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Loss of function variants in human *PNPLA8* encoding calcium-independent phospholipase $A_2\gamma$ recapitulate the mitochondriopathy of the homologous null mouse

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Abstract

Mitochondriopathies are a group of clinically heterogeneous genetic diseases caused by defects in mitochondrial metabolism, bioenergetic efficiency, and/or signaling functions. The large majority of proteins involved in mitochondrial function are encoded by nuclear genes, with many yet to be associated with human disease. We performed exome sequencing on a young girl with a suspected mitochondrial myopathy that manifested as progressive muscle weakness, hypotonia, seizures, poor weight gain, and lactic acidosis. She was compound heterozygous for two frameshift mutations, p. Asn112HisfsX29 and p. Leu659AlafsX4, in the *PNPLA8* gene, which encodes mitochondrial calcium independent phospholipase $A_2\gamma$ (iPLA$_2\gamma$). Western blot analysis of affected muscle displayed the absence of PNPLA8 protein. iPLA$_2$s are critical mediators of a variety of cellular processes including growth, metabolism, and lipid second messenger generation, exerting their functions through catalyzing the cleavage of the acyl groups in glycerophospholipids. The clinical presentation, muscle histology and the mitochondrial ultrastructural abnormalities of this proband are highly reminiscent of *Pnpla8* null mice. Although other iPLA$_2$–related diseases have been identified, namely infantile neuroaxonal dystrophy and neutral lipid storage disease with myopathy, this is the first report of *PNPLA8*-related disease in a human. We suggest *PNPLA8* join

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the increasing list of human genes involved in lipid metabolism associated with neuromuscular diseases due to mitochondrial dysfunction.

Keywords

PNPLA8; mitochondrial dysfunction; phospholipase; dystonia

The myriad important roles assumed by mitochondria in cellular function, including energy production, metabolism, signaling pathways and the regulation of apoptosis, are reflected in the clinical diversity of human mitochondrial diseases. Significant pleiotropy exists for these disorders, which frequently present with neurologic and myopathic phenotypes, often involving multiple organ systems. The organs most reliant on mitochondrial energy production are most susceptible to such defects, such as the extraocular muscles and pancreas. Typical clinical features often include, but are not limited to, myopathy, diabetes mellitus, liver failure, ataxia, seizures, encephalopathy, sensorineural deafness and bone marrow failure (DiMauro and Schon, 2003). Given this complexity, the extensive display of locus and allelic heterogeneity present in mitochondriopathies is not surprising, with over 1,300 nuclear genes implicated in mitochondrial function and 265 disease genes identified to date (Pagliarini, et al., 2008). Mitochondrial diseases may be caused by maternally inherited or sporadic defects of the mitochondrial genome (mtDNA) or by defects in nuclear genes, which may be inherited in an autosomal recessive, dominant or X-linked manner (Thorburn and Dahl, 2001). Collectively, mitochondrial diseases are a significant source of morbidity and mortality, with a conservative estimate for prevalence being 1 in 5000 live births (Skladal, et al., 2003). The diverse clinical presentation of mitochondrial disease in conjunction with the large number of both nuclear and mitochondrial gene disease targets make molecular diagnoses of mitochondrial diseases challenging. With the growing use of whole exome sequencing (WES), definitive genetic diagnoses of mitochondriopathies are now possible, with important implications for prognosis and clinical management.

Herein, we report the use of exome sequencing to identify a novel human mitochondrial disease in patient CMH193, a 7 year old female who was evaluated for a suspected mitochondrial myopathy with dystonia, abnormal gait and abnormal muscle biopsy. This patient was a term baby born to a G4P4 mother by spontaneous vaginal delivery following an uneventful pregnancy, weighing 3.1 kg (25^{th} percentile). She was asymptomatic in the first year, meeting all developmental milestones; however, the parents noted toe-walking. At age 2, she was noted to have a positive Gowers sign, and by the age of 3 years could no longer walk. Independent sitting was restricted to 10 minutes due to fatigue. Her fine motor skills declined and speech became dysarthric. She had proximal muscle weakness which was more pronounced proximally, tight heel cords, subtle rightward deviation of the tongue, and generalized mildly increased tone. These symptoms have progressed over the past several years, with increased proximal muscle weakness and dysmetria. Hypotonicity progressed to spasticity of different opposing muscle groups. She recently developed complex partial seizures associated with recurrent left temporal lobe spikes on EEG, and slow wave discharges exacerbated by sleep. Treatment with a multivitamin containing iron, Carbidopa-Levodopa and leucovorin initially improved symptoms. Supplementation with CoQ10

increased appetite without subsequent weight gain, and persistence of a very lean body habitus. In addition, she developed significant lactic acidosis, requiring bicarbonate supplementation. Relevant positive family history includes a maternal history of migraines and renal stones, a paternal grandmother with early stroke (age 45), and multiple family members with late onset cardiac problems. Complete blood count, metabolic panel, AST, ALT, liver function, creatine phosphokinase have been performed multiple times and are normal, with the exception of a consistently elevated anion gap (17 mmol/L) since age 5. Karyotype, microarray, mtDNA sequence analysis for tRNAs, and mtDNA depletion studies were performed at age four and were normal. Neurotransmitter studies in cerebrospinal fluid performed at the age of 6 showed deficiency of 5-methyltetrahydrofolate. At age 7, elevated lactic and pyruvic acid was demonstrated in blood on multiple occasions (lactic acid 3.8-5.6; n=0.7-2.1 mmol/L and pyruvic acid 0.18-0.32; n=0.08-0.16 mmol/L) with ratios of 17.5-21.1 (n=<20); plasma acylcarnitine profile was normal. Initial MRI brain scan at 33 months of age showed subtle increased signal intensity involving the medial globus pallidus bilaterally which was no longer visualized two years later at the second study, suggesting maturation of myelination. MR spectroscopy at 33 months showed metabolites within the normal range including choline, creatine and NAA. CT of the head was also unremarkable. Accurate cognitive assessment of this patient is limited due to frequent seizures, level of fatigue, and oral motor dystonia, however she attends Elementary school and receives physical therapy, occupational therapy, and speech therapy.

Exome sequencing was performed on patient CMH193 and her two healthy parents (CMH466 & CMH467) following informed consent to participate in a study approved by the Institutional Review Board (IRB) of Children's Mercy Hospital (CMH). DNA was prepared utilizing the KAPA Biosystems library preparation kit (KAPA Biosystems, Woburn, MA) followed by Illumina TruSeqExome enrichment (Illumina, San Diego, CA). Enrichment efficiency was verified using quantitative real-time PCR. Samples were sequenced on an Illumina HiSeq 2000 instrument with TruSeq v3 reagents, yielding paired end 100 nucleotide reads. Alignment and variant calling were performed as previously reported (Bell, et al., 2011; Saunders, et al., 2012). Briefly, gapped alignment to reference sequences (Hg19 and GRCH37) was performed with GSNAP and the GATK. Nucleotide variants were genotyped and annotated utilizing RUNES (Saunders, et al., 2012). Variant analysis for all samples was completed utilizing Viking (Saunders, et al., 2012). The proband was sequenced to a depth of 7.7 gigabases resulting in median target coverage of 135×, which identified ∼170,000 nucleotide variants. The mitochondrial genome was represented at an average depth of 1835×. Variants were filtered to 1% minor allele frequency in an internal database of 1913 samples, then prioritized by ACMG categorization, OMIM identity and phenotypic assessment. No diagnostic genotypes were found in previously reported disease genes, however the patient was compound heterozygous for two frameshift variants, c.334_337delAATT (p.Asn112HisfsX29) and c.1975_1976delAG (p.Leu659AlafsX4) in *PNPLA8* (MIM# 612123; nomenclature based on NM_001256011.1; +1 as the A of the ATG initiation codon in the reference sequence; initiation codon as codon 1), which encodes iPLA$_2\gamma$. The two variants were confirmed by Sanger sequencing and submitted to the LOVD database (http://databases.lovd.nl/shared/transcripts/16455). Segregation analysis confirmed that the variants were inherited from carrier parents, consistent with an autosomal

recessive inheritance pattern. In addition, genotyping of two healthy siblings revealed they were negative for both variants. Both *PNPLA8* variants were absent from the NHLBI Exome Sequencing Project (EVS; http://evs.gs.washington.edu/EVS/) and an internal variant database of 1913 samples. Truncating *PNPLA8* variants were absent from the internal dataset and rare in the EVS, and homozygous truncating variants were absent.

Mammalian iPLA$_2$s serve critical roles in cellular signaling, growth, lipid homeostasis, second messenger generation and ion channel function (Mancuso, et al., 2000) by catalyzing the cleavage of acyl groups from glycerophosholipids resulting in the generation of free fatty acids and lysolipids. Released polyunsaturated fatty acids can be further metabolized by a variety of cycloxygenases, lipoxygenases and P450 enzymes generating potent lipid second messengers of signal transduction. Previous studies using both pharmacologic and genetic approaches have demonstrated the prominent roles of iPLA$_2$s in neurotransmitter release, long-term potentiation, and cognition (Mancuso, et al., 2009; Wolf, et al., 1995). In mitochondria, the products of iPLA$_2$s and their downstream metabolites play key roles in mitochondrial bioenergetics, signaling and the opening of the mitochondrial permeability transition pore (Moon, et al., 2012a). Of the 9 known PNPLA family members, four have previously been identified that differentially regulate various aspects of mitochondrial bioenergetics and signaling, including *PNPLA2* (MIM# 609059), *PNPLA3* (MIM# 609567), *PNPLA6* (MIM# 603197) and *PNPLA9* (MIM# 603604). Variants in three of these genes have been reported in association with human disease: *PNPLA2*, which encodes an adipose triglyceride lipase, is associated with Neutral Lipid Storage Disease with Myopathy, (MIM# 610717) (Fischer, et al., 2007; Janssen, et al., 2013; Reilich, et al., 2011), *PNLPA6* defects cause a broad spectrum of neurodegenerative diseases including Boucher-Neuhäuser and Gordon Holmes syndromes (MIM#s 215470, 612020) (Deik, et al., 2014; Synofzik, et al., 2014a; Synofzik, et al., 2014b; Topaloglu, et al., 2014; Wortmann, et al., 2014; Yoon, et al., 2013.) *PNPLA9* is associated with Infantile Neuronal Axonal Dystrophy (MIM# 603604) (Illingworth, et al., 2014; Khateeb, et al., 2006; Morgan, et al., 2006; Salih, et al., 2013; Tonelli, et al., 2010).

PNPLA8, the predominant phospholipase in mammalian mitochondria, has been extensively characterized using genetic loss of function and gain of function mouse models in conjunction with enantio selective mechanism-based inhibition (Elimam, et al., 2013; Mancuso, et al., 2007a; Mancuso, et al., 2000; Mancuso, et al., 2009; Mancuso, et al., 2007b; Moon, et al., 2012a; Moon, et al., 2012b; Yan, et al., 2005). The alterations in mitochondrial ultrastructure and function in the *Pnpla8* null mouse model bears a striking resemblance to CMH193. The *Pnpla8* -/- mouse has mitochondrial dysfunction, impaired learning, decreased exercise endurance, enhanced insulin sensitivity, a thin body habitus (from energy dissipation by mitochondrial uncoupling), and cold intolerance (Mancuso, et al., 2009).

In addition to the similarities in phenotypes, there are unique morphologic changes observed in mitochondria of the *Pnpla8* -/- mouse, which closely match those observed in the right quadriceps muscle of CMH193 at 4 years of age (Fig. 1). Specifically, mitochondria from CMH193 exhibit marked disarray of cristae and globular dense osmiophilic inclusions by EM analysis. In addition, subsarcolemmal aggregates of abnormal mitochondria with

concentric lamellar membranes and dense round or oval, strongly osmiophilic inclusions were observed, suggestive of degenerating mitochondria (Fig. 1b, c). No large aggregates of mitochondria were observed. In addition, variable fiber size was found with isolated small atrophic fibers of all subtypes observed, but with no ragged red fibers (Fig. 1a). Interestingly, there was a significant increase in secondary lysosomes containing residual bodies that appeared to be derived from mitochondria, which raised the possibility of lipofuscinosis. However, no pathogenic variants were found in lipofuscinosis genes. Test results supporting mitochondrial dysfunction in CMH193 include an elevated anion gap and moderately elevated lactate acid levels in blood requiring bicarbonate supplementation. In the absence of an elevated lactate: pyruvate ratio, these results suggest mitochondrial dysfunction rather than an electron transport chain defect. Consistent with this concept, oxidative enzyme reactions (SDH and COX) showed normal activity in muscle (Supp. Figure S1), and electron transport chain function was not affected (Supp. Table S1). In addition, CSF neurotransmitter studies in CMH193 showed decreased 5-methyltetrahydrofolate, and secondary CSF folate deficiency is seen in many disorders of mitochondrial function (Garcia-Cazorla, et al., 2008). The genotype observed in CMH193 (two frameshift variants) was expected to generate a null phenotype, secondary to a truncated or degraded product. To confirm this, Western blotting against iPLA$_2\gamma$ protein was performed on a muscle biopsy from the patient as well as three de-identified samples with known unrelated diagnoses (CPT2 deficiency, Duchenne muscular dystrophy, and pulmonary hypoplasia; Fig. 2). As predicted, Western blotting of tissue from the patient exhibited a dramatic decrease in multiple immunoreactive iPLA$_2\gamma$ bands known to be absent in the *Pnpla8* -/- mouse. In addition, a novel 110 KD band was present in “control” patients, but absent in CMH193. Due to the surprising coordinate disappearance of both the known iPLA$_2\gamma$ bands and the novel immunoreactive 110 kDa band in CMH193, further studies were undertaken to determine the identity of the latter. Protein was extracted from a cadaveric gastrocnemius muscle at 10hr post mortem and purified by sequential DEAE anion exchange and FPLC Mono Q chromatographies. The resultant highly purified 110 kDa protein was electrophoresed on SDS PAGE, visualized by silver staining, and the 110 kDa band was excised from the gel and subjected to trypsinolysis. Resultant peptides were identified by LC-MS/MS using an LTQ-Orbitrap. The results demonstrated the 110 kD band to be heat shock protein family member 4 (HSPA4) (Supp. Table S2). Furthermore, the 110 kDa band did not contain any peptides corresponding to those present in iPLA$_2\gamma$, either by traditional LC-MS/MS, or through the enhanced sensitivity provided by multiple reaction monitoring of known transitions of previously identified peptides in iPLA$_2\gamma$ (PNPLA8). Collectively, these results indicate that the “immunoreactivity” of the 110 kDa protein detected by Western blotting was due to a non-specific interaction of HSPA4 with the antibody. Previously, we reported the co-chromatography of a 63kDa iPLA$_2\gamma$ isoform and another member of the heat shock protein family that was distinct from HSPA4 (Yan, et al., 2005). We speculate that the concomitant disappearance of iPLA$_2\gamma$ and the 110 kDa band is likely due to the absence of a transcriptional activator that may be derived from intronic DNA in the *PNPLA8* gene, as has previously been described for other protein-chaperone systems (Moabbi, et al., 2012).

Thus, *PNPLA8* may be added to an increasing list of genes involved in lipid metabolism associated with neurodegenerative diseases (Boukhris, et al., 2013; Martin, et al., 2013; Schuurs-Hoeijmakers, et al., 2012; Tesson, et al., 2012). Other $iPLA_2$-specific disorders include *PLA2G6*, associated with infantile neuroaxonal dystrophy 1, *PNPLA2*, associated with neutral lipid storage disease with myopathy and *PNPLA6*, associated with Boucher-Neuhäuser and Gordon Holmes syndromes (Deik, et al., 2014; Synofzik, et al., 2014a; Synofzik, et al., 2014b; Topaloglu, et al., 2014; Wortmann, et al., 2014) as well as spastic ataxia and hereditary spastic paraplegia with or without motor neuropathy (Synofzik, et al., 2014a; Yoon, et al., 2013). In the view of this case report, and given that mitochondrial disorders are clinically heterogeneous diseases, there appears to be a variable spectrum in $iPLA_2$-related disorders. While patients with variants in *PNPLA2* share a recognizable phenotype characterized by slowly progressive adult onset proximal muscle weakness affecting the upper and lower limbs, distal muscle weakness is also present. In addition, half of the affected patients show cardiomyopathic changes as adults (typically around 40 years of age). Other variable features include diabetes mellitus, hepatic steatosis, hypertriglyceridemia, and possibly sensorineural hearing loss (Reilich, et al., 2011). Elevated serum transaminases and creatine are clinical markers of *PNPLA2* disease, but were not found in CMH193.

Children with infantile neuroaxonal dystrophy have a severe progressive psychomotor disorder with typical onset within the first 24 months of life and rapid progression of hypotonia, hyperreflexia, tetraparesis leading to loss of ambulation within 5 years, pyramidal signs and seizures. Skin biopsies showed axonal spheroids. Although brain iron accumulation is variable, MRI studies have demonstrated areas of increased intensity, early degenerative processes, and cerebellar cortical atrophy. Death usually occurs prior to age ten (Morgan, et al., 2006). *PLA2G6* variants were predicted to result in an almost complete lack of enzyme activity, although gain of function could not be excluded, as missense variants have been reported in families with adult-onset dystonia-parkinsonism (PARK14; MIM# 612953) and *PLA2G6* protein is active as a tetramer (Morgan, et al., 2006). As reported in our patient, *PLA2G6* affected patients have dystonia (Tonelli, et al., 2010). The biochemical activity of the respiratory chain complexes I-IV were normal in all patients with $iPLA_2$ variants examined thus far. Moreover, histopathological analysis of skeletal muscle in $iPLA_2$-related diseases revealed some similar findings (Khateeb, et al., 2006; Reilich, et al., 2011). In *PLA2G6*-related diseases, the degeneration of the mitochondrial inner membrane is well characterized and proposed to be the primary pathogenic mechanism (Illingworth, et al., 2014). This may also be the case with *PNPLA8*-related disease, where proteins involved in electron transport and ATP synthesis located in the inner membranes would be secondarily affected by altered membrane homeostasis but not the primary pathogenic mechanism.

Boucher-Neuhäuser and Gordon Holmes syndromes are defined by early-onset ataxia and hypogonadism plus chorioretinal dystrophy (Boucher-Neuhäuser syndrome) or brisk reflexes (Gordon Holmes syndrome), associated with variants in *PNPLA6* (Synofzik, et al., 2014a; Synofzik, et al., 2014b; Topaloglu, et al., 2014). In addition, *PNPLA6* pathogenic variants are also associated with spastic ataxia and hereditary spastic paraplegia, suggesting

a broad spectrum of neurodegenerative *PNPLA6*-related diseases (Synofzik, et al., 2014a; Yoon, et al., 2013). PNPLA6 catalyzes the hydrolysis of membrane phosphatidylcholine into fatty acids and lysophosphatidylcholines; defects may lead to disturbance of membrane homeostasis at the synaptic junctions in a variety of neuronal networks. *PNPLA6* shares 41% amino acid sequence identity with the Drosophila 'Swiss Cheese' (Sws) protein, and has been implicated in the regulating interactions between neurons and glia in the developing fly brain (Beck, et al., 2011). Secondly, it may affect cellular signaling in the nervous system by impacting biosynthesis of acetylcholine or other neurotransmitters (van Tienhoven, et al., 2002). Although there is an intriguingly widening range of phenotypes and variable severity associated with alteration in these genes, it is also possible that in addition to the loss of the *PNPLA8* protein, the absence of the unknown chaperone detected by immunoblotting (110-kD band) could contribute to the pathogenesis in our *PNPLA8* patient.

In summary, a causal link between the proband's phenotype and *PNPLA8* variantsis supported by the parallel alterations in neuromuscular dysfunction, cognitive deficits and the striking similarities in dysmorphic and degenerating mitochondria present in the *Pnpla8* -/- mouse. It appears that many metabolic and genetic factors may account for the clinical heterogeneity of iPLA2-related diseases. The quantitative importance of phospholipids in the nervous system makes the brain and peripheral nerves a critical target for dysfunction in cellular growth, signaling and lipid homeostasis (Lamari, et al., 2013; Mancuso, et al., 2009; Wortmann, et al., 2014). It remains to be seen whether other (milder) variants in this gene result in a different iPLA$_2\gamma$-related phenotype and whether the 110 kD heat shock protein can indeed provide additional insights into the chemical biology of iPLA$_2\gamma$ mediated mitochondrial dysfunction and its downstream sequelae.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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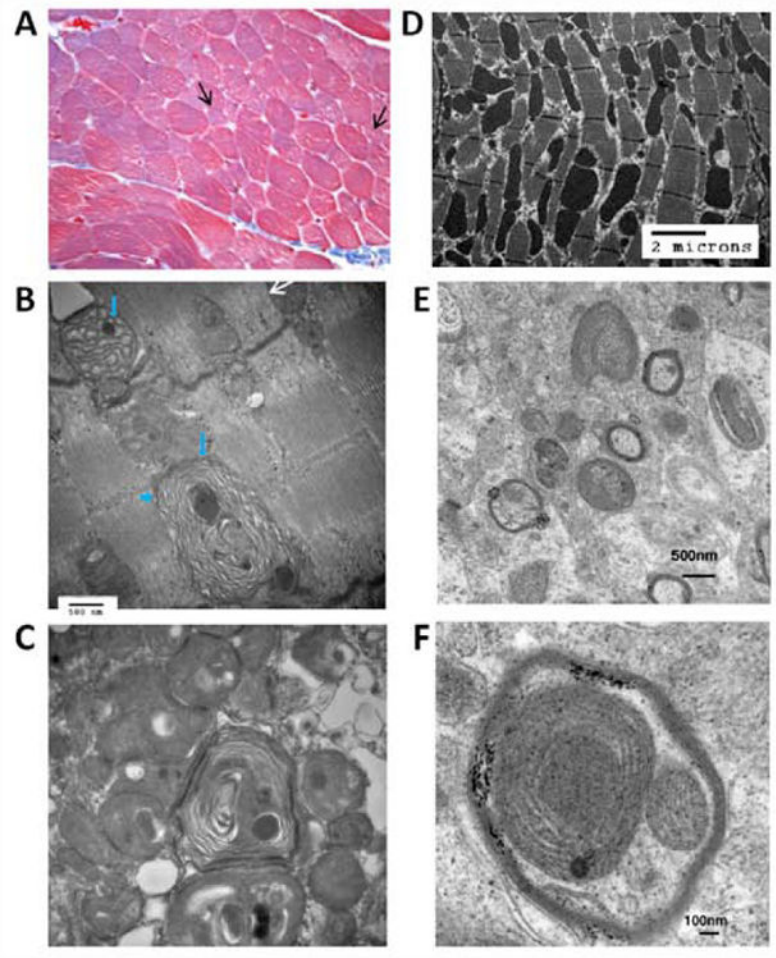

Figure 1. Comparison of Histological and EM findings in *PNPLA8*-affected tissues from human and mice

Panels (A)-(C) show paraffin-embedded muscle biopsy from patient CMH193. Trichromestaining (A) (magnification 400 ×) revealed variability in fiber size with isolated smallatrophic fibers (arrows). Normal fibers average 35 microns. ATPase stains (not shown) revealed that atrophic fibers were type 1 and 2 fibers. Ragged red fibers were not found. Oxidative enzyme reactions (SDH and COX) were normal and no large aggregates of mitochondria were demonstrable.

(B) EM photograph of muscle biopsy from CMH193 at 48000 magnification. Mitochondria show abnormal concentric disarray of internal cristae (arrow head) and globular dense osmiophilic inclusions (short arrows). White arrow shows a mitochondrion close to normal. (C) EM photograph of muscle biopsy from CMH193 at 36000 magnification, exhibiting subsarcolemmal aggregates of abnormal mitochondria with concentric lamellar membranes and dense round to oval osmiophilic inclusions. D) Electron micrograph of myocardium from the *Pnpla8* -/- mouse; E) Electron micrograph of the cerebellum from Pnpla8 -/- mouse exhibiting dysmorphic mitochondria; F) High power view of the cerebellum from the *Pnpla8* -/- mouse.

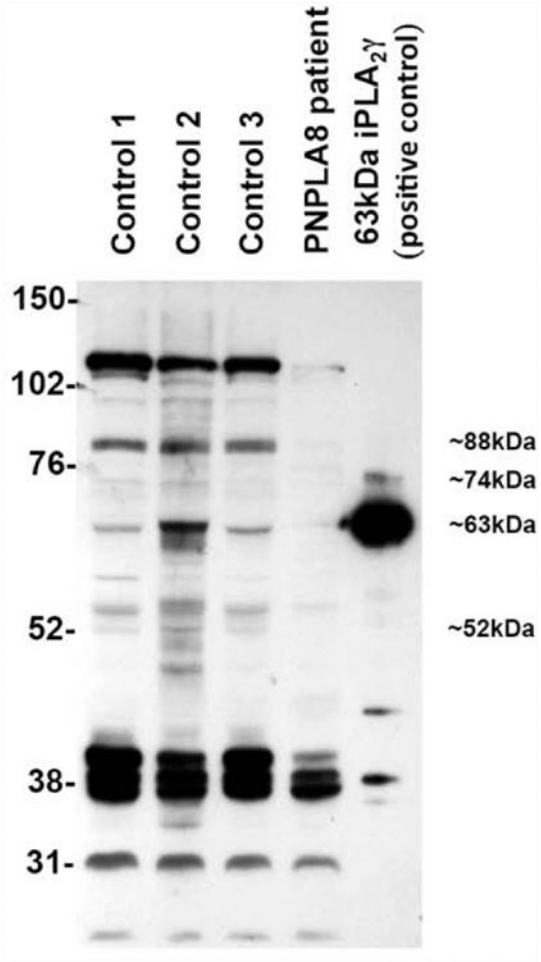

Figure 2. Near absence of PNPLA8 protein in CMH193

Western blot showing dramatic decrease of the 88, 63, and 52kD PNPLA8 protein (NP_001242940.1) in the muscle from CMH193, compared to muscle from three control samples with known non-mitochondrial diagnoses.