodology demonstrated similar abnormalities. A third sample, analyzed by MALDI-TOF (Emory Genetics Laboratory) appeared consistent with a type I glycosylation defect.

Figure 5.1: Mutations in patient 1 and 2 segregate with disease



(A) Pedigree shows segregation of mutations in the family.

(B) Patient photograph:

Dysmorphic features include microcephaly, bi-temporal narrowing, metopic ridging, sloping forehead, supraorbital hypoplasia, wide nasal bridge with short nose, short columella, up-turned nasal tip, anteverted nares, long philtrum, mild retrognathia.

(C) Alternative splicing of *GFM*:

The c.689+908G>A *GFM*-001 mutation (depicted in red) leads to the inclusion of 19 amino acids resulting in the preferential expression of *GFM*-004. The splice donor and acceptor sites of the cryptic exon 5b are depicted in blue.

By 4.5 months of age P1 developed seizures, characterized as infantile spasms, and was started on antiepileptic medication (Topiramate). At 6 months of age he had a right inguinal hernia repair and percutaneous endoscopic gastrostomy tube placement secondary to persistent dysphagia and recurrent emesis. At 7 months of age, renal tubular acidosis was suspected based on persistent non-anion gap metabolic acidosis with simultaneous urine pH greater than 7.0. Treatment with citric acid/sodium citrate

(Bicitra®) at 3 mEq/kg/day was initiated. Hepatomegaly and persistent mild elevation of transaminases with normal bilirubin and coagulation factors were noted at 8 months of age with intermittent low albumin levels. During his frequent hospitalizations due to intercurrent illnesses associated with emesis, it was found that his lactic acidosis improved markedly with high glucose infusion rates exceeding 8 mg/kg/minute. At 2 years of age frequent nose bleeds were suggestive of coagulopathy and he was found to have type 1 Von Willebrand disease with a ristocetin level of 39. His bleeding symptoms have been stable with monthly human von Willebrand factor complex infusions and aminocaproic acid. Due to his family history, cardiovascular status was closely monitored; and at 4.5 years an echocardiogram revealed borderline left ventricular hypertrophy with normal left ventricular function. However, follow up echocardiograms showed resolution of his ventricular enlargement without any specific treatment. Over time, the clinical condition for P1 has improved, with reduced frequency, severity, and duration of hospitalizations and improved tolerance to gastrostomy tube feedings. He has remained seizure free on treatment with Topiramate. Liver synthetic function is borderline abnormal (albumin 3.7 mg/dL, normal reference > 3.8 mg/dL; International Normalized Ratio 1.08 – 1.18, normal reference < 1.15; partial thromboplastin time 36.6 – 41.9 seconds; normal reference < 38 seconds) with persistently borderline elevated transaminases (AST 43 – 102 units/L, normal reference < 46 units/L; ALT 34 – 94 units/L, normal reference < 35 units/L) and mild hepatomegaly. At 7 years of age, his vision and hearing appear intact and he continues to make small developmental gains, is interactive, able to say simple words and sit unassisted.

5.3.2 Case report patient 2

At birth, patient 2 (P2) was found to have symmetric IUGR, cleft palate, and hypospadias. He developed metopic forehead ridging, laryngomalacia, concentric left ventricular hypertrophy, transient hepatomegaly, bilateral renal pyelectasis, and spasticity. Severe feeding intolerance eventually required placement of a jejunostomy tube. Biochemical testing was unrevealing, except for persistent lactic acidosis and an acylcarnitine profile with elevated C4OH, which was interpreted as consistent with ketosis. A defect in pyruvate carboxylase was suspected, but enzyme activity in fibroblasts was normal. P2 had multiple hospitalizations, with the last one taking place at 8 months of age that eventually progressed to multi-organ failure and death at 10 months of age. At the time no specific diagnosis was established for P2.

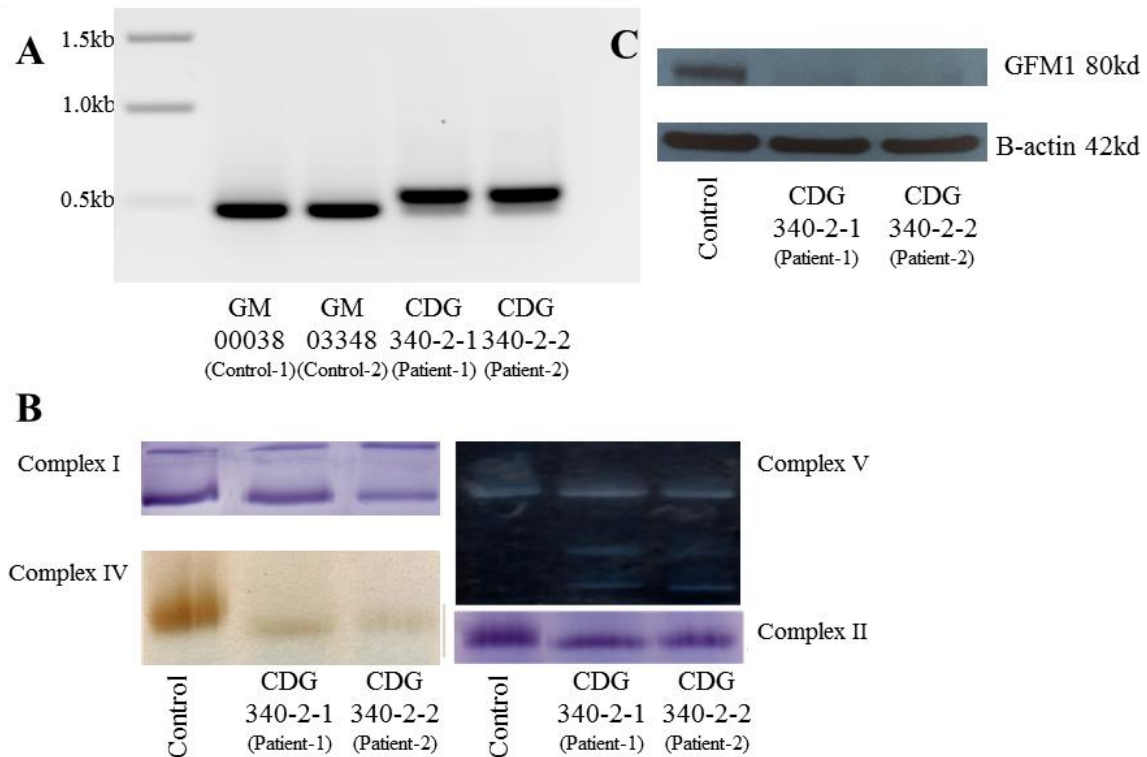
Medical record review and photography were performed after family provided consent under CHOC Institutional Review Board Protocol #130990. Due to the abnormal ESI-MS/MS CDT testing and unusual clinical and biochemical phenotype of patient 1, whole exome sequencing (WES) was performed by the University of Washington Center for Mendelian Genomics (Bamshad et al. 2011). However, no candidate variants were identified in known or putative CDG genes. The analysis did however demonstrate a previously reported mutation in *GFM1* (ENST00000486715), at position c.748C>T, p.Arg250Trp (Smits et al. 2010a). The mother of both patients was found to be a carrier of the mutation. This finding prompted additional analyses of sequencing data which revealed a second, intronic variant in *GFM1*, inherited from the father at position c.689+908 G>A, p.Gly230_231Glnins19 (ENST00000486715). The two sequence variants segregated with the disease: P2 was found to also be compound heterozygous

for both mutations, and the healthy sister bore neither mutation (Figure 5.1A). For cDNA numbering, the use of +1 represents the A of the ATG translation initiation codon in the canonical GFM1 transcript ENST00000486715. All variants have been deposited in the LOVD3.0 (<http://databases.lovd.nl/>).

5.3.3 c.689+908 G>A induces the activation of a cryptic splice site

The effect of the intronic variant at position c.689+908 G>A on mRNA splicing was assessed. Isoform 1 of *GFM1* (GFM1-001 ENST00000486715) is comprised of 18 exons which code for a protein of 751 amino acids (UniProtKB: Q96RP9), while the noncanonical isoform 2 (GFM1-004 ENST00000264263) includes an additional exon between exons 5 and 6 (exon 5b) of GFM1-001 and codes for a protein of 770 amino acids (UniProtKB - Q96RP9). The intronic variant observed in both affected males hypothetically affects the splice donor of exon 6 of the noncanonical GFM1-004 at position c.746+4 G>A (Figure 5.1C). The human splice finder software predicts a significant increase in the strength of the splice donor GT of GFM1-004 intron 6/7 from 88.33 to 97.67 (Desmet et al. 2009). To investigate the variant's effect on GFM1 mRNA splicing in our patients, we reverse transcribed RNA isolated from the fibroblasts of P1 and P2 and amplified the exon/intron junction of GFM1-001 exons 5 and 6 via reverse transcriptase polymerase chain reaction. These experiments revealed an amplicon not observed in two independent controls of increased size (Figure 5.2A).

Figure 5.2: Mutations in *GFM1* are associated with abnormal splicing and result in decreased levels of *GFM1* protein and abnormalities in complexes of the ETC



(A) mRNA analysis for *GFM1*:

Using a forward primer in exon 5 and a reverse primer in exon 6 of *GFM1*-001 shows increased band size as compared to controls. Sanger sequencing of the band reveals an “in frame” insertion of 19 amino acids amplifying *GFM1*-004. The shorter band is significantly decreased in the patient.

(B) Blue native polyacrylamide gel electrophoresis (BN-PAGE) with in gel activity staining: Left panel: In gel staining for mitochondrial complexes reveal decreased levels of complex I and complex IV for patient 2 and complex IV for patient 1. Activity staining of complex II, which does not have any mtDNA encoded subunits, serves as a loading control. Right Panel: BNG for complex V shows additional, lower molecular weight bands indicating incomplete assembly of the complex.

(C) Immunoblotting: Levels of *GFM1* protein are decreased in both patients

5.3.4 Sequencing analysis of amplified cDNA reveals an inframe insertion:

Sequencing analysis of the isolated band revealed an addition of 57 nucleotides, which are identical to exon 6 of *GFM1*-004. We therefore conclude that in primary fibroblast the *GFM1* variant c.689+908 G>A (p.Gly230_231Glnins19) induces the expression of the

noncanonical isoform GFM1-004 rather than canonical GFM1-001 from this allele which extends the G' domain of EFG1 by 19 amino acids.

5.3.5 Blue native polyacrylamide gel electrophoresis with in-gel activity stain reveals a mitochondrial complex V assembly defect and decreased activity for complex I and IV:

Due to the molecular findings in *GFM1*, functional analyses to assess the activity and integrity of the mitochondrial respiratory chain complexes were performed for both patients. Using blue native polyacrylamide gel electrophoresis (BN-PAGE) with in-gel activity stain of mitochondrial lysates from cultured fibroblasts showed P2 had decreased activity for mitochondrial complex I and IV; while in P1 showed decreased activity for complex IV but normal activity for complex I (Figure 5.2B). Both patients showed incomplete assembly of mitochondrial complex V via the presence of additional lower molecular weight bands, a finding consistent with defects of complex V assembly as well as defects in mitochondrial transcription or translation (Figure 5.2B) (van Coster et al. 2001; Smet et al. 2006; Smet et al. 2009)

5.3.6 Western blot for *GFM1* shows decreased protein levels:

Immunoblotting with anti-GFM1 (Abcam, ab176786) antibody corresponding to aa581-742 (Q96RP9) on whole cell lysates from P1 and P2 showed significantly decreased levels of GFM1 protein in both patients as compared to control (Figure 5.2C). Anti-Beta Actin (Abcam) was used to show equal loading. This further supports our findings that the mutations have functional consequence.

5.4 Discussion

In 2004, Coenen et al reported the first patient with *GFM1* mutations. Since then, a total of 15 patients in 12 families have been published (Table S5.1). Prevalent features in these patients include early onset (sometimes neonatal) encephalopathy, liver disease, feeding difficulties, failure to thrive, and lactic acidosis. Liver failure was present in seven of 13 patients; all of them deceased at less than 2 months of age. Intrauterine growth retardation (IUGR), microcephaly, and minor dysmorphic features have also frequently been described. Notably, cardiomyopathy and renal tubular defects, which are relatively common manifestations of mitochondrial diseases, have not been reported in these patients. This has been attributed to tissue specific differences in stability of *GFM1* mutant protein as well as expression levels of the mitochondrial translation factors (Antonicka et al. 2006). Similar to the previously described cases our patient also had early onset of disease, showed dysmorphic features, mild liver involvement and persistent lactic acidemia. P2 had left ventricular hypertrophy but overt cardiomyopathy did not develop in P1. The finding of a pathogenic *GFM1* mutation together with the phenotype of our patients therefore provided enough evidence to warrant additional studies into this gene.

The previously reported missense variant at position c.748C>T (p.Arg250Trp) was found to be homozygous in a patient with mitochondrial encephalopathy (Smits et al. 2010a). The girl, who died at age 2 years, developed seizures at 8 weeks of age while seizures did not start until 4.5 months of age in P1. Like our patient she was hypotonic, had feeding problems, nystagmus and was borderline microcephalic but unlike our patient was not dysmorphic. Her MRI was abnormal for a small frontal cortex, delayed myelination and thinning of the corpus callosum, while patient 1 discussed in this report

had normal MRI at 5 months of age. Similar to our patients her lactate levels were consistently elevated however, in contrast to our case and other reported patients, her liver function tests were repeatedly normal. Functional mitochondrial tests for the girl revealed normal oxidative phosphorylation levels in muscle homogenate, while the same test in fibroblasts showed severely decreased activity of mitochondrial complex I and complex IV and moderate decrease for mitochondrial complex III. This is consistent with mitochondrial analyses on fibroblasts for our patients via blue native polyacrylamide gel electrophoresis (BN-PAGE) with in-gel activity stain which demonstrated decreased activity for mitochondrial complex I and IV for patient 2 but only a decrease in complex IV for patient 1 (Figure 5.2B). Both patients showed complex V assembly defects expected for a mitochondrial transcription or translation defect.

Prognosis for patients with *GFM1* mutations is strikingly poor, as all patients died in infancy, with the exception of one milder case reported to be alive at 6 years of age (Brito et al. 2015). This patient's mild case could not be explained by mutation type since she is compound heterozygous for one nonsense and a previously reported severe missense mutation. The authors emphasize that their patient shares many features with other *GFM1* cases with early onset failure to thrive, encephalopathy and seizures stating at 7 months of age as well as persistent lactic acidosis with a maximum of 4.87mmol/L but lacks liver involvement. Our report now adds a second patient with long-term survival and stable clinical course at age 7 years. In contrast his older brother died at 10 months of age from multiple organ failure. Since the difference in severity for the brothers described here cannot be explained by mutation location in *GFM1* we speculate that it is

due to differences in modifier genes, exposure to infections at a vulnerable age or levels of care.

The finding for P1 of intermittent abnormal glycosylation may shed additional light on an underlying cause for variability in disease severity and expression. Mitochondrial diseases share with CDGs the challenge that both are extremely difficult to diagnose due to their multisystemic involvement, as well as extensive clinical and locus heterogeneity (Freeze et al. 2015). In fact, CDG patients are very likely to be underdiagnosed or even misdiagnosed as mitochondrial disease (Helander 2004). We present a kindred with multisystemic, infantile onset disease who initially was thought to have a type I CDG due to abnormal transferrin glycoforms. Abnormal protein glycosylation is not a common finding associated with mitochondrial disease, although CDT testing may not be routinely performed in patients suspected of having mitochondrial disorders. Only one of the currently published reports on *GFM1* describes testing for CDG, which were found to be normal in an infant with progressive hepatoencephalopathy at 4 months of age (Balasubramaniam et al. 2012). In contrast, our patient did not show abnormal liver functions until 8 months of age, three months after the first abnormal CDT test was obtained. Our findings follow the report of another patient initially diagnosed with a CDG, but eventually found to have mutations in the mitochondrial DNA helicase Twinkle (Bouchereau et al. 2016). Pregnancy history for this patient was significant for a maternal dengue virus infection during the first trimester. The patient developed vomiting in the first weeks of life and by 5 months hypotonia and psychomotor regression were noted. This patient had repeated abnormal glycosylation profiles which were suggestive of type I CDG. It is interesting that our patient as well as the patient reported by Bouchereau both

showed defects in N-glycosylation. Biosynthesis and pre-processing of N-glycans occurs in the endoplasmic reticulum (ER) and is continued in the Golgi. It has long been known that the ER and mitochondria share important membrane contact sites termed “MAM” (Mitochondrial Associated ER Membranes), which are heavily involved in calcium exchange and signaling (Rizzuto et al. 1998). ER calcium levels control the manufacture of many important secretory enzymes including those involved in N-glycosylation (Michalak et al. 2002). Disruption of MAM integrity has recently been shown as a frequent finding of neurodegenerative disease development (Brito, Scorrano 2008; Raturi, Simmen 2013; Stoica et al. 2014). MAM also plays an important role in the immune defense response to RNA viruses since the Mitochondrial Anti-Viral Signaling co-factor MAVS is located at MAM (Horner et al. 2011). MAVS forms prion like aggregates on the outer mitochondrial membrane in response to RNA virus infections (Hou et al. 2011). It may therefore be speculated that remodeling of MAM during intermittent illnesses may affect glycosylation. While the possibility cannot be ruled out that WES sequencing performed for this as well as the patient reported by Bouchereau missed a mutation in a CDG gene it suggests that some mitochondrial diseases may manifest with disruption of glycosylation. In fact, perhaps abnormal markers of N-glycosylation should result in additional molecular and biochemical evaluations for mitochondrial electron transport chain defects. Our findings also reinforce the limitations of exome sequencing technologies and should bring awareness of such to the clinicians interpreting patient reports. The ExAc database is an aggregate data set of 60,706 human exome sequences. Therefore variant frequency information for exons of canonical transcripts is on average comprised of 120,000 alleles. The low Ex-Ac coverage of only 12,324 alleles for the

c.689+908 G>A mutation demonstrates that most exome capture methods do not include (or cover) this region of *GFM1*.

5.5 Conclusion

Our report adds new information to the mutational spectrum of *GFM1* and expands the phenotype to include long-term survival in what are now two cases in this hepatocerebral mitochondrial disorder. Our findings also suggest that patients with *GFM1* mutations are most vulnerable at an early age and then recover and can be relatively stable, a clinical course that has been observed previously in other mitochondrial protein synthesis defects. (Boczonadi et al.) Early detection of the disease due to improved sequencing technologies may therefore lead to improved supportive treatment, slowing decline in health and reducing severity of symptomatology.

5.6 Acknowledgements

We thank the family for providing samples and consenting to this study.

5.7 Contribution

Mariella Simon performed the consenting, provided genetic counseling, performed the SDS PAGE for *GFM1*, and co-wrote the paper.

Table S5.1: Clinical phenotypes associated with mutations in *GFM1*

Publication	Mutation	Patients Age of Onset: (Death)	Biochemical Presentation	Clinical Presentation
[1]	[c.521A>G;p.Asn174Ser]+ [c.521A>G;p.Asn174Ser] homozygous	Sibling 1: In utero (27days) Sibling 2: <7weeks (5months)	Sibling 1: COXPD(f) partial CO-I and CO-IV deficiency(m), Histology(m): normal Sibling 2: Severe CO-I and CO-IV deficiency(f)	Neonatal liver failure, IUGR, microcephaly, stiffness, severe lactic acidosis, basal ganglia/corpus callosum abnormalities Liver failure (7 weeks), stiffness, elevated lactate, abnormal corpus callosum,
[2]	[c.961T>C; p.Ser321Pro]+ [c.1765-2_1765-1delAG;p.Gly589Profs*19]	Sibling 1: In utero (9days) Sibling 2: In utero (neonatal)	COXPD(m) CO-IV deficiency(f), GFM1 protein decreased	Dysmorphic , IUGR,microcephaly, neonatal liver failure, lactic acidosis,coagulopathy
[3]	[c.139C>T;Arg47*] + [c.1487T>G; Met496Arg]	< 1 month (16 months)	COXPD (m,f) Histology(m): abnormal	Dysmorphic features, short tibia, feeding difficulties, vomiting,lactic acidosis, elevated pyruvate, abnormal MRI putamen, globus pallidus mesencephalon, microcephalia,

[4]	[c.748C>T, p.Arg250Trp]+ [c.748C>T, p.Arg250Trp] homozygous	<1week (2years)	COXPD(f) CO-III deficiency(m) GFM1 protein absent(f)	Hypotonia, microcephaly, small frontal cortex, thin corpus callosum and delayed myelination, encephalopathy, lactic acidosis
[5]	[c.539delG;p.Gly180Alafs*11]+ [c.688G>A;p.Gly230Ser)	<1week (8 months)	CO-I+IV deficiency(f)	Liver failure, IUGR, encephalopathy, lactic academia,inc pyruvate, ketosis, hypoglycemia, vomiting, abnormal MRI: putamen globus pallidi. Nystagmus, cystic kidneys, mild coagulopathy, normal transferrin
[6]	[c.720delT;p.Glu241Asnfs*2]+ [c.2011C>T; p.Arg671Cys]	<1year	CO-IV deficiency(f) GFM1 mRNA and protein decreased	Encephalopathy, hypersarrhythmia, FTT, dystonia, squint
[6]	[c.720delT;p.Glu241Asnfs*2]+ [c.910A>G; p.Lys304Glu]	<1week	COXPD(l) mtDNA proliferation, GFM1 mRNA and protein decreased	Encephalopathy, developmental delay, seizures, hypotonia, episodic metabolic acidosis

[7]	[c.2011C>T;p.Arg671Cys]+ [c.2011C>T;p.Arg671Cys] homozygous	<1week (4years)	CO-IV(m) CO-I+IV deficiency(f) Histology(m): abnormal GFM1 protein(f) decreased	Neonatal encephalopathy, feeding difficulties vomiting, reflux, microcephaly coarse facies, hypotonia, dystonia, polyneuropathy, mild lactate elevations, abnormal MRI
[7]	[c.1193T>C, p.Leu398Pro]+ [c.1193T>C, p.Leu398Pro] homozygous	<1week (20months)	Partial CO-VI deficiency(m), COXPD(f)	Neonatal liver failure, spasticity, contractures, hypoglycemia, acidosis, high urinary lactate, ketosis, microcephaly, dysmorphic features
[8]	[c.130_137delins;p.Glu44_Ile46delinsLysLysLys]+ [c.130_137delins;p.Glu44_Ile46delinsLysLysLys]	Sibling 1: In utero (4months) Sibling 2: (14days)	CO-I+IV deficiency(m) GFM1 protein(f) absent CO-IV deficiency(m) GFM1 protein(f) absent	IUGR, dysmorphic,cortical atrophy, lactic acidosis, hypoglycemia,coagulopathy,liver failure

[8]	[c.964G>A;p.Glu322Lys]+ [c.1655T>G;p.V552G]	<1week (3months)	COXPD(l) CO-IV deficiency(f,m,l) GFM1 protein(f) decreased	Small head circumference, liver failure,lactic acidosis, leukodystrophy, seizure
[9]	[c.1404delA;p.Gly469Valfs*84]+ [c.2011C>T;p.Arg671Cys]	2months Alive at 6years	COXPD(f), GFM1 protein(f) decreased	Severe encephalopathy, spastic-dystonic tetraparesis, FTT, seizures, lactic academia, thinning corpus callosum, white matter alteration
This publication	[c.748C>T, p.Arg250Trp]+[c.689+908 G>A, p.Gly230_231Glnins19]	Patient 1: 3months, alive at 7years Patient2: In utero (10months)	COXPD(f), GFM1 protein(f) decreased	IUGR, dysmorphic, microcephaly, lactic acidosis, abnormal carbohydrate deficient transferrin, coagulopathy. IUGR, cleft palate, dysmorphic, transient hepatomegaly

Abbreviations: Combined respiratory deficiency: COXPD, Fibroblast:(f), Muscle:(m), Liver(l), Intrauterine growth deficiency: IUGR, Mitochondrial respiratory chain complex I-IV:COI-IV

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CHAPTER 6

Novel Mutations in The Substrate Binding Domain of the Mitochondrial Matrix Protease *Lonp1* are a Cause of Mitochondrial Disease

6.1 Introduction

Mitochondria are home to key pathways of bioenergetic metabolism and therefore directly or indirectly impact every aspect of cellular physiology (Wallace 2005). This is reflected by the diversity of the mitochondrial proteome, which comprises a group of approximately 1000 biomolecules in its inventory (Cotter et al. 2004). A busy and dynamic place, the organelle never rests: It constantly folds and unfolds, imports and exports, produces and degrades, sends and receives signals and powers up and powers down. Overshadowing the endurance of this heavy workload is the generation of reactive oxygen species (ROS) (Harman 1956). Although important for cell signaling, free radicals create a heavy burden of damaged and aggregated proteins on the mitochondrial machinery, challenging redox and proteome homeostasis (Droge 2002). An extensively regulated mitochondrial quality control system has evolved to ensure the integrity of the organelle, most of which is intrinsically connected to the organelle's dynamic nature (Andreux et al. 2013). The process of fission and fusion provides a mechanism to ensure that the organelle may quickly isolate damaged components within its network for subsequent autophagosomal and lysosomal degradation (Mouli et al. 2009). The term mitophagy has been coined specifically for this clearance function, which is cell type specific and has many different mediators. In neurons, for example, mitophagy is conferred via the PTEN induced putative kinase PINK. This serine-threonine kinase mediates recruitment of the ubiquitin ligase Parkin to a depolarized outer mitochondrial membrane as to initiate degradation by the autophagosome (Greene et al. 2012). Demonstrating the importance of mitochondrial protein homeostasis is the fact that mutations in *PINK* and *PARKIN* can lead to monogenetic forms of autosomal recessively inherited early onset Parkinson's disease

(Am Pickrell, Youle 2015). Much like mitophagy is involved in the turnover and recycling of mitochondrial waste in bulk, the mitochondrial proteolysis machinery minds the integrity of mitochondrial biomolecules one protein at a time. Mitochondrial proteases, together with the mitochondrial antioxidant defense system, juggle the difficult task to ensure integrity of the mito-proteome. Three known AAA (ATPase Associated with diverse cellular Activities) proteases, also termed ATP-dependent proteases, namely LonP1, ClpXP and the inner membrane bound *m*-AAA, control protein homeostasis in the mitochondrial matrix. Unlike ClpXP and *m*-AAA, LonP1 retains its two catalytic functions of ATP hydrolysis and peptidase activity on the same protein chain (Goldberg 1993). In humans, the molecule assembles into a barrel shaped hexamer with a N-terminal substrate binding domain, an ATPase domain and a protease domain, which confers protease activity via a serine-lysine dyad at its active center (Venkatesh et al. 2012). Together with CLpXP, LonP1 participates in the mitochondrial and endoplasmic reticulum mitigated unfolded protein response (UPR)^{MT}, (UPR)^{ER}, elicited by retrograde signaling to and from the nucleus (Bezawork-Geleta et al. 2015; Fiorese et al. 2016). The exact mechanism for UPR^{MT} related LonP1 regulation in humans had remained elusive, since human LonP1 does not carry the mitochondrial promoter response elements MURE1 and MURE2, known for example to be involved in UPR^{MT} related regulation of ClpP in COS-7 cells (Aldridge et al. 2007; Venkatesh et al. 2012). However, LonP1 is the main protease that turns over targets such as damaged ornithine transcarbamylase, a known (UPR)^{MT} activator (Bezawork-Geleta et al. 2015). A recent report by Fiorese et. al demonstrates, that the transcription factor ATF5 regulates UPR^{MT} in mammalian cells and is an activator of *LonP1*. Much like the UPR^{MT} regulator ATFS- in worms, ATF5 accumulates within

mammalian mitochondria during basal conditions but translocates to the cytosol and the nucleus during periods of high oxidative stress, where it activates expression of UPR^{MT} related genes (Fiorese et al. 2016). Only a few LonP1 substrates have been clearly identified in mammalian cells, but they affect all facets of mitochondrial metabolism. A well-known target is the TCA cycle enzyme aconitase (ACO2), which is sensitive to oxidative modification. LonP1 has revealed itself as an oxygen sensor since it will only degrade mildly oxidated ACO2, while highly aggregated ACO2 may no longer be processed by LonP1 (Bota, Davies, Kelvin J A 2002). One often overlooked, but essential, function of mitochondria is the synthesis of the steroid progenitor pregnenolone. Without intact, functioning mitochondria, generation of steroid hormones like estrogen and testosterone would cease. Pregnenolone is synthesized from cholesterol, which needs to be imported into the mitochondria by the steroidogenic acute regulatory protein (StAR) (Granot et al. 2007b). LonP1 is responsible for the turnover of StAR and thus its activity is related to hormone synthesis. It has recently been shown that high levels of StAR in the mitochondrial matrix can induce upregulation of LonP1 expression via the transcription factor Nrf-2 (Granot et al. 2007a). This indicates again a cross talk between mitochondria and the nucleus to fine tune the expression of this important protease.

LonP1 is also involved in mtDNA maintenance and binds preferably to the control region of mtDNA. Abnormal LonP1 function therefore may lead to mtDNA depletion, increased mtDNA error rates and mtDNA rearrangements compromising energy output (Chen et al. 2008). Such mtDNA instability would result in decreased expression and translation of mitochondrial respiratory complex subunits and abnormal mitochondrial electron transport. At the cellular level, compromised electron transport would manifest in

decreased availability of ATP and increased ROS production (Lu et al. 2007). Furthermore, LonP1 also degrades human mitochondrial transcription factor alpha (TFAM, covered in chapter four) in the phosphorylated state. TFAM is responsible for initiation of mitochondrial DNA transcription and is involved in mtDNA replication. Disruption of TFAM degradation may therefore affect mtDNA as well as mt-mRNA levels and quality and thus negatively affect mitochondrial respiration (Lu et al. 2013). Packaging of mtDNA into nucleoids (mtDNA/protein complexes), assures efficient mtDNA compaction and distribution throughout the mitochondrial tubules and TFAM is the main protein component of mitochondrial nucleoids (Gilkerson et al. 2013). Abnormal TFAM degradation may therefore lead to disruption in nucleoid formation and organization (Lu et al. 2012). After TFAM, LonP1 is the second most abundant protein of mitochondrial nucleoids and due to this, mutations in *LonP1* may affect nucleoid formation two fold: Via faulty processing of TFAM and/or via abnormal LonP1 levels. Since mitochondrial nucleoids play an additional organizational role in structuring mitochondria through contributions to mitochondrial fission and fusion as well as determination of cristae morphology (Cogliati et al. 2013), LonP1 is therefore indirectly involved in mitochondrial morphology and dynamics as well. LonP1 is best known for its protease activity, but LonP1 is also an essential mitochondrial chaperone, assuring the correct assembly and insertion of subunits of the mitochondrial respiratory chain. LonP1 is therefore directly responsible for the integrity of the mitochondrial respiratory chain through chaperone, protease, replication and translation functions and assures metabolic homeostasis in the matrix (Lee, Suzuki 2008). In summary the functions of LonP1 affect in a direct or indirect

way all aspects of mitochondrial metabolism including respiration, production of biomolecules, mtDNA replication, mitochondrial morphology and UPR^{mt}.

The importance of mitochondrial proteases for the health of the organelle has also been demonstrated by the fact that mutations in all three matrix proteases have been associated with human disease (Venkatesh et al. 2012). Interestingly, the syndromes present with a completely different clinical picture, depending on which protease they are associated with. Mutations in *CLPP*, which codes for the peptidase subunit of caseinolytic mitochondrial matrix protease, CLpXP, have been associated with Perrault syndrome, while mutations in the paraplegin matrix-AAA peptidase subunit *SPG7* cause autosomal recessive spastic paraplegia type 7. The distinct phenotypic expression of these two syndromes has been linked to varying cellular functions of the proteases, their location in the organelle as well as the identity of their targets (Jenkinson et al. 2013; Casari et al. 1998).

Although Lon protease has been highly studied in model organisms, mutations in human *LonP1* had not been associated with disease until 2015 when Strauss et al. and Dikoglu et al. identified LonP1 mutations in individuals with a rare, multi-system disorder defined by cerebral, ocular, dental, auricular and skeletal abnormalities -CODAS (Strauss et al. 2015; Dikoglu et al. 2015). We have identified novel, pathogenic *LonP1* mutations in two unrelated patients that interestingly do not fit the CODAS phenotype. Patient 1 has compound heterozygous mutations at positions p.Pro122Leu (paternal) and p.Ile636Met (maternal) while patient 2 is homozygous for mutations at position p.Pro125Leu. p.Pro122Leu and p.Pro125Leu are the first reported *LonP1* mutations in the N-terminal domain of the gene, which may explain the difference in phenotype. In this study we

present functional studies, investigating the role of the N-terminal domain, and we discuss how the difference in mutation location may have affected the phenotype and potential disease mechanisms.

6.2 Results

6.2.1 Clinical Description: LonP1 patient phenotypes differ from previously described CODAS patients

Patient 1 was born at 36 weeks of gestational age by induction due to observed intrauterine growth retardation (IUGR). Birth weight was 4 pounds, 14 ounces and a small placenta was observed after delivery. The infant was hypotonic and failed her newborn hearing screen (x2) but was able to go home with parents on day three. By six months of age she was still not able to sit crawl or roll over and subsequently did not achieve other early milestones like holding a bottle or standing. She had feeding difficulties early on and poor weight gain with a history of paradoxical movement of the vocal chord and risk for dysphagia and aspiration, which prompted placement of a gastrostomy tube. Due to low truncal strength and weak muscle tone she is presently non-ambulatory at age six years old and is vocal but speaks no words. She has strabismus, nystagmus, and ptosis. Sensorineural hearing loss was confirmed and has been addressed with hearing aids at five years of age. At 3 years of age, metabolic testing for plasma amino acids was normal, creatine kinase was mildly elevated and urine organic acids revealed low-level elevations of TCA cycle intermediates. A carbohydrate deficient Transferrin study, ferritin TIBC and very long chain fatty acids were all normal. General genetics studies such as karyotype, microarray and Prader-Willi/Angelman methylation studies were normal. MRI was abnormal at 6 months showing cerebellar atrophy, generalized hypomyelination of

the cerebral hemispheres and thinning of the corpus callosum. MR Spectroscopy at 3.5 years showed few scattered lactic acid metabolites. The above findings were rather unspecific and not necessarily indicative of a mitochondrial disease patient.

The Patient's family history on the maternal side reveals a healthy 17-year old maternal half-sister and several maternal cousins with learning disabilities including a maternal first cousin with "selective mutism", a second maternal first cousin who has learning difficulties and "toe walks", two maternal first cousins (siblings) with possible autism spectrum disorder and one with attention deficit disorder. These findings may be either coincidental, suggestive of maternal (mtDNA) inheritance, or represent autosomal dominance with incomplete penetrance leading to a syndrome associated with learning and behavioral difficulties. Due to the unspecific clinical and family history, targeted gene panel testing was not performed. Whole exome sequencing (WES) revealed compound heterozygous novel variants in the *LonP1* gene at positions c.365 C>T, p.Pro122Leu (paternal) and c.1908 C>G, p.Ile636Met (maternal). This prompted a mitochondrial focused clinical laboratory workup including muscle biopsy. Mitochondrial DNA (mtDNA) sequencing and a mtDNA copy number assay in muscle tissue was normal. Mitochondrial respiratory chain enzyme analysis in skeletal muscle homogenate was elevated but normal when adjusted for elevated citrate synthase, which suggested mitochondrial proliferation (Table 6.1). Oxidative phosphorylation (Oxphos) studies on patient fibroblasts were abnormal with decreased oxidation through mitochondrial complex I and III and normal at mitochondrial complex IV suggesting a block in electron transport at complex III of the mitochondrial respiratory chain (Table 6.2). The same study also suggested that fatty acid oxidation was unaffected. Histology on muscle was essentially

normal with few scattered fibers with absent COX activity. Electron microscopy has not yet been performed. The patient has several overlapping symptoms with the CODAS phenotype, specifically prenatal onset, cerebellar atrophy, sensorineural hearing loss, hypotonia, short stature, paroxysmal movements of the vocal chord and feeding difficulties. At six years of age she has not developed cataracts and since thorough skeletal imaging ruled out skeletal dysplasia she therefore does not fit the CODAS criteria (Table 6.3). The physicians were therefore not sure, whether mutations in LonP1 are responsible for the patient's disease and referred the family for follow up studies to the research lab.

Table 6.1: Electron transport chain studies for muscle homogenate

Complex	Enzyme Analyzed	Patient 1	Patient 2
Complex I	NADH-Ferricyanide Dehydrogenase	89%	N/A
Complex I	NADH-Ferricyanide Reductase	N/A	133%
Complex I→III	NADH-Cyt. C Reductase (Rotenone Sensitive)	97%	15%*
Complex II	Succinate Deyhydrogenase	91%	99%
Complex II	Succinate Q Reductase (TFFA Sensitive)	N/A	105%
Complex II	+Coenzyme Q1 (TFFA Sensitive)	N/A	98%
Complex II→III	Succinate-Cyt. C Reductase (Antimycin Sensitive)	121%	32%*
Complex III	Decylubiquinol-Cyt. C Reductase	N/A	182%
Complex IV	Cytochrome C Oxidase	108%	62%
	Citrate Synthase	100%	100%
	Citrate Synthase	174%	110%

Clinical assessment of electron transport (ETC) in homogenate from frozen muscle biopsy tissue in patient 1 (Baylor Genetics) and patient 2 (Center for inherited disorders of energy metabolism "CIDEM"). All values for ETC enzymes are expressed as %of mean of control samples and normalized by citrate synthase (100%) a mitochondrial marker of mitochondrial hyper proliferation. To indicate whether hyperproliferation was present citrate synthase values expressed as %of mean of control samples is reported in the last row. *results in bold are considered normal-at the low end of the control range per report since a quality control sample which is included in the "Complex I→III" assay is also low at 60% and indicative of an endogenous inhibitor or that the homogenate itself influences the assay.

Table 6.2: Oxphos analysis for patient 1

Substrate/Inhibitors	Significance	Patient 1
Pyruvate + Malate	Basal Respiration (Intact Cells) I→IV	71%
Digitonin	Basal Respiration (Permeabilized Cells)	42.5%
ADP	State 3 Respiration* I→IV	23%
+Glutamate	Glutamate ¹ Stimulation	19%
+Succinate	Complex II→IV, activity	21%
+FCCP	Maximal Respiration	29%
+Rotenone	Isolated Complex II activity	47%
Uncoupled Complex I		15%
+AntimycinA	Non mitochondrial oxidation	233%
+TMPD	Isolated Complex IV	76%
	Citrate Synthase	100%
	Citrate Synthase	120%

Clinical evaluation of oxidative phosphorylation in fibroblast cell line from patient 1 (CIDEM). Respiration is expressed as %of mean of control samples and normalized by citrate synthase (100%) a mitochondrial marker of mitochondrial hyper proliferation. To indicate whether hyperproliferation was present citrate synthase values expressed as %of mean of control samples is reported in the last row.*State 3 Respiration is attained by administration of ADP “active respiration”. 1 Glutamate stimulation would indicate block of Pyruvate or Malate metabolism rather than electrontransport. Values in bold are considered below the control range.

Table 6.3: Comparison of phenotype for patient 1 and 2 vs CODAS syndrome

Symptoms	Patient 1	Patient 2	CODAS
Cerebral			
<i>Hypotonia</i>	+	+	+
<i>Delay in Motor Milestones</i>	+	+	+
<i>Intellectual Disability</i>	+	+	+/-
<i>Developmental Delay</i>	+	+	+
Ocular			
<i>Cataracts</i>	-	-	+
Dental			
<i>Delayed Tooth Eruption</i>	-	-	+/-
Auricular			
<i>Scapha and Helix Dysplasia</i>	-	-	+/-
<i>Conductive/Sensorineural-Hearing Loss</i>	+	-	+/-
Skeletal			
<i>Short Stature</i>	+	?	+/-
<i>Subluxation of Hips</i>	-	-	+/-
<i>Epiphyseal Hypoplasia</i>	-	-	+
<i>Delayed Ossification</i>	-	-	+/-
<i>Vertebral Coronal Clefts</i>	-	-	+/-
<i>Genu Valgus</i>	-	-	+/-
Facies			
<i>Flat</i>	+	?	+/-
<i>Grooved Nasal Tip</i>	-	?	+/-
Other			
<i>Laryngomalacia</i>	+	?	+/-
<i>Umbilical Hernia</i>	-	-	+/-
<i>Cerebellar Hypoplasia</i>	+	+	+/-
<i>Ptosis</i>	+	?	-
<i>Nystagmus</i>	+	+	-
<i>Liver Failure</i>	-	+	-
<i>Regression</i>	-	+	+/-
<i>Vocal Cord Paresis</i>	+	-	+

+/- indicates symptoms were present in some CODAS patients but not all. ? indicates either not assessed or not clear at this time

Patient #2 comes from a consanguineous family of Egyptian descent: the parents are first cousins. The male infant was reportedly normal at birth with normal birthweight and did not develop symptoms until one year of age after getting sick on a trip to Egypt, which caused an acute decompensation and loss of previously attained milestones. He is now three years old, he makes babbling sounds but no longer says specific words and can no longer stand unsupported. He has abnormal eye movements, dystonia and mild dysmorphic features. A feeding tube was placed to ensure sufficient nutrition. MRI revealed cerebellar atrophy, hypomyelination, and abnormal formation of the splenium of the corpus callosum. On cerebral spinal fluid (CSF) he had decreased 5-methyl tetrahydrofolate (10nM, reference range 40-187), a unspecific finding associated with mitochondrial disease. Biochemical testing for aminoacids in plasma revealed moderately elevated valine, isoleucine and leucine suggesting protein catabolism, which is normally seen after prolonged periods of ketosis or starvation. Urine analysis showed metabolites of branched chain amino acids, ethylmalonic acid, 3-methylglutagonic acid and orotic acid indicative of mitochondrial dysfunction. Muscle biopsy was examined by light microscopy and was normal for mitochondrial markers like increased glycogen or ragged red fibers. NADH and ATPase staining was also normal but COX staining was negative in scattered fibers. Electron microscopy was suggestive of mitochondrial disease with unusual mitochondrial inclusions representing osmiophilic, granular deposits within mitochondria as well as large, round, dense inclusions that nearly replace the mitochondria. Muscle tissue was also submitted for electron transport chain (ETC) studies which were reported at the low end of normal for electron flow from complex I through complex III onto cytochrome c and electron flow from complex II through complex III onto cytochrome c.

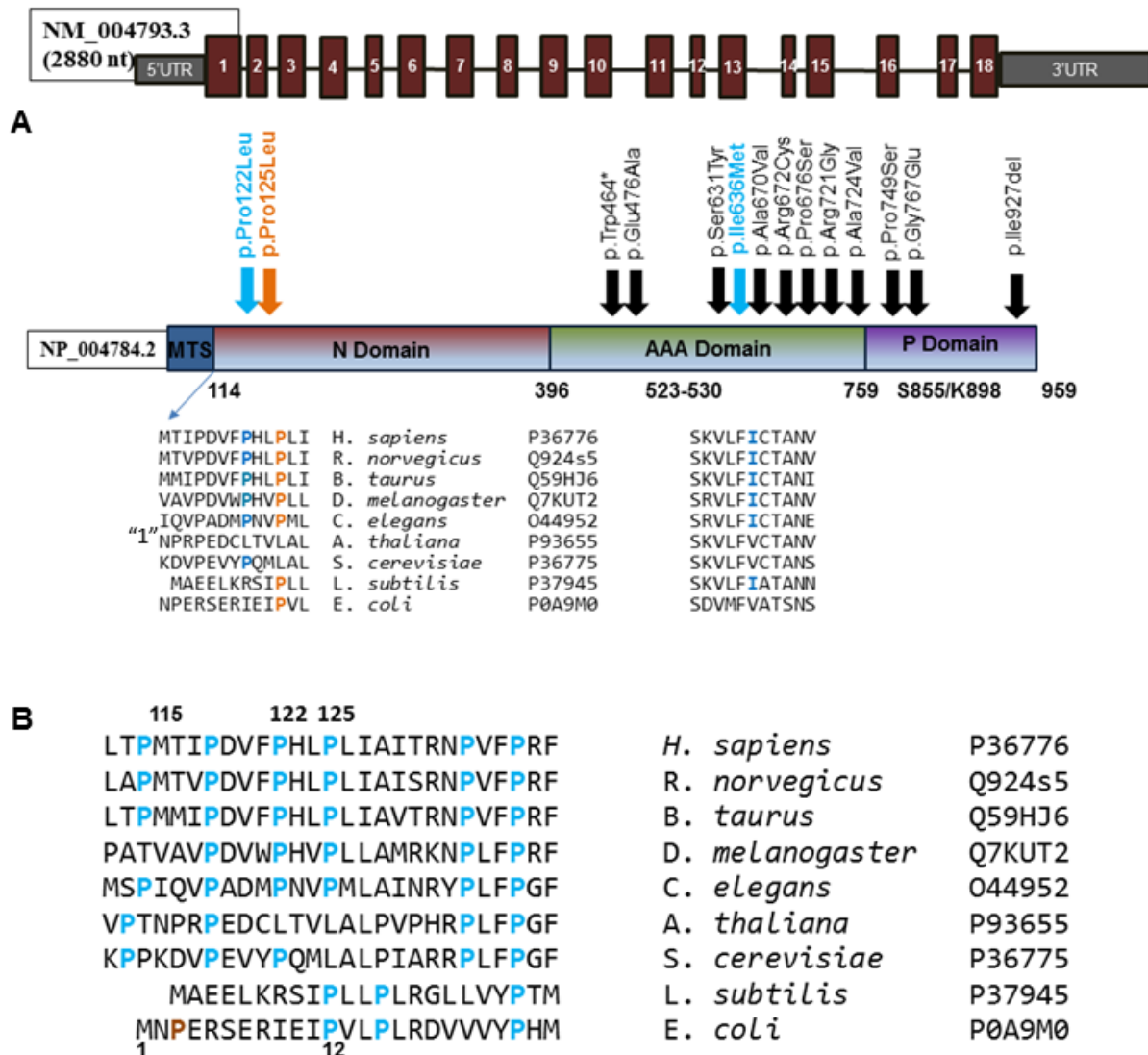
The results did not show a clear defect of but were suggestive of compromised mitochondrial electron transport. The patient also developed liver involvement with elevated transaminases, which precipitated a liver biopsy to assess mitochondrial content via mtDNA copy number in that tissue. Results were indicative of mitochondrial disease with decreased mtDNA copy number at 35% of control. Although this result is considered low, it is not consistent with a primary mtDNA depletion disorder. Additional testing also revealed significantly elevated copper in liver, which usually is suggestive of Wilson's disease, caused by mutations in copper-transporting ATPase 2 (*ATP7B*), but this was ruled out by DNA sequencing. A targeted mitochondrial disease gene testing panel was ordered, which revealed a single heterozygous pathogenic mutation in the methylmalonic aciduria cblC type, with homocystinuria gene (*MMACHC*) which causes cobalamin deficiency and another single heterozygous pathogenic mutation in the urea cycle enzyme carbamoyl phosphate synthetase I (*CPS1*) gene, which most likely explains the observed elevation in orotic acid. Since these are recessive conditions and could not explain the patient's phenotype, whole exome sequencing was ordered. Results showed homozygous novel variants in *LonP1* at position p.Pro125Leu. Since the patient does not have phenotypic overlap with CODAS syndrome patients a clear diagnosis could not be made and the case warranted follow up in the research lab.

6.2.2 Homology modeling for LonP1 correlates mutation location with impact on protein stability and functionality

After the referral was made and families had formally consented to participate in functional studies in the research lab, we first took a closer look at mutation locations and

their correlation with protein function. LonP1 has three key domains as well as a mitochondrial targeting sequence (aa1-114), which is cleaved upon mitochondrial import (Figure 6.1A). The LonP1 N-terminal domain (residue 115-396) is involved in protein recognition and binding. The central ATPase (AAA) domain is responsible for the unfolding of substrates prior to cleavage by the proteolytic domain. This domain is also thought to be involved in mtDNA binding with an active site at position 523-530 (García-Nafria et al. 2010). The serine protease activity of LonP1 is conferred by the P domain (residue 759-959) with a “Serine-Lysine” dyad at positions p.Ser855 and p.Lys898 (Figure 6.1A). Since the cleaved mitochondrial targeting sequence is 114 amino acids long we can see that the patient mutations (p.Pro122Leu) and (p.Pro125Leu) actually represent the 8th and 11th amino acid in the mature protein respectively (Figure 6.1B).

Figure 6.1: LonP1 mutations cluster in the AAA domain



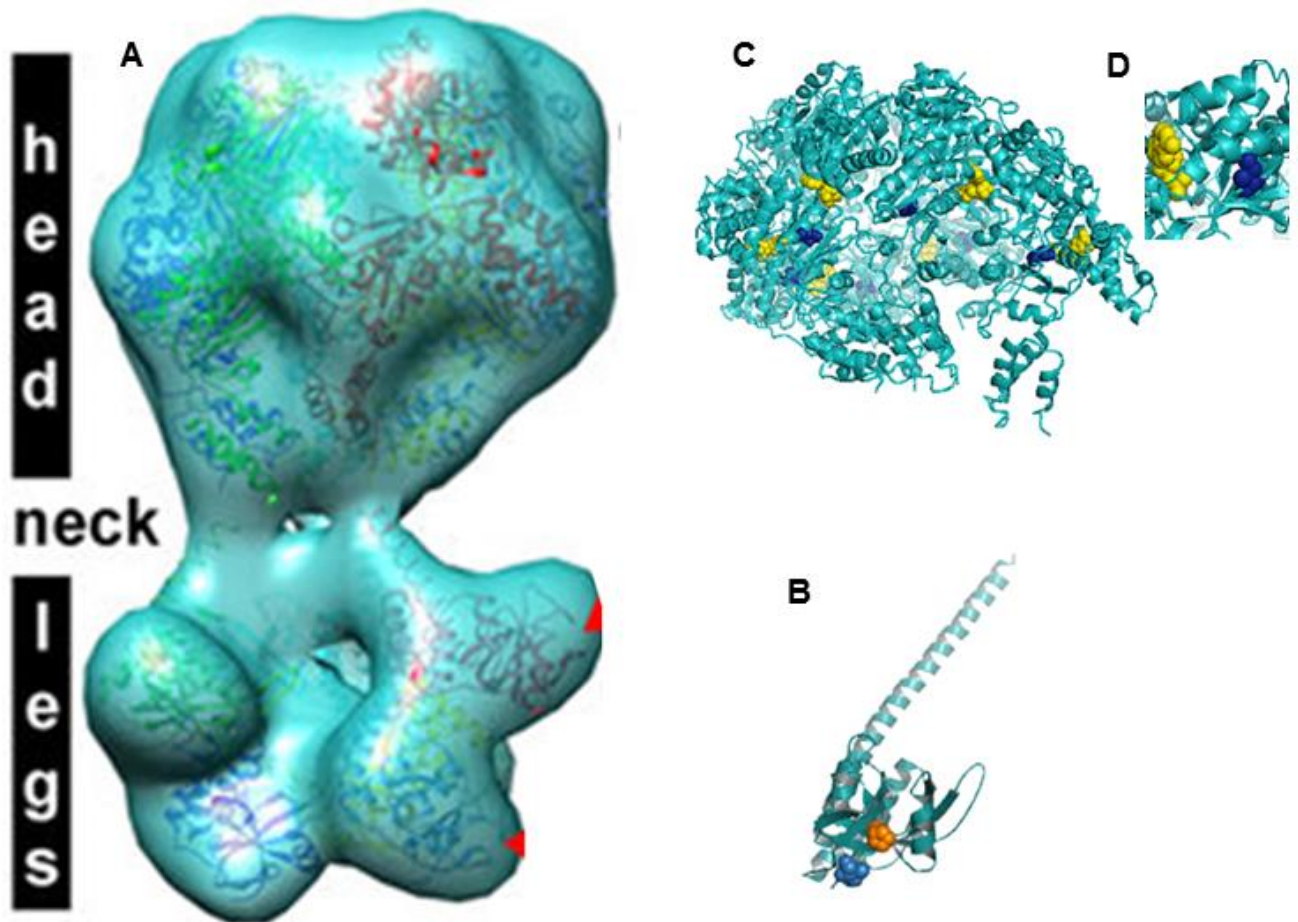
(A) Top: Gene structure of LonP1 coding region NM_004793.3. Bottom: LonP1 mutations and their alignment/conservation between 9 species. Mutations of patient 1 are depicted in blue and mutation of patient 2 is depicted in orange. CODAS mutations are in black font. The active sites of the AAA domain are at position 523-530 and are involved in mtDNA binding. The active sites in the P domain conferring the Serine Lysine protease activity are depicted at residue p. Ser855 and p.Lys898. (B) Homology modeling between 9 species reveals a region of 6 Prolines at the very N terminus of the imported LonP1 Protein. After import and clipping of 114 amino acids, Proline #122 is the eighth amino acid in the chain and Proline # 125 is the 11th amino acid in the mature protein chain.

A recent report reveals a 3D model structure of LonP1 using cryo-electron microscopy with a head, neck and legs domain (Kereïche et al. 2016) (Figure 6.2A). The study confirms the hexameric barrel conformation of the ATPase and P domains (head) but

reveals a different conformation of the N-domain (legs), which is arranged as a trimer of dimers. Currently there is no crystal structure for the N-terminal domain of human LonP1 and we therefore mapped our patient mutations to the structure of *E. coli* Lon protease (EcLon). The region reveals a highly conserved group of prolines, which provide rigidity to the N-terminus of the molecule and most likely facilitate the entry of targets through an open state with fewer hydrogen bonds. The high hydrophobicity of the N-terminus is suggestive of direct interaction with the hydrophobic degrons of target proteins. Residue p.Pro125 (mutation location of patient 2) is conserved in that organism and corresponds to p.Pro12Leu of *EcLon*. Mutation modeling suggests the loss of van der Waals forces between Pro12 and its neighbor Ile11. Although it might be argued that these residues are so close to the N terminus in a disorganized region and may not be of structural importance, it has been shown that a deletion construct of the first 154 amino acids of hLonP1 results in loss of proteolytic activity (Kereiche et al. 2016). Homology alignment for the second mutation in patient 1, p.Ile636, reveals a highly conserved residue through *Bacillus subtilis*. Although in budding yeast the residue itself is not conserved, it is replaced by isoleucine, an amino acid with very similar properties. Homology modeling for the p.Ile636Met mutation was done using the crystal structure of Lon from *B. subtilis*. P.Ile636 in hLonP1 corresponds to p.Ile474 in *B. subtilis* Lon. The model demonstrates the interaction of Ile636 with the ligand ADP. The residue is located in a beta sheet immediately across from the ATP binding site. In a study by Duman et al., the crystal structure of *EcLon* reveals the ATP binding site to be directly at the interface between the six Lon monomers (Duman, Lowe 2010). p.Ile636Met may therefore have an effect on

ATP binding and additionally could affect the tertiary structure of the protein. It may therefore be possible that the p.Ile636M variant interrupts oligomerization of the protein.

Figure 6.2: Modeling of patient mutations onto LonP protein structure



(A) 3D model of full length human LonP1 adapted from Kereiche et al 2016 and reproduced under creative commons license 4.0: <https://creativecommons.org/licenses/by/4.0/>. The N terminal domain is overlapping with the legs and neck and the ATPase and P domain is overlapping with the head. (B) Model of the N domain of LonP1 with location of mutation for patient 1 labeled in blue and patient 2 labeled in orange using *E.coli* crystal structure PDB 3ljc. (C) Shown is a model of the ATPase domain from the side with ATP labeled in yellow and Ile636 in blue using the *B. subtilis* crystal structure PDB 3M6A. (D) Zoomed in look at the interaction between Ile636 and ATP.

To further interrogate the variants and their potential to be disease causing, variants were submitted to Annovar online (wAnnovar) for pathogenicity prediction based on 7 algorithms interrogating homology models. The p.Pro122Leu and p.Pro125Leu variants

found in patient 1 and 2 respectively are predicted pathogenic in five and six out of seven algorithms, respectively. The Ile636Met variant found in patient 1 is predicted pathogenic by all 7 algorithms (Table 6.4). Neither variant has been reported in the Exac database interrogating 120,000 alleles.

Table 6.4: Insilico pathogenicity prediction for *LonP1* variants

<i>In silico</i> Pathogenicity Prediction Tool	p. Pro122Leu 5/7 Damaging	p. Pro125Leu 6/7 Damaging	p.Ile636Met 7/7 Damaging
¹ PolyPhen-2 HDIV	Probably Damaging (0.997)	Probably Damaging (1.000)	Probably Damaging (0.999)
² PolyPhen-2 HVAR	Probably Damaging (0.942)	Probably Damaging (0.999)	Probably Damaging (0.997)
³ LRT	Deleterious (1.000)	Deleterious (1.000)	Deleterious (1.000)
⁴ Mutation Taster	Disease Causing (1.000)	Disease Causing (1.000)	Disease Causing (1.000)
⁵ SIFT	Deleterious (0)	Deleterious (0)	Deleterious (0)
⁶ FATHMM ⁴	Tolerated (0.325)	Tolerated (0.180)	Damaging (0.542)
⁷ RadialSVM	Tolerated (-1.066)	Damaging (0.022)	Deleterious (1.075)
⁸ EXAC	0/120,000	0/120,000	0/120,000

PolyPhen-2HDIV Scores range from 0-0.452 (Benign) 0.453-0.956 (possibly damaging) 0.957 -1 (probably damaging) PolyPhen-2HVAR scores range from 0-0.446 (Benign) 0.447-0.909 (possibly damaging) 0.909 -1 (probably damaging) LRT scores range from 0-1; > score signifies that the codon is more constrained and variant is more likely to be deleterious. Mutation Taster scores denote the certainty of the prediction:1-p value. Sift scores >0.05 are Tolerated, Scores <0.05 are Deleterious. FATHMM scores range from 0-1: higher score is more likely deleterious. Radial SVM/Meta SVM scores use odds ratios ExomeAggregation Consortium ExAC indicates frequency of variants interrogated on 10/17/2016

6.2.3 Correlation of mutation location with phenotype reveal all mutations associated with CODAS syndrome cluster in the ATPase and P domain of *LonP1*

To further assess whether the *LonP1* mutations in our patients could explain their disease we took a closer look at the clinical manifestations in CODAS syndrome and whether any

mild or unusual cases had been observed. A report by Strauss et al., delineates four different mutations in ten affected individuals from Amish (Swiss), Mennonite (German) and mixed European descent. All four mutations cluster in the ATPase domain close to the ATP binding site. Eight patients from the old order Amish community were homozygous for the p.Arg721Gly mutation. All of them had severe abnormalities of the vocal chord and three died from a laryngeal obstruction during the first days of life and one died early due to pneumonia. The four surviving children developed cataracts at 2 months, had varying levels of hearing loss and skeletal dysplasia. Interestingly none of the children had increased lactate, alanine or abnormal TCA cycle intermediates. The second report on CODAS syndrome comprises seven patients from five families and eight causal mutations, none of them overlapping with the previous report. Again mutations clustered in the ATPase domain, with a few are in the P domain (Table 6.2). All patients had early onset cataract and spondylo-epiphyseal dysplasia but only one of the patients had sensorineural hearing loss and three were without cognitive involvement, which demonstrates some phenotypic heterogeneity. Since this report covered individuals with varying ethnic backgrounds rather than from genetic isolates it is not surprising that the phenotypes associated with LonP1 mutations varied as compared to the report by Strauss et al. Further more ascertainment in both reports was for CODAS syndrome patients with a focus on skeletal dysplasia clinics in the second report. Our patients do not fulfil the criteria for CODAS syndrome which may be attributed to location of the mutation in the protein since both patients have mutations in the N-terminal domain of LONP1. Patient #1 who shares hypotonia, swallowing difficulties, vocal chord abnormalities and sensorineural hearing loss with CODAS patients is compound heterozygous for one

mutation in the N domain but the second mutation is in the ATPase domain clustering with the CODAS mutations. The second patient who does not have any mutations in the ATPase or P domain does not have any overlap with classical CODAS syndrome but fits the mitochondrial disease etiology of sudden onset after illness with subsequent regression and metabolic abnormalities. We will have to see once additional patients with mutations in LonP1 are ascertained whether this phenotype genotype correlation will be consistent.

6.2.4 Modulating nutrient source in fibroblast culture is an important tool as to observe cellular abnormalities affecting mitochondrial metabolism

To assess the effect of LonP1 mutations on mitochondrial respiration we established fibroblast cultures from patient 1 and her parents. We also obtained fibroblasts for patient 2 from our collaborators. We investigated the impact of culture media and nutrient availability on respiration in glucose vs. galactose experiments with and without the addition of pyruvate. These assays interrogate two different phenomena of cellular metabolism. Respiration deficient cells, much like cancer cells, tend to shift their ATP production from mitochondrial complex V to glycolysis. The galactose challenge prevents the cell from relying heavily on glucose since galactose needs to be converted to glucose via the Leloir pathway before the cell can use it for aerobic glycolysis. This process is inefficient and involves steps with slow enzyme kinetics. Also, the expression of enzymes of the Leloir pathway is cell type specific and is predominantly observed in liver cells. Additionally hexose transporters of the cytoplasmic membrane have a much lower affinity for galactose than glucose and may not be expressed at all in some cell types (Colville et al. 1993, Elkalaf et al. 2013, Heidenreich et al. 1993). Cells cultured in galactose,

therefore, depend on respiration rather than aerobic glycolysis to generate enough ATP to support cellular processes including ATP dependent proteolysis via AAA proteases like LonP1. Thus, in normal healthy cells, respiration in galactose would be increased over glucose, while in dysfunctional respiratory deficient cells respiration would be decreased as compared to normal controls. The pyruvate challenge, on the other hand, interrogates a different phenomenon of cellular metabolism: The addition of pyruvate results in oxidation of superfluous NADH to NAD, via lactate dehydrogenase to restore the NADH/NAD balance (Fujii et al. 2014). Respiration deficient cells tend to accumulate NADH since its electrons can no longer be donated to mitochondrial complex I and increased NADH levels cause inhibition of many metabolic pathways (Baugh et al. 1967). In most cases pyruvate is therefore beneficial in respiration deficient cells. Thus normal healthy cells, will display normal respiration even when cultured without pyruvate while dysfunctional respiratory deficient cells are stressed when cultured without pyruvate. Recently it has been shown that the addition of pyruvate significantly lowers the availability of glutamate and alpha ketoglutarate in the mitochondrial matrix (Chen et al. 2016). The addition of pyruvate therefore presents a challenge for cells that heavily depend on the oxidation of pyruvate rather than glucose or fatty acids.

6.2.5 Patient cell lines react differently to the absence of pyruvate in culture medium depending on carbon source but respiration is abnormal in both

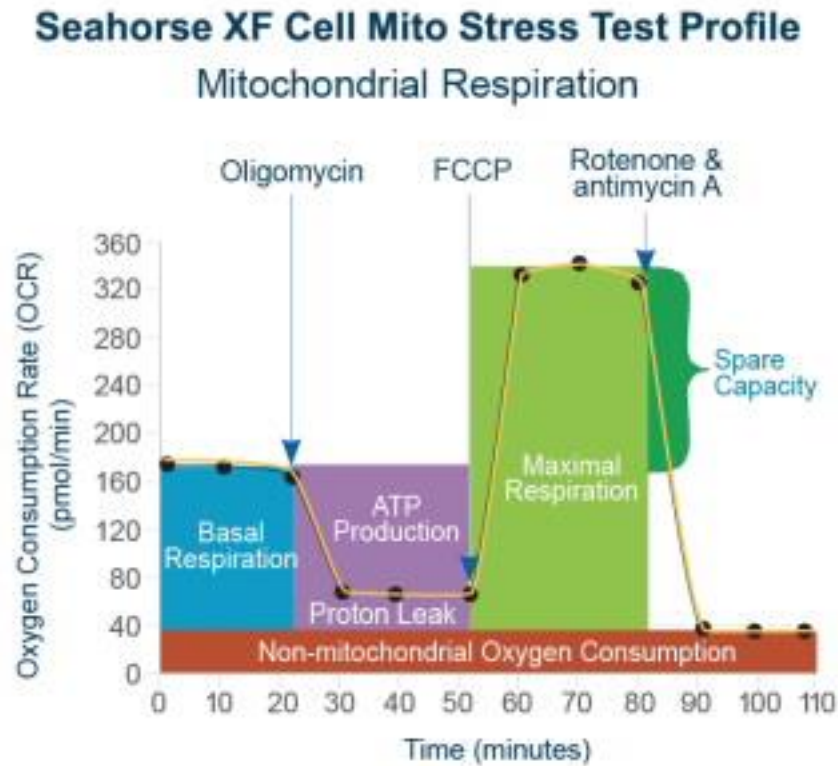
We assessed mitochondrial respiration using the Seahorse Biosciences “mitostress kit” (Figure 6.3). In this assay, several basal respiration (BR) measurements are made, before the inhibitor oligomycin is added as to block oxidative phosphorylation at mitochondrial

complex V. The remaining oxygen consumption reflects proton leak plus non-mitochondrial respiration. Subsequently the uncoupler FCCP is added and maximal respiration (MR) is assessed. Finally the last injection contains the inhibitors rotenone and antimycin, which block mitochondrial complex I and III, respectively. Any remaining oxygen consumption comprises solely non-mitochondrial respiration. The ability of the respiratory chain to accommodate increased energy demand is reflected by the parameter “spare respiratory capacity” (SRC), which is calculated by subtracting basal respiration from maximal respiration (Figure 6.3).

Mitostress assay results show a BR of 120% of control for both patient cell lines when grown in glucose plus pyruvate and a BR of only 80% of control for patient cells cultured in glucose without pyruvate (Figure 6.4A). This indicates that cell lines from LonP1 patients can maintain levels of respiration above normal during basal conditions, as long as pyruvate had been present during culture, but are respiration deficient when cultured without pyruvate. Both patient cell lines cultured in glucose plus pyruvate are also able to attain MR levels comparable to normal control MR (Figure 6.4B), but when cultured without pyruvate MR drops for patient 2 cells to a level of 70% of control. Cells from patient 1 however can sustained normal MR as compared to control even without pyruvate in the culture media (Figure 6.4B). When calculating SRC it was interesting to find that the parameter is above control levels for cells from patient 1, when cultured in glucose without pyruvate while this is not the case for cells from patient 2 (Figure 6.4C). This finding indicates that cells from patient 1 are better adapted to respond to high energy needs, as simulated by the addition of FCCP, when having been cultured without pyruvate. Cells from patient 2, which react with decreased SRC after having been cultured

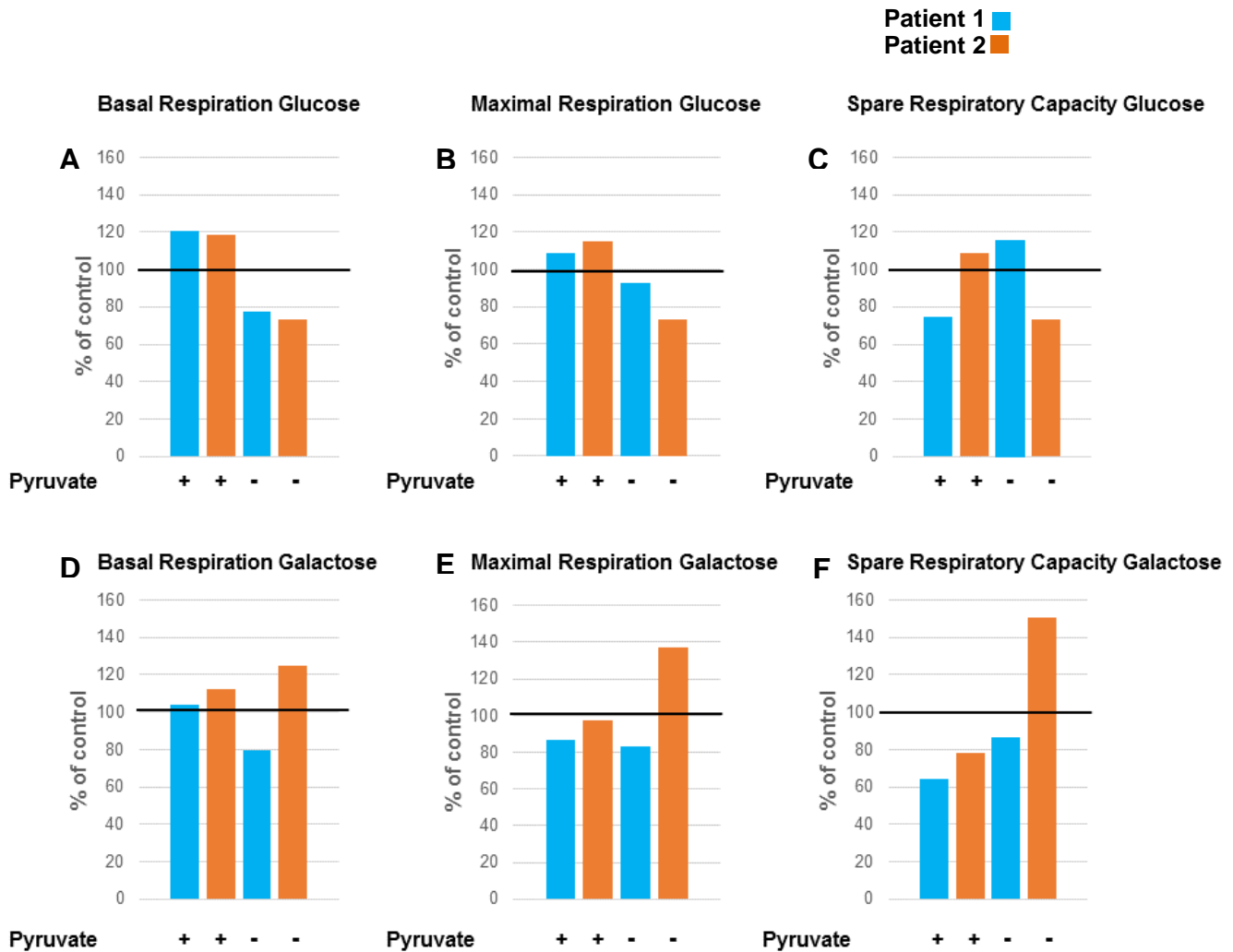
without pyruvate do not show a positive effect during high energy demand due to pyruvate starvation. When we repeat the same experiments in galactose (Fig 6.4D-F), we observe increased SRC for patient 2 in galactose without pyruvate but not for cells from patient 1 (Figure 6.4F). This indicates that in galactose, when cells rely heavily on respiration over aerobic glycolysis, cells from patient 2 have adapted more readily to respond to increased energy demand when pyruvate is withheld. These combined SRC results most likely indicate that the different LonP1 mutations have different downstream effects on metabolism in fibroblasts from the two patients.

Figure 6.3: Seahorse XF Cell Mito Stress Test allows for assessment of spare respiratory capacity



Shown is a trace of mitochondrial respiration generated by sequential application of mitochondrial inhibitors provided in the Seahorse XF Cell Mito Stress Test (Seahorse Bioscience.com). Basal respiration is calculated by subtracting non-mitochondrial oxygen consumption from resting rate. ATP production is the amount of respiration coupled to oxidative phosphorylation and calculated by subtracting proton leak from basal respiration. Maximal respiration is achieved via the uncoupler FCCP, which allows protons to flow back into the matrix through an impaired inner membrane. The spare respiratory capacity represents the cell's ability to react to increased energy need. It is calculated by subtracting non-mitochondrial oxygen consumption from maximal respiration.

Figure 6.4: Patient cell lines have decreased spare respiratory capacity depending on availability of pyruvate



(A-F) Healthy control and patient cells were assayed using a 24 well format using the Seahorse bioanalyzer. Inhibitors of the mitochondrial respiratory chain were injected sequentially (Oligomycin, FCCP, AntimycinA+Rotenone) using the “Seahorse Mitostress” assay kit. The first two bars in each graph represent a single assay with 6 replicates per cell line (wells) from cells cultured with pyruvate. Bar three and four represent a single assay with 6 replicates per cell line (wells) from cells cultured without pyruvate. Normalization was performed by adjusting for total protein. Relative respiration is expressed as % of normal control from cells plated on the same assay plate. (A-C) BR, MR and SRC from cells cultured in glucose. (D-F) BR, MR and SRC from cells cultured in galactose.

6.2.6 Patient cell lines are more susceptible to stress via exposure to antibiotic/antimycotic than control cells

We also wanted to test the effects of cellular stress on the LonP1 patient cell lines using the Seahorse assay. For this we chose to add antibiotic/antimycotic to glucose as well as galactose culture media. In this case both media were supplemented with pyruvate, thus we did not test the effect of pyruvate withdrawal on cells exposed to antibiotic. We specifically used the common tissue culture additive “Anti-Anti” (antibiotic-antimycotic), which contains penicillin (beta-lactam antibiotic), streptomycin (aminoglycoside antibiotic), and Amphotericin B (antifungal – binds to ergosterol). All three agents have previously been shown to increase oxidative stress in addition to the well-known inhibition of translation of mtDNA encoded proteins associated with aminoglycosides (Zholudeva et al. 2015; Blatzer et al. 2015; Kohanski et al. 2007).

Increased generation of ROS has been observed in conjunction with antibiotic use in mammalian culture, which inhibited respiration as much as 40%. The negative effects of the antibiotic could be ameliorated by co-administration of the antioxidant N-acetylcysteine (Kalghatgi et al. 2013). We postulated that LonP1 dysfunction would render patient cells more susceptible to oxidative stress generated by exposure to antibiotic/antimycotic as compared to control. We speculated that such an effect would result in decreased SRC. We also anticipated that this effect would be more severe if cells were pushed to generate ATP via respiration rather than aerobic glycolysis by the use of galactose media.

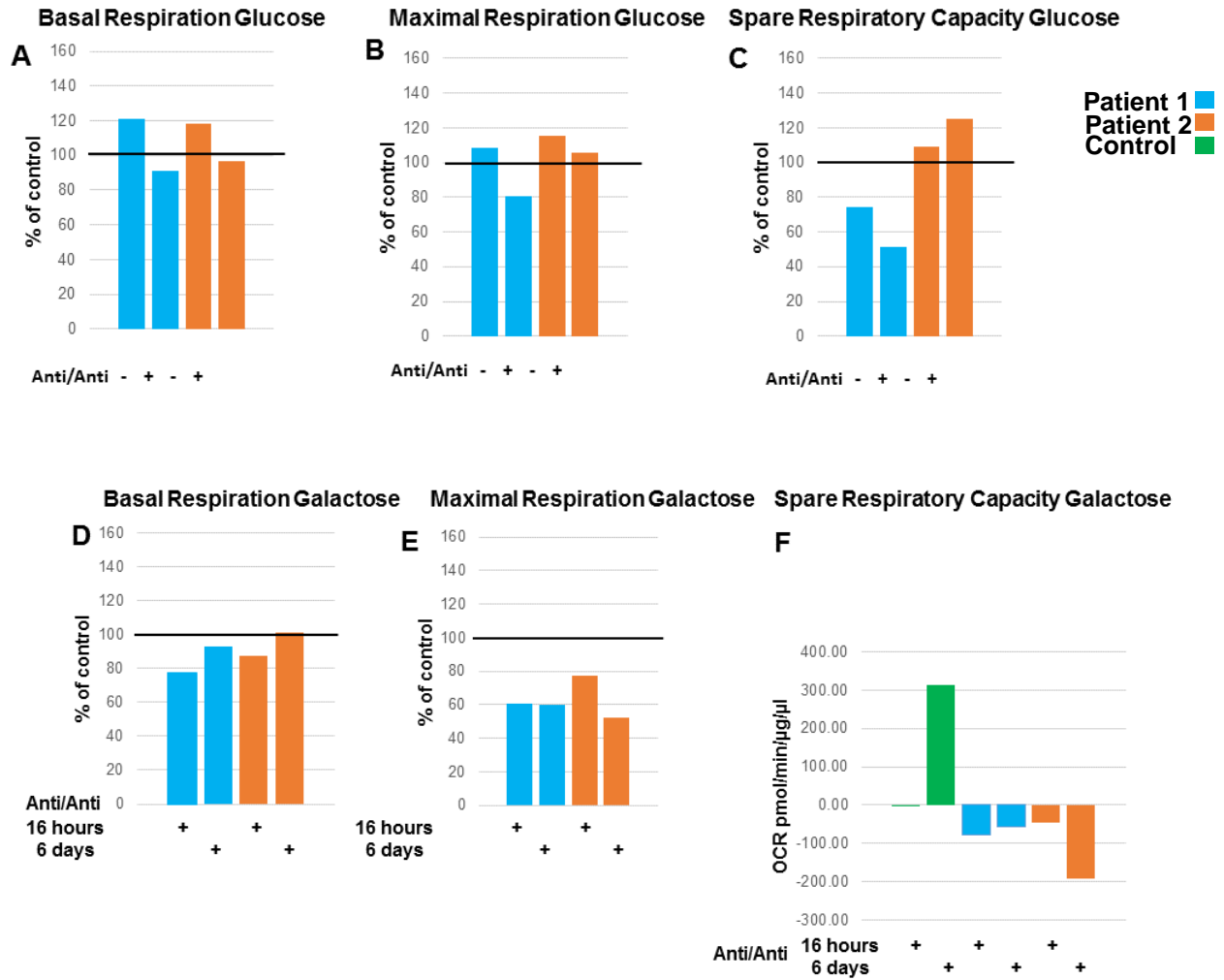
For cells from patient 1 we observed the expected result of increased sensitivity to stress with decreased BR, MR and SRC after addition of antibiotic/antimycotic to the

glucose media. BR was reduced from 120% of control without antibiotic to 90% of control with antibiotic/antimycotic, MR decreased from 110% of control to 80% of control and SRC decreased from 75% of control to 50% of control with antibiotic (Figure 6.5A-C). This indicates that antibiotic/antimycotic exposure negatively affects respiration in cells from patient 1 both under basal conditions as well as during high energy demand conditions. We were however surprised to find that the use of antibiotic in glucose media actually increased SRC in cells from patient 2 (Figure 6.5C). This suggests that the exposure to antibiotic/antimycotic ameliorated cellular defects caused by dysfunctional LonP1 in cells from patient 2 (Figure 6.5C).

We then repeated the antibiotic/antimycotic challenge in galactose media. We previously had added antibiotic to the culture media for 6 days before plating cells for the Seahorse assay. Since we were concerned that cells may not survive the additional stress of antibiotic/antimycotic in galactose media we measured respiration after only 16 hours of exposure and observed drastic effects on SRC in both patients (Figure 6.5F). In fact, MR in galactose plus antibiotics was lower than the BR and thus the SRC could not be calculated since it would be negative. This was also true for the control cell line. Brand and Nicholls document in their hallmark paper: "Assessing mitochondrial dysfunction in cells" that this is a common finding associated with the combination of the galactose challenge and additional exposure to stress (Brand, Nicholls 2011). In these situations, in order to assess SRC, oligomycin and FCCP need to be administered in two separate assays (Brand, Nicholls 2011), which will be performed in our future studies. We therefore wanted to find out whether cell lines in galactose plus antibiotic/antimycotic could recover from cellular stress over time or whether the inhibitory effect of the antibiotic on respiration

was permanent. We therefore assessed respiration again after the cells had been exposed to antibiotic/antimycotic for 6 days in galactose culture (Figure 6.5D-F). Indeed control cells were able to recover SRC after prolonged culture in galactose plus antibiotic/antimycotic while patient cells were not (Figure 6.5F). To better demonstrate these results we present data for SRC in Figure 6.5F not as percent of control but as normalized oxygen consumption. This finding may indicate that normal control cells can upregulate metabolic pathways or defense mechanisms to regain sufficient respiration while cells from LonP1 patients cannot.

Figure 6.5: Patient cell lines have decreased spare respiratory capacity depending on carbon source in response to addition of Antibiotic/Antimycotic



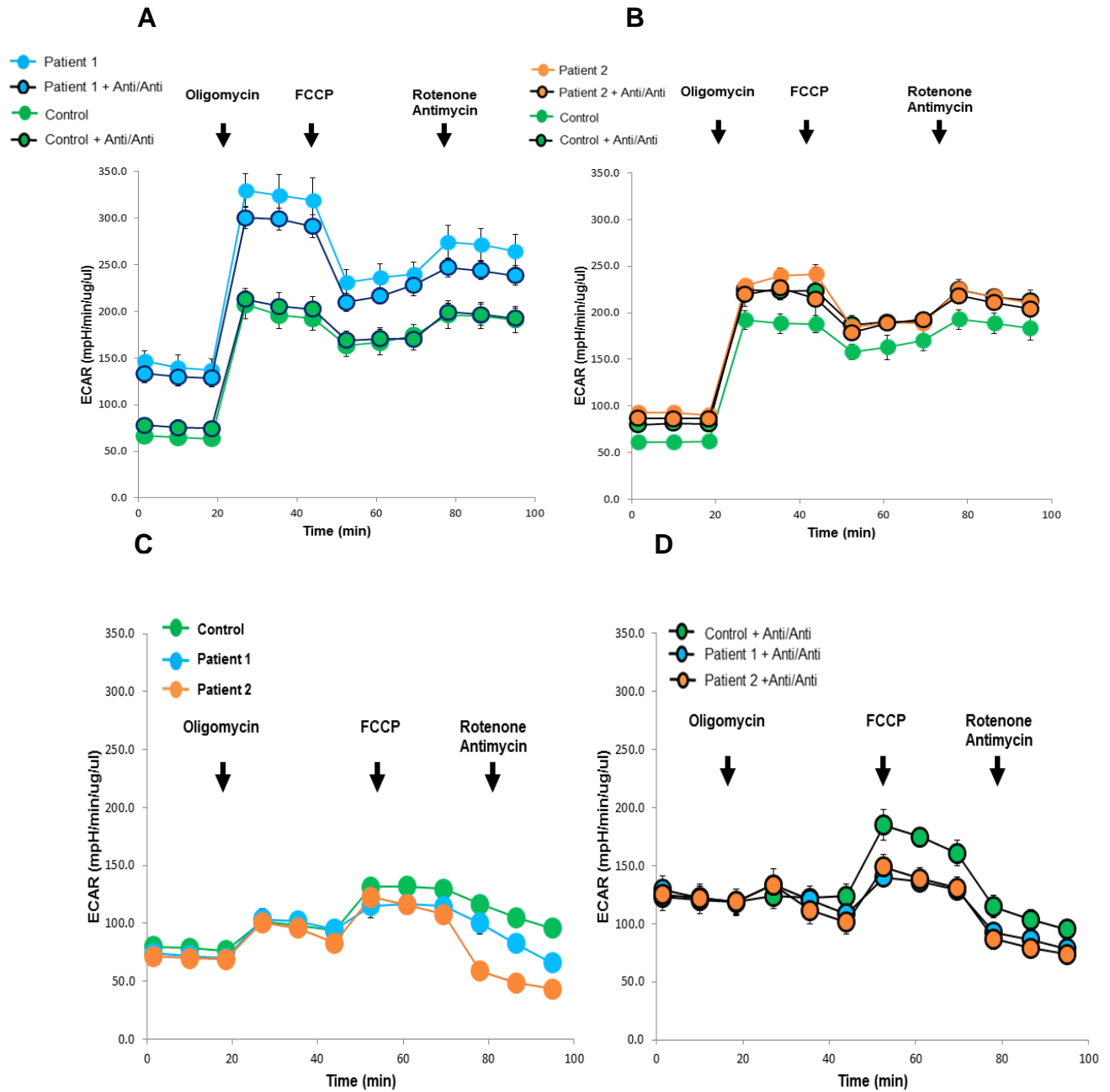
(A-F) Cell lines from patient 1 and 2 were cultured in media without and with antibiotic/antimycotic. (A-C) BR, MR and SRC from cells cultured in glucose. (C) Spare respiratory capacity was calculated by subtracting basal respiration from maximal respiration expressed as % of control. (D-F) BR, MR and SRC from cells cultured in galactose. Experiment #1 (first and third bar) represents exposure to antibiotic/antimycotic for 16 hours and experiment #2 (second and fourth bar) represents exposure for 6 days. (F) Spare respiratory capacity was calculated by subtracting basal respiration from maximal respiration and is negative for patient cell lines since BR>MR. The value is therefore not expressed as % of control.

6.2.7 The modulation of extracellular acidification rate (ECAR) in patient cell lines is dependent on culture media

ECAR (extracellular acidification rate) represents the change in pH in the assay medium during a Seahorse run. The first “Mitostress” assay inhibitor oligomycin inhibits the mitochondrial ATP synthase and ECAR grows to its maximal rate to generate ATP via glycolysis and gives information about the glycolytic capacity of the cell. ECAR therefore represents the rate of generation of lactate via lactate dehydrogenase from pyruvate when respiration is disrupted or when oxygen tension is low. The expectation for respiration deficient cells is therefore to see increased ECAR as a compensatory mechanism as to ensure sufficient ATP generation.

As expected results show elevated ECAR for cells from patient 1 when cultured in glucose compared to control (Figure 6.6A), indicating that the cells rely more heavily on ATP generation via aerobic glycolysis than respiration. Cells from patient 2 show similar ECAR as compared to control, indicating that the cells do not use more aerobic glycolysis when cultured in glucose (Figure 6B). In both patient cell lines ECAR is not affected by the use of antibiotic (Figure 6.6A and B). When cells are cultured in galactose (Figure 6.6C and D), ECAR is generally lower than in glucose and this is true for all three cell lines. Also since patient as well as control cell lines do not show any glycolytic reserve in response to administration of oligomycin in galactose we are not sure if cells are able to metabolize galactose at all and should repeat the assay with increased galactose concentration.

Figure 6.6: Extracellular Acidification Rate (ECAR) is differently modulated in patient 1 and 2



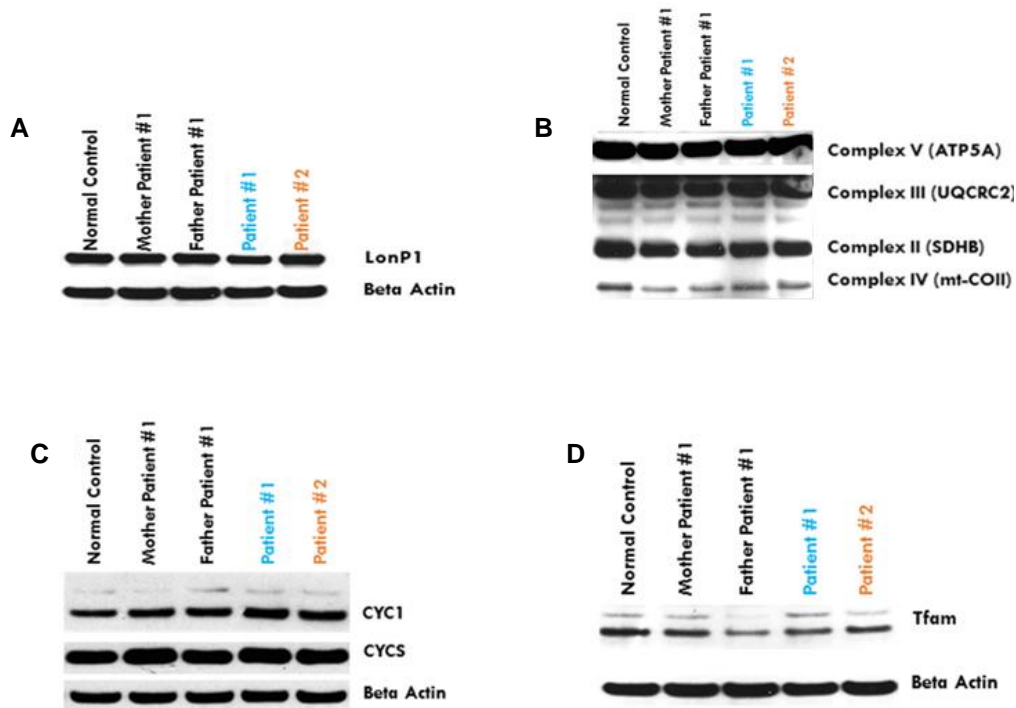
(A-B) Cells were run on a 24 well Seahorse bioanalyzer using the “Mitostress Kit” and pH change in response to inhibitors was assessed. At time point 20 minutes oligomycin is injected to block mitochondrial complex V and increase in ECAR indicates glycolytic capacity. (A) ECAR for cells from patient 1 and control cultured in glucose media with and without antibiotic. (B) ECAR for cells from patient 2 and control cultured in glucose media with and without antibiotic. (C) ECAR for both patient cell lines cultured in galactose without antibiotic. (D) ECAR for both patient cell lines cultured in galactose with antibiotic.

6.2.8 SDS PAGE reveals decreased levels of LonP1 in patient 1 and normal levels for patient 2. Protein levels of LonP1 targets are not decreased

To assess whether the LonP1 mutations in our patients have affected the levels of LonP1 protein itself or of LonP1 targets we performed SDS PAGE on lysates from patient fibroblasts, which were grown in glucose media containing pyruvate and antibiotic/antimycotic. We will repeat these experiments in the other media conditions described in Figures 6.4 and 6.5 in the future. We repeatedly found that patient 1 has decreased LonP1 levels, both compared to normal controls and compared with other mitochondrial disease patient samples (data not shown). In contrast SDS PAGE for patient 2 showed normal LonP1 protein levels (Figure 6.7A). We also interrogated protein levels of several LonP1 targets including ETC subunits ATP5A, UQCRC22, SDHB and mt-COII, which were assessed using an antibody cocktail (Figure 6.7B). None of these targets displayed any change in protein levels. Lymphoblastoid cell lines from CODAS patients had demonstrated an absence of mt-COII via western blot due to aggregated mt-COII protein complexes, but this was not the case in our patient cell lines. We were also particularly interested in the nuclear-encoded mitochondrial complex III subunit UQCRC22, since we had seen a block in electron transport through mitochondrial complex III for both patients in either ETC or Oxphos assays (Table 6.1 and 6.2). Since UQCRC22 is intrinsically sensitive to assembly defects of mitochondrial complex III we were surprised to find normal levels for both patients for this protein (Figure 6.7B). We then assessed the protein level for Ubiquinol-Cytochrome-C Reductase (CYC1), the mitochondrial complex III subunit, which catalyzes the last step in electron transfer from mitochondrial complex III (Figure 6.7C). Since CYC1 was normal in both patients and

since CYC1 donates electron to the electron carrier cytochrome C (CYCS) we also assessed CYCS protein levels, which were normal as well (Figure 6.7C). Since clinical findings for patient 2 were suggestive of mtDNA depletion in liver (Clinical description patient 2) and since TFAM is a direct target of LonP1 and associated with mitochondrial depletion syndrome, we also interrogated TFAM via western blot. We found that protein levels were normal other than for the father of patient, 1 whose cells had low levels of TFAM, which we cannot explain at this time (Figure 6.7D).

Figure 6.7: Western blot for LonP1 and its targets reveals low levels of LonP1 for patient 1



(A) SDS Page of protein lysates from fibroblasts of patients 1, 2, and age matched control as well as parental samples from patient 1. Cells were cultured in DMEM containing 15mM glucose and 1mM Pyruvate supplemented with 15%FBS, 1x non essential amino acids and 1xAntibiotic-Antimycotic. 30ug of protein lysates were run in each lane with beta actin as a loading control. (A) Anti-Lonp1 antibody hybridization reveals decreased LonP1 levels for patient 1. (B) Oxphos antibody cocktail reveals normal results for both patients including mt-COII. (C) Due to a block in oxphos at mitochondrial respiratory complex III seen previously we tested the protein levels of CYCS and CYC1 which were normal. (D) TFAM levels are low for the father of patient 1 with uncertain significance.

6.2.9 mtDNA copy number is differentially regulated in cells from patient 1

vs cells from patient 2 in glucose vs galactose media

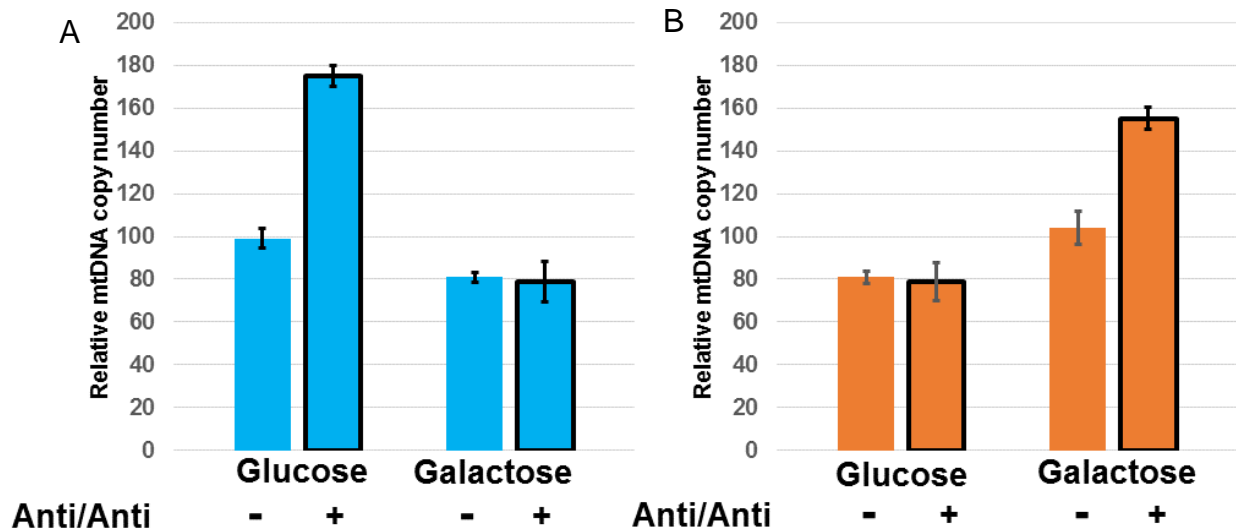
Clinical studies in liver sample from patient 2 demonstrated mtDNA depletion at 35% of control while mtDNA copy number for patient 1 was normal in muscle. Citrate synthase, a marker of mitochondrial content was assessed in muscle from both patients and was increased, at 174% of control for patient 1 suggesting mitochondrial proliferation, while this marker was normal for patient 2 (Table 6.1). We therefore suspected that binding

properties of LonP1 to mtDNA were differentially affected by the patient mutations and interrogated mtDNA copy number in patient fibroblast. Since mtDNA copy number is also a direct indicator of a regulatory response to levels of respiration and mitochondrial stress, we assessed mtDNA copy number in fibroblasts from both patients in glucose and galactose media with and without antibiotic; pyruvate was always present in these media (Giordano et al. 2014). We hypothesized that mtDNA copy number would be increased in cells from patient 1 as suggested by the ETC findings (mitochondrial proliferation) (Table 6.1), a response often seen in respiration deficient cells. We also hypothesized that mtDNA would be decreased in cells from patient 2 (mtDNA depletion) as suggested by clinical findings in liver tissue. Such an mtDNA depletion would then suggest a defect in mtDNA replication.

As expected we found that cells from patient 1 can upregulate mtDNA copy number in response to stress as long as nutrient conditions are favorable (Figure 6.8A and 6.8B). This is indicated by an almost two fold increase in mtDNA copy number in cells from patient 1 in response to the use of antibiotic (Figure 6.8A). In galactose medium the cells no longer can upregulate mtDNA copy number in response to stress caused by the culturing with antibiotic/antimycotic (Figure 6.8A). Cells from patient 2 on the other hand show no change in mtDNA copy number in response to culture in glucose medium with antibiotic (Figure 6.8B). These findings are consistent with results shown previously in Figure 6.5, which indicated that antibiotic use in glucose medium did not cause compromised respiration for cells from patient 2. In galactose medium however cells from patient 2 had shown compromised respiration in response to addition of antibiotic/antimycotic. As expected mtDNA copy number was therefore found increased

in cells from patient 2 in galactose plus antibiotic/antibiotic (Figure 6.8B). At this time we can not explain why cells from patient 1 are not able to respond with mitochondrial proliferation to stress in galactose medium while cells from patient 2 can.

Figure 6.8: Relative mtDNA copy number is modulated by the use of antibiotics in cell culture



(A-B) shown is relative mtDNA copy number from both patient cell lines cultured in glucose vs galactose without and with the use of antibiotic and compared to mtDNA levels of normal control. Quantitative PCR was performed using beta 2 microglobulin as the nuclear reference gene and mt-ND1 as the mtDNA encoded gene to generate mtDNA/nDNA ratio via delta delta CT as previously described (Bonnen et al. 2013) Black frames indicate culture in media with antibiotic/antimycotic.

6.2.10 Cells from patient 1 show aggregated nucleoids in cells cultured in glucose media without antibiotic

LonP1 plays an important role as a mtDNA binding protein and, together with TFAM, is a major component of the mitochondrial nucleoid, a mtDNA protein cluster, which affords packaging of mtDNA (Venkatesh et al. 2012). Aggregated nucleoids have been observed in mouse models with abnormalities in mitochondrial fission protein Drp1 (Ishihara et al. 2015). Aggregated nucleoids have also recently been observed in fibroblasts from a patient with mutations in *TFAM*, leading to low levels of TFAM protein and mtDNA

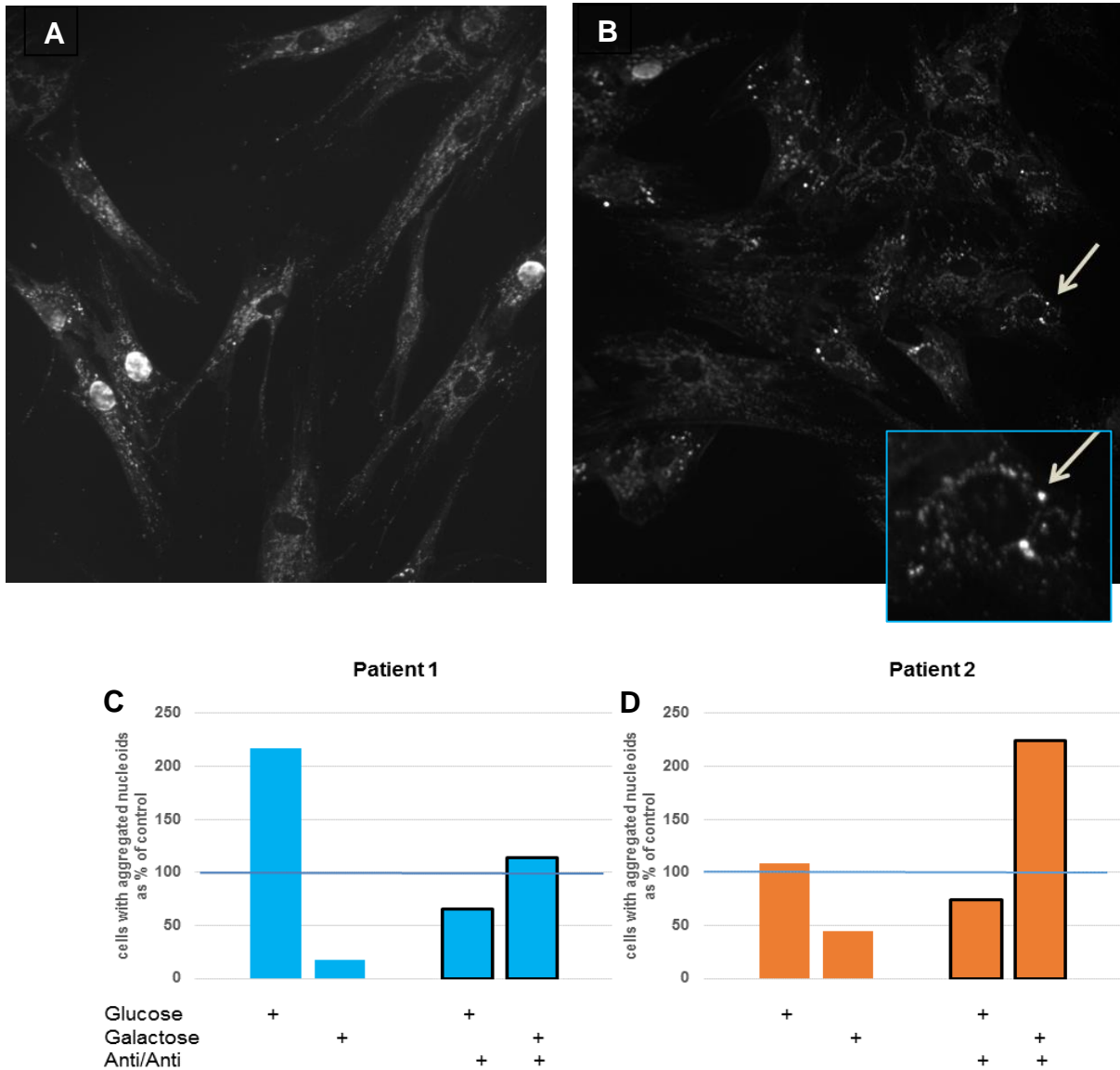
depletion (Stiles et al. 2016). In vitro experiments with recombinant TFAM have shown that abnormal nucleoid formation may be due to dysregulated mtDNA-protein stoichiometry since increased TFAM/mtDNA ratio resulted in aggregation of mitochondrial nucleoids (Farge et al. 2014).

The decreased LonP1 protein levels observed on western blot for cells from patient 1, together with the findings of increased mtDNA copy number in response to culture conditions suggested a possible altered ratio of mtDNA to LonP1 protein. We therefore suspected that nucleoid formation might be affected and performed immunohistochemistry for fibroblasts of patient 1, grown with and without antibiotic/antimycotic, in glucose and galactose media (Figure 6.9). We found high levels of abnormal aggregated nucleoids predominantly in cells from patient 1 grown in glucose without antibiotic/antimycotic (Figure 6.9B) while cells from patient 2 did not show an increase of aggregated nucleoids in this condition. In galactose culture without antibiotic/antimycotic aggregated nucleoids cells from neither patient showed and increase in aggregated nucleoids (Figure 6.9C). In galactose culture with antibiotic/antimycotic however cells from patient 2 showed increased numbers of aggregated nucleoids while cells from patient 1 did not. These findings indicate that the decreased protein levels of LonP1 in patient 1 may have directly affected nucleoid formation. The results also indicate that increased mtDNA copy number in cells from patient 2 in response to antimycotic/antibiotic in galactose may have led to dysregulated LonP1/mtDNA stoichiometry and increased aggregated nucleoids.

Aggregated nucleoids may also be a result of dysregulation and altered processing of LonP1 targets, which bind to mtDNA but have not been interrogated via SDS PAGE

for modulated levels. Overall results show that altered mtDNA levels in conjunction with altered levels of LonP1 and its target leads to aggregated nucleoid formation and that the incidence is correlated with culture conditions.

Figure 6.9: Large aggregated nucleoids are observed for patient 1 via immunohistochemistry in glucose media without antibiotic



(A) Depicted are nucleoids from control fibroblast, cultured in glucose and pyruvate (no antibiotic) visualized via immunohistochemistry using an anti-DNA antibody. (B) Depicted are nucleoids from patient 1, cultured in glucose and pyruvate (no antibiotic). The arrow points to swollen, aggregated nucleoids. The zoomed in image shows that aggregates are mainly perinuclear. (C) Cells were counted per each image (>50) and a ratio of cells positive for aggregated nucleoids vs total cell number was calculated. This is expressed as percent of ratio found in cells from control. Dark outline on bars indicates use of antibiotic.

Table 6.5: Summary of main Seahorse study findings contrasted with mtDNA copy number and aggregated nucleoids

A

Summary patient 1	BR	SRC	mtDNA copy #	Cells with aggregated nucleoids
Glucose + Pyruvate	Increased	Decreased	Normal	Increased
Galactose + Pyruvate	Normal	Decreased	Decreased	Decreased
Glucose no Pyruvate	Decreased	Normal	N/A	N/A
Galactose no Pyruvate	Decreased	Normal	N/A	N/A
Glucose + Pyruvate Antibiotic/Antimycotic	Normal	Decreased	Increased	Decreased
Galactose + Pyruvate Antibiotic/Antimycotic	Normal	Decreased	Decreased	Normal

B

Summary patient 2	BR	SRC	mtDNA copy #	Cells with aggregated nucleoids
Glucose + Pyruvate	Increased	Normal	Decreased	Normal
Galactose + Pyruvate	Normal	Decreased	Normal	Decreased
Glucose no Pyruvate	Decreased	Decreased	N/A	N/A
Galactose no Pyruvate	Increased	Increased	N/A	N/A
Glucose + Pyruvate Antibiotic/Antimycotic	Normal	Increased	Decreased	Decreased
Galactose + Pyruvate Antibiotic/Antimycotic	Normal	Decreased	Increased	Increased

Summary of results from Seahorse studies for both patients in glucose vs galactose media with and without pyruvate and with and without antibiotic/antibiotic. Results are contrasted with findings for mtDNA copy number and aggregated nucleoids. All mtDNA copy number testing and immunohistochemistry for nucleoids was done in cells with the addition of pyruvate to culture media. (A) Results of patient 1. (B) Results for patient 2. N/A indicates that experiment was not performed

6.3 Discussion

Mitochondrial disease has been closely associated with findings of tissue specificity and phenotypic heterogeneity, although the underlying processes governing these phenomena remain unexplained. The ubiquitous expression of LonP1 and its involvement in many aspects of cellular metabolism would again have predicted a progressive multi-systemic disorder of energy deficiency. Recent findings, including results from this study, reveal LonP1 as another mitochondrial disease gene, exhibiting many distinct clinical pathologies. LonP1 mutations have been associated with CODAS syndrome, a disease with little phenotypic overlap with the patients presented here. Protein degradation via LonP1 is a domain specific process, involving protein recognition, protein unfolding and protein degradation. The ability of LonP1 to bind mtDNA, modulating mtDNA replication and compaction into nucleoids, is also domain specific (García-Nafria et al. 2010). We therefore suggest that the specialized activity of the respective protein domains and the domain specific location of mutations is what underlies the varying phenotypic expressions of the disease.

CODAS mutations cluster around the ATP binding site and therefore may lead to abnormal proteolysis of select targets but may display normal enzyme activity of others. This is demonstrated by Strauss et al. through decreased protease activity of recombinant LonP1 protein with CODAS mutations towards steroidogenic acute regulatory (StAR) protein, while proteolysis of TFAM is not affected. The ATPase mutations therefore most likely modulate a certain set of LonP1 targets in a characteristic developmental and tissue specific manner, resulting in the development of CODAS. In this report we describe

clinical findings for Patient 1 who has only one mutation in the CODAS critical region, while the other mutation is in the N-terminal domain. Although oversimplified, her symptomatology does suggest an intermediate phenotype between CODAS patients and patient 2, who has both mutations in the N-terminus. This is manifested by in utero growth retardation, sensorineural hearing loss and vocal cord abnormalities. In contrast to CODAS patients she does not present with cataracts or skeletal abnormalities. We predict for patient 1 that a portion of assembled LonP1 hexamers will predominantly be ATPase mutant protein while other hexamers will predominantly contain N-domain mutant subunits. The exact percentage and balance of oligomerization can of course not be predicted, since subunit composition of the molecules in vivo depends on steric properties of the subunits and the stability of the individual chimeric molecules. Additionally, assembly and stability properties of proteins have been shown to be tissue specific and therefore can't be well-predicted outside of a model organism (Ravn et al. 2015; Antonicka et al. 2006).

For patient 2 both LonP1 alleles carry the same N-domain mutation, resulting in the homo-oligomerization of a homogeneous mutant hexamer. The patient's biochemical findings are classical for mitochondrial disease with unspecific patterns of TCA cycle intermediates, reduced methyl-tetrahydrofolate, increased methylglutaric, and lactate peaks on MR spectroscopy. Onset of disease was sudden and precipitated by an infection followed by a period of regression - all common patterns observed with syndromes of mitochondrial energy deficiency. Liver involvement, also a common finding, precipitated a biopsy with a puzzling finding of elevated copper. It is possible that altered LonP1 function is responsible for the copper deposits with abnormal processing of copper

containing mitochondrial biomolecules. Mitochondrial complex IV's catalytic core contains copper atoms and LonP1 has been shown to degrade the nuclear encoded respiratory chain subunit cytochrome c oxidase subunit IV isoform 1 (COX4-1). However, this hypothesis is not consistent with the finding of normal mitochondrial complex IV, assessed by ETC assay in muscle (Table 6.1) and western blot for mt-COII in fibroblasts (Figure 6.7B). Another interesting target to consider is superoxide dismutase 1 (SOD1), which binds copper and is an essential player in the mitochondria's antioxidant defense system. The finding of increased copper may also be liver specific and since liver tissue also displays decreased mtDNA copy number, indicating faulty mtDNA replication, future experiments investigating LonP1 disease pathology specifically in the liver may be warranted. The differentiation of hepatocytes from patient 2 via induced pluripotent stem cells (hiPSC's) would be informative to further investigate the source of the copper deposits as well as the loss of mtDNA in a liver-specific manner.

Although our findings for patient 1 and 2 as well as findings for CODAS patients vary significantly with respect to phenotype and outcome of functional studies, a striking common finding for patient 2 and CODAS patients is abnormal mitochondrial ultrastructure as assessed by electron microscopy (EM). Mitochondria from CODAS patients reportedly display swollen, vesiculated cristae with electron-dense aggregations on lymphoblastoid cell lines. Similarly, EM on muscle from patient #2 reveals numerous "unusual" mitochondrial inclusions represented by osmiophilic, granular deposits that nearly replace the mitochondria. EM to assess mitochondrial ultrastructure on muscle from patient 1 is currently being pursued. Disrupted cristae morphology in patient 2 may be secondary to swollen nucleoids since cristae disruption has been associated with the

presence of nucleoids (Busch et al. 2014). We will therefore be curious to see if cristae morphology is also disrupted in EM on muscle from patient 1 since we saw large aggregated nucleoids in patient 1 fibroblasts (Figure 6.9B).

Since clinical findings and *in silico* mutation analysis and protein modeling left us with more questions than answers we pursued extensive metabolic studies in fibroblasts from patient 1 and 2. This was done to delineate the effects of LonP1 mutations on nutrient source dependency and response to cellular stress. When we withdrew pyruvate from a glucose culture medium, cells from both patients displayed similarly decreased BR and MR while response to pyruvate withdrawal in a galactose medium was significantly different in the two cell lines. In glucose, cells from both patients showed decreased BR in response to pyruvate withdrawal but only cells from patient 1 showed increased SRC without pyruvate. In contrast cells from patient 2 showed increased SRC in galactose when pyruvate was withheld while cells from patient 1 did not (Figure 6.4). Pyruvate has recently been shown to induce synthesis of aspartate, which is essential for growth of cells with respiratory chain defects. We therefore anticipated the addition of pyruvate to support cellular metabolism in LonP1 patient cells and consistent with this hypothesis we did observe sustained above normal basal respiration in glucose as well as galactose media for both patient cell lines when pyruvate was present (Figure 6.4A and 6.4D). But why would cells from patient 2 actually attain higher respiration when pyruvate is withheld in galactose media (Figure 6.4D-F)? A recent metabolomics study interrogating the effect of pyruvate in HeLa cells found that the addition of pyruvate lowers glutamate availability in the mitochondrial matrix (Sabatini 2016). Limited glutamate availability may in fact explain the negative effect of pyruvate on respiration, particularly in a galactose medium

where nutrient availability is limited. Other than glutamate availability, the finding could also be explained by a block in the TCA cycle of patient 2 since this would lead to abnormal NADH/NAD ratios depending on the location in the cycle. A compromised TCA cycle in cell lines would be consistent with clinical findings for patient 2 who has increased TCA cycle intermediates via urine organic acids analysis. This may have preferably affected cells from patient 2 due to the mutation specific effects on LonP1 and its targets. In future studies we will interrogate glutamate dependency for both patient cell lines and perform western blot for Aconitase to investigate these hypotheses.

Since LonP1 protects the cell from negative effects during periods of oxidative stress we also exposed the cells to antibiotic/antimycotic, known to negatively affect mitochondria. The use of antibiotic/antimycotic in glucose media resulted in significantly lowered SRC in cells from patient 1 but not for patient #2 (Figure 6.5C) and in fact SRC for patient 2 was 120% of normal control in glucose with antibiotic. This indicates that as long as cells from patient 2 have adequate levels of glucose, increased cellular stress does not negatively affect respiration. It may be the case that the well documented effect of inhibition on protein translation of mtDNA encoded subunits by the antibiotic streptomycin actually lowered the load of unfolded proteins slated for a mutated LonP1 protease in cells from patient 2 but this is speculative at this time and should have also held true for patient 1.

Since we saw modulated respiration in response to the use of antibiotic we wanted to see if the observed results may have been confounded by changes in mtDNA copy number (See Table 6.5 for Summary). Indeed, we found significantly increased mtDNA copy number in response to antibiotic in both patients but in interestingly the patient cells

responded again differently in glucose vs galactose. Patient 1 displayed increased mtDNA copy number in response to antibiotic/antimycotic in glucose media while patient 2 demonstrated increased mtDNA in response to antibiotic in galactose media. This indicates that cells from patient 2 do not experience cellular stress in glucose media and this is consistent with findings of SRC which was also normal for patient 2 in culture with antibiotic/antimycotic. Cells from patient 1 on the other hand had decreased SRC in glucose plus antibiotic/antimycotic and this was true despite significantly increased mtDNA levels (Table 6.5A). These findings suggest that cells from patient 1 are significantly negatively affected by the use of antibiotic and that the observed toxic effects may have clinical significance. Studies have shown that the administration of antioxidants like N-acetylcysteine (NAC) in conjunction with administration of bactericidal antibiotics like the ones used in this study can be ameliorated (Kalghatgi et al. 2013). The investigators treated mice with clinically relevant doses of ciproflaxin, ampicillin or kanamycin. Glutathione levels were measured as an indicator of oxidative stress and oxidative tissue damage was assessed by protein carbonylation. The observed toxic effects could be rescued with NAC treatment. Since NAC is an FDA approved drug and considered safe this may be a good option for patients with mitochondrial disease in general which need to have antibiotic therapy.

Our study also showed large aggregated nucleoids in patient 1 in glucose media without antibiotic. We suggest that this finding is due to abnormal stoichiometry of LonP1 to mtDNA copy number since patient 1 has decreased levels of LonP1 protein and normal levels of mtDNA. Another possibility is that the abnormally aggregated nucleoids observed in patient fibroblasts are a downstream effect of disrupted TFAM. TFAM is

essential for mitochondrial nucleoid formation and LonP1 has been shown to regulate mtDNA levels mainly through its interaction with TFAM (Kukat et al. 2015). Although TFAM protein levels in our patient cell lines are normal we will repeat SDS PAGE for cell lysates with comparable culture conditions. LonP1 has also been shown to have direct impact on nucleoid formation besides its interaction with TFAM. LonP1 has also been shown to bind less tightly to mtDNA in response to oxidative stress (Lu et al. 2007). The study shows that mtDNA binding to the control region of mtDNA in HeLa cells is not affected by oxidative stress but that overall less LonP1 molecules are bound to mtDNA during periods of oxidative damage. Oxidative stress may therefore be another confounding factor in nucleoid formation and it would be interesting to see in future experiments how stoichiometry of mtDNA to LonP1 is modulated secondary to oxidative stress in our patient cells.

In summary we have identified two unrelated patients with novel mutations in the mitochondrial matrix protease *LonP1*, which are the first patients with mutations in the substrate binding or “N” domain of LonP1. Patient 1 is compound heterozygous with one N domain mutation while patient 2 is homozygous and therefore has both mutations in the N domain. Our studies performed in patient derived primary dermal fibroblasts show that LonP1 protein levels are decreased in patient 1 while they are normal in patient 2. Using a microplate respirometry assay we find that the two cell lines respond differently to nutrient source. When we withdraw pyruvate from a glucose culture medium SRC is significantly increased in patient 1 and decreased in patient 2. When we withdraw pyruvate from a galactose culture medium SRC is not significantly changed in patient 1 but increased in patient 2. When we challenge the cells by exposure to an

antibiotic/antimycotic in glucose media we observe severely decreased spare respiratory capacity for patient 1 and increased spare respiratory capacity for patient 2 in glucose media. When culturing cells in antibiotic/antimycotic we also observed increased mtDNA copy number for patient 1 in glucose and for patient 2 in galactose. For patient 1 we also demonstrate aggregated nucleoids in glucose media while this is not observed for patient 2. The observed abnormalities in patient fibroblasts are consistent with previously reported cellular functions of LonP1. We propose that mutations in the substrate binding domain of LONP can cause mitochondrial disease of varying severity. We also propose that homozygous mutations in the substrate binding domain have a different effect on mitochondrial metabolism as compared to the compound heterozygous mutations with only one mutation in the substrate binding domain.

6.4 Materials and Methods

6.4.1 Patients

Informed consent was obtained from all research participants or their legal guardians under the CHOC Childrens Hospital Institutional Review Board #130990 (PI Jose Abdenur) and University of California Human Subjects Protocol #2015-1831 CMU Reliance #43 (PI Susanne Rafelski).

6.4.2 Cell culture

Primary human skin fibroblasts were established from 3mm punch biopsy. Tissue was grown on plates coated with fibronectin (10ug/ml of PBS) in alpha MEM with 1x nonessential aminoacids (NEAA), Primocin to prevent mycoplasma contamination and

15% FBS (Hyclone). Established cultures were initially maintained in alpha MEM, 1x NEAA, 1x Antibiotic/Antimycotic and 15% FBS (Hyclone) at 37C in 5% CO₂. Cultures were then transferred to the respective media conditions.

Base media: DMEM no glucose (Gibco- ThermoFisher cat# 11966026) contains 4mM Glutamine, supplemented with 10% FBS Fetal Clone 3 Hyclone (GE Healthcare H30109.03) and 1X (0.1mM) non-essential amino acids (NEAA100x =10mM each Glycine, L-Alanine, L-Asparagine, L-Aspartic Acid, L-Glutamic acid, L-Proline, L-Serine)

Glucose media: Base media plus addition of D-glucose to 5mM (+/- 1mM Pyruvate final concentration)

Galactose media: Base media plus addition of D-galactose to 5mM (+/- 1mM Pyruvate final concentration)

Antibiotic/Antimycotic: “Anti-Anti” (antibiotic-antimycotic-Gibco 100x #15240062) contains 10,000 units/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL Amphotericin B

6.4.3 Microtiter plate respiration assay

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements were performed on a Seahorse Bioscience XFe-24 bioanalyzer using the Seahorse XF Cell Mito Stress Test. Cells were seeded in XF24-well microplates in the respective media at 30K cells/well in 100ul of media after counting in triplicate with a hemacytometer. After 1 hour cells were transferred to a CO₂ buffered incubator for 16 to 20 hours. The following day growth medium was replaced with unbuffered assay medium supplemented with glucose or galactose 5mM, 1mM pyruvate and 4mM

glutamine final concentration. Media PH was adjusted to 7.4 and sterile filtered. Cells were equilibrated prior to Mitostress testing with unbuffered medium for 45 min at 37 °C in a CO₂-free incubator. Upon assay completion cells were rinsed in ice cold PBS and lysed with 30µl RIPA Buffer (Pierce) supplemented with Protease inhibitor cocktail 50ul/ml (Sigma) for 30min agitated at 4⁰C followed by scraping/mixing with a 200ul pipette tip. Protein levels were determined for each well in triplicate using DC protein microplate assay (Biorad). Values were read on an Elisa Plate reader (Thermo) and used for normalization of OCR and ECAR as per ug/ul of protein.

Data was analyzed using XF Excel Mito Stress Test Report Generator, Wave desktop software and statistical analysis was performed using Graphpad Prism.

6.4.4 mtDNA copy number determination in fibroblasts

Genomic DNA was extracted from 1x10⁶ cells after lifting the cells with trypsin and rinsing in PBS. A Puregene DNA extraction kit was used according to the manufacturer protocol. Quantitative PCR assay was performed in triplicate on a CFX Connect Real-Time PCR Detection System using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories). Relative mtDNA copy number was determined via delta delta CT method using CT values for the mt-ND1 gene vs the nuclear housekeeping gene beta-2microglobulin (B2M) as compared to an age matched normal control. Primers were used as previously described (Bonnen et al. 2013).

6.4.5 Immunoblotting

Cells were lysed on ice in RIPA Buffer (Pierce) supplemented with Protease inhibitor cocktail 50ul/ml (Sigma) for 30min at 4⁰C and cleared at 16,000 rpm for 20min. Protein concentration was determined in triplicate via DC protein assay (Biorad). Lysates were stored in Nupage LSD sample buffer at -20⁰C (Life Sciences). SDS PAGE was performed using NuPage Bis-Tris Gels, NuPage MOPS or MES running buffer respectively in SureLock XCell system (Invitrogen). Proteins were blotted onto PVDF membrane (Life) and blocked for 30min at RT with 5% Nonfat Dry Milk in TBS-T or 5% BSA in TBS-T or Fast Blocking Buffer (Pierce). Membranes were incubated with primary antibody as recommended by the manufacturer together with 1:100,000 beta actin primary antibody at 4⁰C overnight. (Anti-LonP1: Protein Tech cat# 15440-1-AP, Anti-TFAM: Abcam cat# 131607, Anti-Cytochrome C: Abcam cat# 133504), Anti-CYC1-cytochrome b_c1: Abcam cat# 137757, Anti-Aconitase: Abcam cat# 129104, Total oxphos human WB antibody cocktail: Abcam cat# 110411). The membrane was washed briefly with 1xTBS-T and incubated with HRP-conjugated goat anti-rabbit IgG at RT for 1hr. Membranes were then washed 3x with 1x TBS-T at RT and Proteins were detected with SuperSignal Chemiluminescent Substrate (Thermo) and imaged on CL-Xposure Film.

6.4.6 Immunohistochemistry for mitochondrial nucleoids

Primary fibroblasts were plated at 100,000 cells/well in a 6 well glass bottom plate (CELVIS) and fixed with 4% paraformaldehyde, permeabilized and blocked with PBS + 0.1% Triton-X100 and 3% normal donkey serum. Anti-DNA (Millipore, clone AC-30-10)

was applied to decorate mtDNA/nucleoids and detected using Alex Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch) and imaged on an Olympus IX70 microscope using a 20x objective, an Optronics MacroFire camera and imageproPlus software.

6.5 Acknowledgements

We thank the families and patients for participation in the study. We also thank the clinical teams at CHOC and MassGeneral for caring for the patients.

6.6 Contributions

Mariella Simon performed all of the experiments except for the antibody hybridization for mitochondrial nucleoids, which was performed by Alex Stover. Shaya Eftkharian provided laboratory support as a volunteer and helped with the figures.

CHAPTER 7

Discussion

7.1 The success of the “bedside to bench and back to bedside” paradigm requires collaborative and multidisciplinary approaches

The successful employment of multidisciplinary methods in the research arena, as demonstrated in this dissertation have led to the discovery of many new genetic syndromes. For example, the combination of next generation sequencing technology with functional molecular and bioenergetic studies resulted in the delineation of three gene defects (*NARS2*, *TFAM* and *LONP1*), which had never been associated with human disease at the time the sequencing results had become available. This type of systems approach now needs to be brought to the clinic. The involvement of mitochondrial pathologies as discussed in this dissertation unite all clinical specialties like metabolics, neurology, cardiology, immunology, ophthalmology, oncology, infectious disease and toxicology to name a few. A new type of collegial teamwork approach to disease with bioenergetics as the nexus will bring new ideas and treatment modalities to the field of mitochondrial medicine. Unfortunately, not all hospital specialties believe in this type of collaboration but once we start to approach disease from a more holistic standpoint boundaries of subspecialties may break down. This already starts with the terminology of disease subspecialties. For example, it has always been puzzling to me, why there should be a distinction of metabolic disease and mitochondrial disease since all mitochondrial disease is metabolic and all metabolic disease affects mitochondria.

An example for a classical metabolic disorder, which leads to well documented secondary mitochondrial abnormalities is methylmalonic acidemia (MMA). MMA is caused by the faulty breakdown of the amino acids methionine, threonine, isoleucine and valine due to deficiency or decreased activity of methylmalonyl-CoA mutase (Matsui et al. 1983). MMA had long been considered a metabolic disorder but since the accumulation of methylmalonate leads to inhibition of mitochondrial oxygen consumption via depletion of alpha ketoglutarate from mitochondria this definition has been revised (Melo et al. 2012). MMA is now considered also a mitochondrial disease by some groups like ours. The experience of mitochondrial abnormalities associated with MMA and other “metabolic disorders” has caused a much needed shift of thought for many in the the metabolic disease field who now embrace many metabolic disorders as potentially “mitochondrial”.

The arbitrariness of the distinction of metabolic and mitochondrial disease can also be directly demonstrated by two cases addressed in this dissertation. In chapter three we describe a patient with mutations in the mitochondrial mtDNA polymerase *POLG*. Initially the infant was thought to have glycogen storage disorder, due to fasting intolerance and abnormal glycogen on liver biopsy – a metabolic disease. As soon as mutations in *POLG* were detected the patient transformed from a presumed metabolic patient into a mitochondrial patient. Of course, this did not change the fact that the patient had abnormal glycogen metabolism, a finding not usually associated with *POLG* related disorders. Another example is the curious case, covered in chapter five of this dissertation with mutations in the mitochondrial elongation factor GFM1, which leads to abnormal translation of mtDNA encoded proteins. This patient was initially diagnosed as a patient with a congenital disorder of glycosylation – a metabolic patient- because the patient

repeatedly demonstrated abnormal glycosylation patterns via transferrin studies. It was only the molecular diagnosis that then brought the patient to the mitochondrial disease clinic. But why did the patient have abnormal glycosylation patterns. In the submitted report we speculate that this was due to abnormalities in the interaction between mitochondrial and endoplasmic reticulum related processes.

So disease truly does illuminate cell biology. As we learn to open mindedly bring the bench and bedside together we will eventually be able to resolve these type of puzzles and help the patients, which have brought them to the bench.

As discussed current disease classifications leave little room for interaction between clinicians and their teams missing opportunities for collaboration and learning. The same is true for basic science cell biologists, which segregate themselves into specialties of the cytoskeleton, the cell cycle, cellular organelles or signaling pathways to name a few, while others are starting to integrate and build the study with a systems biology approach. These are the scientist we need to excite and get to participate actively at the bedside. In the end, no matter what goes wrong with the cell it will eventually lead to a pathology and dysregulated metabolism and bioenergetics. It would therefore be beneficial to approach the study of metabolic disease from a “pathway to cellular dysfunction” perspective.

It has been three years now that I first stepped into Dr. Susanne Rafelski’s office wanting to continue my clinically focused doctoral studies in her laboratory. Having come from the department of pathology it seemed like a big leap of faith to transfer my mitochondrial disease project into the department of developmental and cell biology and to join the laboratory of a quantitative, budding yeast, cell biologist. After two months in the lab, I attended my first American Society of Cell Biology (ASCB) meeting

and was intrigued to learn how fluorescent markers combined with laser confocal microscopy had brought the inner workings of the cell to light. These techniques not only allow the study of mitochondrial dynamics and morphology but also the quantification of metabolites, the detection of pH and calcium fluxes and the assessment of membrane potential and enzyme activities all in live cells. During the meeting, I searched for posters with a clinical theme and found only a handful, disappearing in the sea of basic science studies, which filled the large exhibit halls. Although I have since become infected with a love for basic science, I had felt that clinical explorations in general are underappreciated and underutilized in the basic science arena. I am therefore excited that this year's ASCB meeting headline reads: "Using Disease to Illuminate Basic Cell Biology at ASCB" which leaves me hopeful that patients in the future will benefit from this shift in focus and appreciation.

The puzzles of the mitochondrial disease clinic certainly ring true to the meaning of that headline. The reverse can be said as well - cell biology truly does illuminate all human disease. In the introduction of this thesis, I gave a historical perspective on the field of mitochondrial medicine. When comparing, the clinical workup performed for Luft's patient to a modern day workup in today's metabolic clinic, surprisingly little has changed while the research methods available to today's bench scientist have however improved dramatically.

7.2 Emerging methods in mitochondrial medicine

Most importantly Luft's patient could not enjoy the power of a genomic workup including mtDNA, nuclear DNA or RNA studies (Luft 1994). With the rise of accessibility

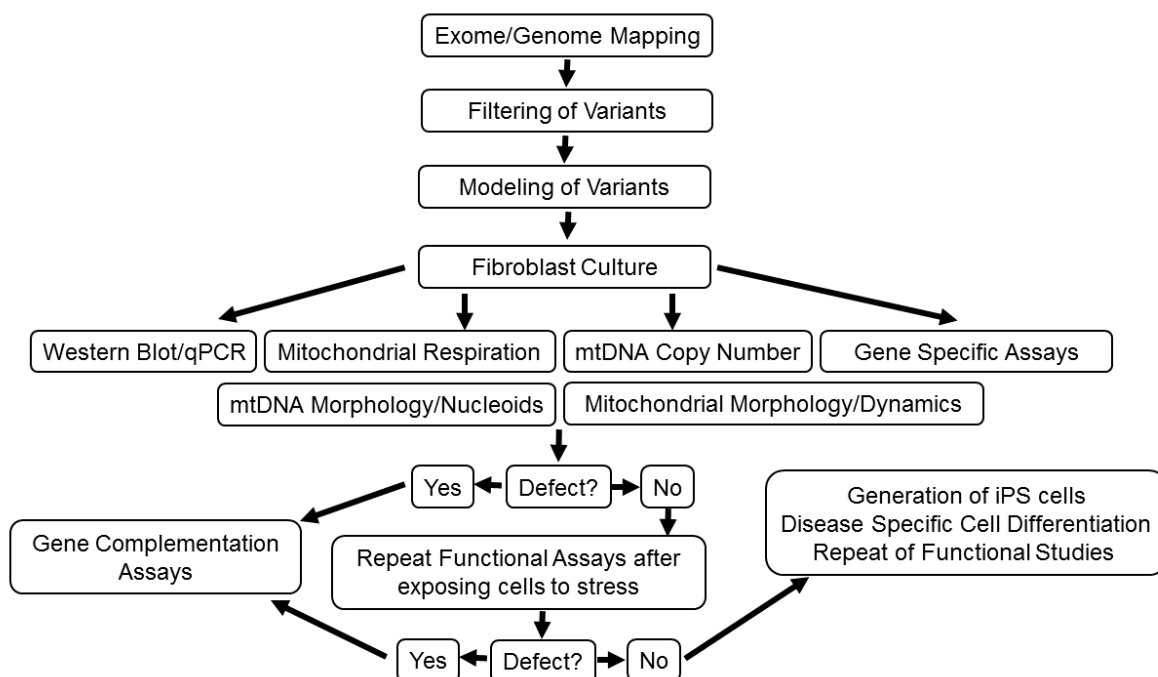
of next generation sequencing methods, exome and full genome sequencing have quickly become the new norm for investigations in the rare disease field and have moved from research to the clinical arena. Such a workup would have saved Luft's patient from having several muscle biopsies and allowed for more targeted treatment modalities, if DNA analysis had been informative. Unfortunately despite the use of comprehensive sequencing technologies, the majority of rare disease patients still go undiagnosed if only molecular studies are employed (Calvo et al. 2012). The need for functional studies, assessing cellular metabolism, employing modern cell culture techniques including the use of induced pluripotent stem cells (iPS) is therefore more pressing than ever (Hamalainen et al. 2013).

The current approach to establish disease causality of variants identified via next generation sequencing methods follows a path from variant filtering to identification of a plausible variant to functional studies (Figure 7.1). After mapping the data to the most current genome build, variant calls are made and subsequently filtered against publicly available databases to eliminate common variants. Next, variants are eliminated, which do not fit the observed pattern of inheritance. For example, if sequencing was performed for the patient as well as the parents (trio analysis) and the pedigree suggests autosomal dominant inheritance, then all variants stemming from the unaffected parent may be subtracted. After this, variants are subjected to *insilico* pathogenicity prediction analysis using established algorithms and variants predicted to be benign are filtered out. Next variants are segregated into specific "patterns of inheritance" categories: Variants on the X chromosome are grouped for potential x linked inheritance, variants with at least two calls in the same gene and inherited in trans (not on the same parental chromosome) are

grouped for autosomal recessive inheritance and single variants are grouped for autosomal dominant inheritance or autosomal recessive inheritance where the second variant call was missed or not covered by the sequencing chemistry. The last filtering step of variants is done to eliminate genes which clearly do not fit the observed disease category and depends heavily on clear gene function associations. This step therefore needs to be regularly repeated and data revisited as new insights regarding gene functions becomes available. Once a plausible gene candidate has been established more detailed *insilico* analysis is being performed to assess conservation of the variant between species, location of the variant within the protein and modeling of the variant's effect on protein structure. Concurrently fibroblast cultures need to be established from patient and potentially also from additional family members. Fibroblasts are then used for a battery of functional tests, establishing a picture of mitochondrial and cellular health. In particular protein and mRNA levels of the gene of interest are interrogated via western blot and quantitative PCR technology respectively. Mitochondrial respiration is assessed in our laboratory via Seahorse bioanalyzer technology and mtDNA copy number is assessed via quantitative PCR. Imaging technology is employed to demonstrate mitochondrial nucleoids using immunohistochemistry and mitochondrial morphology and dynamics is interrogated via live cell microscopy. Gene specific assays assessing the pathogenic potential of the observed variants are also employed. Once a functional defect can be demonstrated, gene complementation assays rescuing such a defect are then undertaken as to establish causality of the variant with the observed disease. In the event that a defect cannot be established through standard culture conditions, cells are then re-cultured, exposed to cellular stress conditions and functional studies are repeated

following the same protocols. If exposure to stress can not demonstrate a defect in patient cell lines the expression of the defect may be tissue specific. For this induced pluripotent stem cells may be generated from the patient fibroblasts and differentiated into a cell type resembling a phenotypically affected tissue (Figure 7.1).

Figure 7.1: Overview of functional studies prompted by delineation of novel gene variants



Exome and full genome sequencing may result in the delineation of novel variants in genes, which may not previously have been associated with disease. This then warrants follow up via functional studies in the research lab to establish causality of the variants underlying the observed phenotype.

If we contrast the research studies outlined above and particularly the assessment of mitochondrial respiration we can demonstrate how far modern science has come in streamlining the process of functional mitochondrial analysis for our patients. For example respiration studies performed for Luft's patient were assessed one sample at a time in a respirometer using extracted mitochondria. Cells from LonP1 patients on the other hand

were investigated using a modern Seahorse Bioanalyzer, which allows assessment of respiration in a multi-well format, in live cells and with the concurrent analysis of respiration and generation of lactic acid. The multi-well format and automation is particularly advantageous for the clinic since several patient samples can be multiplexed within a short time frame, making respiration analysis accessible and common place for the metabolic laboratory.

A powerful technique by itself, a microplate respiration assay becomes even more meaningful when combined with metabolomic analyses, employing mass spectrometry technology. As discussed in the chapter on LonP1 the underlying biology of the response to culture conditions, including nutrient source or exposure to cellular stress could not be fully interpreted and therefore warrants further interrogation of cellular and mitochondrial metabolism. This option becomes particularly exciting for the mitochondrial disease field when mitochondrial metabolism may be assessed in in the mitochondrial matrix compartment, since it has been shown that “whole cell dynamics” may often not capture the flux of mitochondrial biomolecules (Birsoy et al. 2015). A recent study by Chen et al. combines a new rapid isolation method of epitope tagged mitochondria with metabolic profiling of mitochondrial matrix metabolites termed the MITObolome. The study’s quantitative analysis of mitochondrial metabolites was only made possible through employing sophisticated modern imaging technology allowing volumetric assessments of matrix contents.

The study revealed several new concepts including the effects of pyruvate on diminished mitochondrial matrix concentration of glutamate and alpha-ketoglutarate, which is directly relevant for the findings observed in cells from patients with LonP1

mutations. The study also revealed mutation specific matrix levels of select mitochondrial aminoacyl tRNA synthetases, which is directly applicable to investigations performed in cells from patients with mutations in NARS2 as discussed in chapter two of this dissertation. Furthermore, the study for the first time gives insight into changes of the MitoObolome in response to individual inhibition of the mitochondrial respiratory complexes and shows for example that acetyl-CoA accumulates in the mitochondrial matrix secondary to inhibition of mitochondrial complex I, but not due to inhibition of mitochondrial complex III. The technology was introduced in HeLa cells but will become more relevant when employed in differentiated iPS cells from patients as to simulate the tissue specific expression of the disease in the patient. This then will allow the direct monitoring of metabolic fluxes in the mitochondrial matrix in response to treatment modalities. The suggested concurrent administration of the antioxidant N-acetylcysteine (NAC) in conjunction with antibiotic, as discussed in chapter six of this dissertation would be such an example. Here the measurement of glutathione in response to NAC would allow to interrogate the efficacy directly in cells or isolated mitochondria of a patient undergoing treatment and this technology is therefore a perfect example of how the cycle of bedside to bench and back to bedside can be applied.

7.3 Tissue specificity - a conundrum of mitochondrial disease

In order to allow for a higher percentage of positive outcomes in the mitochondrial disease clinic, with effective and targeted treatment modalities, we need to also better understand the underlying cellular pathologies and pathways that lead to mitochondrial disease. One extremely important concept for this is to further understand the underlying patterns of

tissue specificity. The studies presented in this dissertation describe several syndromes associated with significant tissue specificity, a hallmark of mitochondrial disease. To explore the underlying processes of tissue specificity is one of the most important and highly pursued areas of the mitochondrial disease field since it's understanding is essential for the development of tissue specific treatment options.

One explanation for why a ubiquitously expressed gene should only result in liver damage and not brain abnormalities, despite the fact that both tissues are highly energy demanding, has been in part explained by the "threshold model". In this model the expression of the mitochondrial defect is modulated in a tissue specific manner due to the differential metabolic expression or differential sensitivity of one of its components. Rossignol et al. explore the concept by the systematic investigation of isolated mitochondria from various tissues and their response to various electron donors and inhibitors. Although the study indeed shows that mitochondria, extracted from different tissues, have varying response curves with respect to electron donors and sensitivities to inhibitors of oxidative phosphorylation the studies do not explain what structurally underlies the threshold effect (Rossignol et al. 1999).

A study by Antonicka et al. is one of the most informative investigation into the subject. The group interrogates tissue specificity in a patient with mutations in the mitochondrial elongation factor *GFM1*, one of three nuclear encoded mitochondrial translational elongation factors which are all needed to progress the growing protein chain through the mitochondrial ribosomes. As discussed in chapter five of this thesis, mutations in *GFM1* result in a mitochondrial hepatopathy while cardiomyopathy is not, or only rarely seen in association with the disease. The group investigates protein levels of

oxphos complexes, a direct output measure of GFM1, in patient liver, heart and skeletal muscle tissues. Since the patient mutations affect a ubiquitously expressed member of the mitochondrial protein translational machinery, levels of mtDNA encoded oxphos subunits should therefore theoretically be affected equally between the different tissues. However, results showed that the translational defect caused significantly lowered levels of oxphos subunits particularly in liver. Liver from normal controls showed no difference in levels of the interrogated oxphos subunits in comparison to heart or muscle. The group then went on to demonstrate that the intensified translational defect in liver was most likely due to modulated expression levels of the three mitochondrial elongation factors, which are all essential for protein translation. For reasons not known at this time the stoichiometry between the three different elongation factors is in fact tissue specific and explains the increased vulnerability of liver tissue in conjunction with mutations in *GFM1* (Antonicka et al. 2006). Large scale mRNA studies on autopsy samples employing RNAseq would be extremely helpful in interpreting the tissue specificity of mitochondrial disease and genetic disease in general. These studies would allow for generation of publicly available databases which could then be interrogated and findings correlated with clinical investigations. Hopefully additional studies will come forward as to elucidate the underlying processes of the phenomenon since much remains unexplained and a complete picture remains elusive. Particularly animal models may prove useful in the elucidation of the phenomenon.

7.3.1 Studying tissue specificity in animal models: The NDUFS4 mouse

The NDUFS4 mouse is a perfect model system to interrogate pathways, which lead to tissue specificity (Kayser et al. 2016). NDUFS4 is a nuclear encoded mitochondrial complex I subunit and knockout mice are used as a model system for Leigh syndrome as introduced in chapter one of this dissertation. Interested in the preferential development of basal ganglia lesions Kayser et al. used the Seahorse bioanalyzer to assess spare respiratory capacity in isolated murine brain mitochondria as well as from mouse synaptosomes. The study showed that compromised spare respiratory capacity was limited to synaptosomes from vulnerable regions of the brain. This proved that the ability of the brain to react to increased energy need by increasing oxphos activity is critical for neuronal survival and is most likely the underlying faulty process of disease pathology. The study also showed that the regional sensitivity to neuronal cell death was restricted to mitochondria from the synapse while non-synaptic mitochondria had similar respiration rates irrespective of their location in the brain. In synaptosomes spare respiratory capacity from olfactory bulb regions was significantly reduced and this was not explained by increased oxidative stress or additional reliance on aerobic glycolysis. The investigators were surprised to find that spare respiratory capacity was not decreased when they used alpha-ketoglutarate/malate rather than pyruvate/malate or glutamate/malate as substrates. In preliminary experiments the group found that alpha-ketoglutarate increased respiration through mitochondrial complex II rescuing the NDUFS4 conferred defect at complex I. We had speculated on a similar mechanism in our LonP1 paper when we saw increased spare respiratory capacity in patient 2 in glucose media without pyruvate. We postulated that the availability of pyruvate lowered the levels of alpha-ketoglutarate

in the matrix in our patient cell lines as shown in a recent mito-metabolomics study in HeLa cells (Chen et al. 2016). In summary the results of the study suggest a cell specific threshold of mitochondrial bioenergetics in response to the *NDUFS4* mutations induced complex I defect. Again the study shed light on some aspects of tissue specificity but most likely many concept's will hold true and cause the phenomena in conjunction with each other.

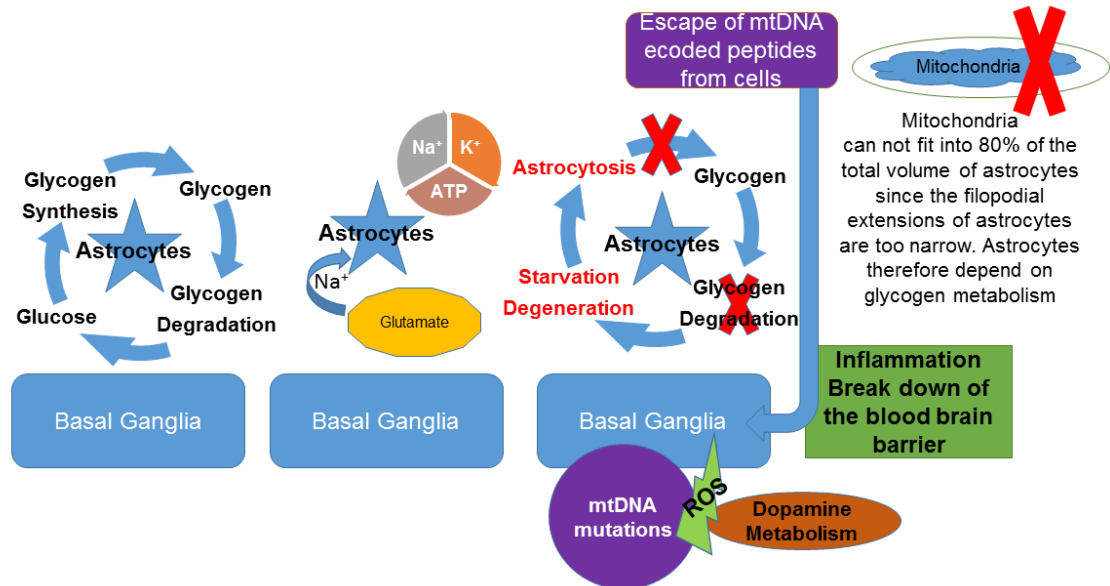
7.3.2 Tissue specificity and the mystery of basal ganglia disease

As introduced in chapter one and further discussed in chapter two (NARS2) tissue specificity in Leigh syndrome is defined by preferential degeneration of the basal ganglia in the central nervous system. The question is what makes basal ganglia more susceptible to mitochondrial dysfunction as compared to other structures in the brain? Since basal ganglia disease is not associated with one particular mitochondrial gene defect but rather may be caused by numerous mtDNA and nDNA encoded lesions, a more global vulnerability of the structures must come into play (Finsterer 2008). Although in no way comprehensive, I would like to introduce several concepts, which describe some aspects of basal ganglia disease pathology and how they might apply to Leigh syndrome patients.

I would also like to bring forward a hypothesis that originates from clinical studies outlined in chapter three of this dissertation (Figure 7.2). Those studies revealed abnormal glycogen metabolism in a child with mutations in *POLG* causing basal ganglia disease and Alpers syndrome. I propose that basal ganglia are structures, which experience increased oxidative stress. I also propose that the oxidative stress negatively

affects glycogen metabolism in astrocytes, which are specialized glia cells of the brain with high energy requirements and interesting cellular shape, since much of their fine end-terminals do not accommodate the size of mitochondria. This then makes the end-terminals particularly vulnerable to disruption of glycogen metabolism and insufficient energy supply, leading to degeneration of astrocyte foot processes. The degeneration of astrocyte feet has been associated with precipitation of autoimmune disease leading to blindness and multiple sclerosis type symptoms (Lucchinetti et al. 2014). Also mitochondria themselves may escape from the cellular compartment when filopodia sheets break down secondary to a nutrient crisis and act immunogenic (Zhang et al. 2010). I therefore propose that degeneration of astrocyte feet, induced by increased oxidative stress and faulty glycogen metabolism is one underlying process of tissue specificity in basal ganglia disease and other mitochondrial disorders. In this next section I will demonstrate a few examples from the literature, which support the possibility of such a cascade.

Figure 7.2: Impaired astrocytic glycogen metabolism may contribute to basal ganglia disorders



Astrocytes are responsible for reuptake of glutamate from the synaptic cleft. This process is energy demanding since the Na^+/K^+ ATPase needs to reestablish the ionic cellular gradient disrupted by co-transport of Na^+ and glutamate into the cell. Glycogen metabolism supplies astrocytes with the needed ATP, particularly in regions of the cell where mitochondria cannot fit and assures astrocytic health throughout the brain. Basal ganglia are suggested as a site of increased mtDNA damage since dopamine is the predominant neurotransmitter in this tissue and dopamine metabolism has been linked to high levels of ROS. Glycogen metabolism may be highly susceptible to increased levels of ROS due to the unique monomeric structure of the glycogen debrancher enzyme with two active sites. Disruption of glycogen metabolism then no longer supports the high energy need for astrocytic glutamate uptake from the synaptic cleft and leads to degeneration of astrocyte feet leading to astrocytosis and a breakdown of the blood brain barrier. Mitochondria and mtDNA encoded peptides as well as other non-mitochondrial proteins like aquaporins, which are usually confined to the astrocytic cytoplasm can then enter the extracellular space and cause an immune response and therefore further aggravate astrocytic inflammation. Mutations in genes which compromise mitochondrial health will further increase oxidative stress in astrocytes and cause a shift from normal states as illustrated on the left of this Figure to astrocytosis as illustrated on the right of this Figure.

Basal ganglia are a group of structures or “nuclei” deep in the brain, which are functionally related, integrating circuits of voluntary and oculomotor movement as well as of formation of emotions and cognition (Bekiesinska-Figatowska et al. 2013). The basal ganglia are comprised of the caudate nucleus, putamen, nucleus accumbens, globus pallidus, subthalamic nucleus, the mesencephalic nuclei of the substantia nigra and the ventral

tegmental area. Two main circuits connect the basal ganglia to the cortex, where the “direct pathway” is concerned with the initiation of movement while the “indirect pathway” is inhibitory and is concerned with the termination of movement (Perez-Costas et al. 2010). It is therefore not surprising that the basal ganglia have been shown to house the majority of dopaminergic neurons and dopaminergic nerve terminals (Camps et al. 1989). In addition to Leigh syndrome, degeneration of basal ganglia is associated with disorders like Parkinson’s and Huntington’s disease, schizophrenia and obsessive compulsive disorder as well as disorders of autoimmunity like pediatric autoimmune neuropsychiatric disorder associated with streptococcus infections (PANDAS) (Coskun et al. 2012; Dileepan et al. 2016).

Studies in aging brains have shown that somatic mtDNA mutations and mtDNA deletions in particular accumulate preferably in the basal ganglia (Corral-Debrinski et al. 1992; Soong et al. 1992). This has been ascribed to increased oxidative stress via H₂O₂ generation due to dopamine degradation via mitochondrial monoamine oxidase as well as other free radical and nitric oxide producing processes (Dias et al. 2013; Barros-Minones et al. 2015). The hypothesis is in agreement with results stemming from an extensive next generation sequencing project of mtDNA, which confirmed a higher rate of somatic mtDNA mutations in brains from patients with Parkinson’s disease, a finding which correlates with increased oxidative stress (Coxhead et al. 2016). But how does the presumed increase in oxidative stress, caused by dopamine metabolism lead to neurodegeneration in the basal ganglia? As introduced earlier, based on the findings, which stem from observations from the bedside via careful and thorough interrogation of a clinical case by CHOC metabolic physicians we have reason to believe that glycogen

metabolism is particularly sensitive to oxidative stress and that disturbed glycogen metabolism may play a role in the tissue specificity of basal ganglia disease.

7.3.2.a Basal ganglia disease and brain glycogen metabolism in the astrocyte

In a patient with Alpers disease caused by mutations in *POLG*, which is discussed in chapter three of this dissertation, we observed that glycogen debrancher enzyme (Amylo-Alpha-1,6-Glucosidase,4-Alpha-Glucanotransferase or *AGL*) activity was completely absent in liver while other enzymes of glycogen metabolism were normal. Sequence analysis of the *AGL* gene was also normal. A review of publicly available mRNA expression data from patients with mitochondrial disease did not suggest lowered *AGL* expression levels associated with mitochondrial pathologies (Simon et al. 2013). We hypothesized that the glycogen debrancher enzyme has increased vulnerability to oxidative stress since it is one of the few known monomeric enzymes with two active sites (Zhai et al. 2016; Frickel et al. 2001). This hypothesis is in harmony with the fact that glycogen accumulation is a common, but often overlooked finding in mitochondrial disease since it requires electron microscopy of tissues with glycogen metabolism (Fan et al. 2013; Roels et al. 2009). Unfortunately, not much attention is paid to glycogen metabolism in the brain, which is restricted to astrocytes. Astrocytes are glia cells with many vital functions in supporting neuronal health and metabolism, in the uptake and recycling of neurotransmitters and in representing key components of the blood brain barrier. Astrocytes are heavily involved in immune response related pathways and produce and release cytokines when “activated”. Astrocyte activation is defined by the astrocytes’ response to any brain insult including oxidative crises like stroke, toxic insults

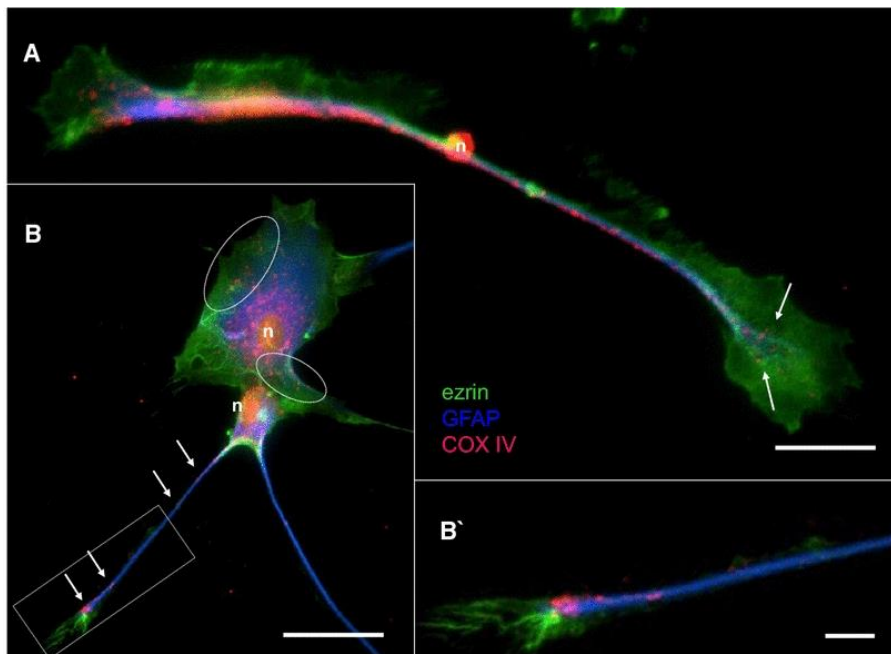
or neurodegenerative disease. Additionally astrocytes are activated when sensing a break-down of the blood barrier (Pekny, Nilsson 2005). Unlike neurons, these glial cells are able to polymerize and store glucose as glycogen (glycogenesis) and to hydrolyze glycogen back to glucose for use in aerobic glycolysis. Astrocytes are also the only cell in the CNS with pyruvate carboxylase activity, which allows fixation of carbon and de novo glutamate synthesis (Morales, Rodriguez 2012).

The glycogen of astrocytes is vital to brain function during intermittent episodes of hypoglycemia, since the brain is completely dependent on glucose metabolism and astrocyte glycogen therefore stalls neuronal death during hypoglycemic episodes or stroke (Suh et al. 2007). If energy deprivation is extensive, like for example during hypoglycemic episodes secondary to diabetes basal ganglia damage is triggered (Jurynczyk et al. 2010). In these situations mobilized glycogen is converted into lactate and released into the extracellular space to be taken up by neurons to sustain axon excitability (Tekkok et al. 2005). The infantile brain goes through a labile period whereas it is particularly vulnerable to hypoglycemic insults resulting in basal ganglia disease during the age of six to twenty two months (Gataullina et al. 2013).

Glycogen metabolism in astrocytes has also been ascribed to support energy needs of the astrocyte itself via aerobic glycolysis. This is particularly important in the astrocytic peripheral astrocyte processes (PAPs) which are so fine that they were presumed to not accommodate mitochondria (Figure 7.3A-B') (Hertz et al. 2007). Energy metabolism is unusually taxing in the PAP since these fine threadlike webbs represent the majority of astrocytic cell volume and are the sites of glutamate uptake from the synaptic cleft (Martin et al. 2015). A recent study of rat cortical astrocytes, combining

immunohistochemistry and high-resolution microscopy has shown that small spherical mitochondria do reside at least in a portion of PAPs (Figure 7.3B) (Derouiche et al. 2015).

Figure 7.3: Immunohistochemistry of astrocytes shows mitochondria in parts of PAP



Fixed and immunostained primary rat astrocyte from culture. Reproduced with permission from Derouiche et al 2015. Shown is the compartmentalization of the cell. Perinuclear cytoplasm and the larger stem processes are glial fibrillary acidic protein (GFAP) positive in blue, the peripheral astrocyte processes which are the sites of glutamate uptake and recycling (with thin lamelli and folopodia) are ezrin positive and colored in green, mitochondria are COX IV positive are colored in red.

This finding supports the notion that PAP metabolism depends on synergy of oxidative and other energy producing pathways, since glutamate recycling is indeed highly energy consuming. The reason for this is the increased need for Na^+/K^+ ATPase activity as to re-establish ion gradients, disturbed by the co-transport of glutamate and Na^+ into the astrocyte via a Na^+ uniporter (Pellerin, Magistretti 1994).

If we assume that basal ganglia disease is precipitated by dysfunctional glycogen metabolism, there should be at least some precedent in the literature, which associates

abnormal CNS glycogen with disease or examples of experiments in animal models linking dysfunctional glycogen metabolism to cognitive abnormalities. It is therefore surprising how little is known about dysfunctional astrocyte glycogen metabolism in childhood disease. However, several adult onset disorders point to involvement of abnormal glycogen metabolism in connection with neurodegeneration. For example in a study by Dalsgaard et al. brain biopsies from patients with temporal lobe epilepsy showed two fold increased glycogen in the affected vs unaffected areas of the brain suggesting abnormal glycogen metabolism in response to CNS insults (Dalsgaard et al. 2007).

Adult onset polyglucosan body disease (APBD) caused by mutations in the glycogen branching enzyme *GBE1* results in a leukodystrophy leading to urinary incontinence, ataxia with spastic gait and peripheral neuropathy (Mochel et al. 2012, Sampaolo et al. 2015). The disease presents usually in the fourth to fifth decade and is characterized by amylo-pectin like polysaccharide deposits (polyglucosan bodies) in the CNS, the peripheral nervous system as well as in muscle tissue. Autopsy of a woman with the disease showed predominance of polyglucosan bodies in astrocytes (Dainese et al. 2013). A similar disease with earlier onset can be caused by mutations in the laforin glycogen phosphatase gene *EPM2A* or the malin E3 ubiquitin ligase gene *NHLRC1*, both of which regulate glycogen synthesis via control of glycogen synthase (Kecmanovic et al. 2016). In Lefora disease poorly branched polymers of glucose termed “Lefora bodies” are seen in axons and dendrites of neurons, which is attributed to the fact that neurons themselves also have glycogen metabolism. The disease presents with seizures and visual loss in early adulthood (Vilchez et al. 2007). It has been shown in malin knockout

mice that Lefora bodies also accumulate in astrocytes and that the disruption of glycogen synthase can rescue the observed defects (Valles-Ortega et al. 2011; Vilchez et al. 2007).

Our current knowledge of the effects of dysfunctional astrocyte glycogen metabolism on mentation stems from experiments in one day old chicks. In this animal model it has been shown that inhibited glycogen turn over in the brain prevents memory formation (Gibbs 2015). The same finding was shown in rats, where injection of the glycogen phosphorylation inhibitor DAB right before or immediately after training inhibited memory formation. However DAB injection 15 minutes before or 1 hour after training, when glycogen metabolism was no longer or not yet inhibited did not disrupt memory formation (Suzuki et al. 2011). A study investigating brain specific glycogen depletion in glycogen synthase knockout mice showed increased susceptibility to epilepsy with greater axon excitability and sensitivity to the glutamate receptor agonists (López-Ramos et al. 2015). Investigators also have shown that glycogenolysis is needed to sustain glutamatergic neurotransmission. In a murine neuronal astrocyte coculture system in which glycogen breakdown was inhibited, the group showed decreased uptake of the glutamate analogue D-[H³] which suggests that glutamate recycling is directly related to the functional glycogen metabolism (Sickmann et al. 2009).

If abnormal glycogen metabolism is one of the precipitating factors of basal ganglia disease remains to be seen but the high density of dopaminergic neurons in basal ganglia generating oxidative stress combined with their high energy need to recycle glutamate from the synaptic cleft may play a significant role in disease pathology. One way to investigate the validity of the outlined hypothesis would be to expose astrocytes or hepatocytes in culture to oxidative stress, followed by enzymatic analyses of glycogen

debrancher activity. If debrancher activity would actually be compromised one could take this experiment further and coculture neurons and astrocytes, generated from iPSCs from a patient with POLG mutations as to interrogate the specific effects of the mutation on glycogen metabolism in vitro. These type of investigations could also be pursued in an animal model which may better simulate what is going on in an organism. It may take some time until investigations into glycogen metabolism in association with basal ganglia disease result in new treatment modalities but at least one type of basal ganglia disease can already be treated today and will be discussed in the next section.

7.3.2.b Biotine-thiamine-responsive basal ganglia disease

Faulty energy generation associated with basal ganglia disease has also been associated with abnormalities in thiamine metabolism. A paper published in 1998 describes the clinical course of 10 patients with biotin responsive basal ganglia disease, a neurological disorder associated with a distinctive CNS involvement characterized by destruction of the caudate heads as well as the putamen (Ozand 1998). The report describes onset of disease in early childhood most commonly precipitated by a febrile illness with loss of motor milestones, confusion and dystonia which if left untreated was shown to lead to severe debilitating disease and early death. The study demonstrates that prompt treatment with biotin may completely revert symptoms and lifelong administration of the cofactor may allow for normal development and a life without handicap. It has later been shown that in some cases biotin treatment alone was unable to rescue the phenotype and that a combination treatment of biotin with thiamine was required. The disorder has since been renamed “biotine-thiamine-responsive basal ganglia disease (BBGD) and

surprisingly revealed itself to be caused by faulty thiamine rather than abnormal biotin transport (Subramanian et al. 2006). The majority of genetic lesions underlying BBGD have been mapped to the thiamine transporter *SLC19A3*, where decreased activity of *SLC19A3* leads to low levels of free thiamine in cerebrospinal fluid in patients with the disease. Since thiamine is an essential cofactor for three key mitochondrial enzymes, pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase and branched chain alpha ketoacid dehydrogenase, thiamine deficiency leads to severe dysfunction of mitochondrial energy metabolism and precipitates a cellular energetic crisis (Kevelam et al. 2013).

The positive outcome of biotin treatment in children with mutations in *SLC19A3* has been associated with the observed upregulation of *SLC19A3* secondary to biotin exposure (Distelmaier et al. 2014). In addition to the observed upregulation of *SLC19A3*, biotin has also been shown to be involved in modulating gene regulation of several energy producing pathways. The group of Velasquez et al. showed via microarray testing that biotin starvation in budding yeast, worms and rodents lead to lowered expression of genes involved in glucose utilization as well as lipogenesis (Velazquez-Arellano et al. 2011). The group also showed that administration of biotin as well as administration of aspartate results in rescue of the observed energy deficits. The group's findings may therefore offer additional explanations for the underlying mechanisms of the efficient rescue of symptomatology in thiamine transporter deficiency via biotin.

Thiamine deficiency is also the underlying cause of Wernicke encephalopathy which is frequently caused by malnourishment and may lead to ophthalmoplegia, ataxia and confusion (Hazell 2009). It is interesting that in Wernicke encephalopathy the loss of

astrocytic glutamate transporters is a major precipitating event of the disease (Jhala et al. 2014). Studies in cultured astrocytes suggest that during thiamine deficiency astrocytes release factors into the tissue culture medium, which can downregulate the expression of astrocytic glutamate transporters in healthy astrocytes exposed to the medium (Hazell et al. 2001; Jhala et al. 2014). Finally it most likely will not matter much to families with BBGD why biotin administration saves the life of their children what matters is that the treating physician recognizes the disease before it is too late. The group of Karenfort et al. therefore recommends that any patient with symmetrical basal ganglia lesions and neurological symptoms be treated with thiamine and biotine until mutations in SLC19A3 have been excluded (Distelmaier et al. 2014).

7.3.3 The road to effective treatment modalities

I have now summarized a lot of concepts, new technologies and interesting studies performed in the mitochondrial disease field. The big question is has this knowledge, has science really helped patients? I have had the opportunity to tell a few families, after searching for years for answers why their children are sick or why they have died, that we have in fact delineated a cause. This is helpful since it brings closure or allows for prenatal diagnosis in future pregnancies. This knowledge, combined with invitro fertilization technology can also help to avoid a pregnancy with a gene defect altogether via preimplantation diagnosis or egg and sperm donation. But how about the children who are sick already and need our help? Have we let them down by just studying and rehashing the same concept's over and over without bringing real treatment options to the bedside? For one, if we truly want to help children who are sick, we need to first

believe that they actually can get well. Second we need to do everything we can to avoid making them sicker. This then would be a topic for another dissertation, which I will not embark. I would like though briefly mention the one outcome of my studies, which I am most proud of, and which could have the potential to be translated to the bedside.

Bioenergetic investigations in fibroblasts for patients with mutations in *LONP1*, which are discussed in chapter six of this dissertation have suggested a significantly increased susceptibility to the use of antibiotic/antimycotic in culture. But our findings are not new and have been investigated prior and a few studies have been published. The use of erythromycin for example, in a patient with the common Lebers Hereditary Optic Neuropathy (LHON) 11778 mtDNA mutation precipitated bilateral vision loss. This prompted cell culture experiments with the 11778 mtDNA mutation in a galactose vs glucose media with and without the use of gentamycin (Luca et al. 2004). In this study, cells with the 11778 mtDNA mutations were no longer able to grow in a galactose plus antibiotic culture media, but were able to grow in a glucose plus antibiotic media. Controls cells only showed slowed growth in galactose plus antibiotic media. These results shows that our findings are not isolated or *LonP1* specific but have application in the mitochondrial disease clinic in general.

The use of antibiotics in mitochondrial patients has previously been associated with ototoxicity particularly in association with bactericidal antibiotics like aminoglycosides, quinolones and beta-lactams (Kalghatgi et al. 2013). Aminoglycoside toxicity has long been known to affect individuals who carry otherwise benign mutations in the mtDNA coding for ribosomal RNA at position mt1555 (Guan 2011). The mt1555 mutation does not cause other health concerns and therefore carriers are usually not

aware of the fact that they have this gene change however, administration of aminoglycoside antibiotics will expose them to the irreversible hearing loss (Francis et al. 2013). Other gene defects also predispose to ototoxicity and it is estimated that 120,000 individuals lose their hearing due to aminoglycosides annually. Novel, designer aminoglycosides no longer target mitochondrial protein synthesis or prevent the antibiotic's entry into sensitive targets like hair cells to benefit mitochondrial disease patients (Shulman et al. 2014). But more importantly the sequencing of individual's DNA to assess their liability to ototoxicity before administering them could avoid the problem altogether. Nevertheless patients are not tested for these mutations before the administration of a systemic aminoglycoside (Huth et al. 2015).

Finally, we can learn much from the study by Kalghatgi et al. where investigators administered antioxidants in conjunction with the use of antibiotics, as described in chapter six of this dissertation (Kalghatgi et al. 2013). Their study is published in the journal *Science*, which suggests stringent peer review. Their findings demonstrate consistently the beneficial effects of co-administration of antioxidant and antibiotic in cell culture as well as animal studies leading to the amelioration of toxic effects caused by bactericidal antibiotics. Since the antioxidant N-aceylcysteine, used in the experiments is FDA approved and has not shown toxicity in patients, this treatment option has the potential to be translated to the clinic rather quickly. It is my hope that our findings in cells from patients with mutations in LonP1, once published, will help to add more strength to this cause.

Web Resources

The URLs for the data presented herein are as follows:

Allen Brain Atlas: <http://www.brain-map.org>

ANNOVAR: <http://annovar.openbioinformatics.org/en/latest/>

wANNOVAR: <http://wannovar.wglab.org/>

ClinVar: <http://www.ncbi.nlm.nih.gov/clinvar/>

DomPred - Protein Domain Prediction Server: <http://bioinf.cs.ucl.ac.uk/dompred>

ExAC- Exome Aggregation Consortium: <http://exac.broadinstitute.org>

GEO -Gene Expression Omnibus Repository: <http://www.ncbi.nlm.nih.gov/geo>

LOVD- Leiden Variant Server: <http://databases.lovd.nl/>

OMIM- Online Mendelian Inheritance in Man: <http://www.omim.org/>

NHLBI - Exome Sequencing Project-Exome Variant Server: <http://evs.gs.washington.edu/EVS/>

Mitophenome: <http://www.mitophenome.org>

NCBI-National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov>

POLG - Human DNA Polymerase Gamma Mutation Database: <http://tools.niehs.nih.gov/polg>

Primer3: <http://frodo.wi.mit.edu/primer3>

PYMOL: www.pymol.org

SHIELD - Shared Harvard Inner-Ear Laboratory Database: <https://shield.hms.harvard.edu/>

STRAP- Software Tool for Researching Annotations of Proteins:

<http://www.bioinformatics.org/strap/>

UCSC Genome Bioinformatics: <http://genome.ucsc.edu>

Unigene: <http://www.ncbi.nlm.nih.gov/unigene/>

WHO- World Health Organization: <http://www.who.int>

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