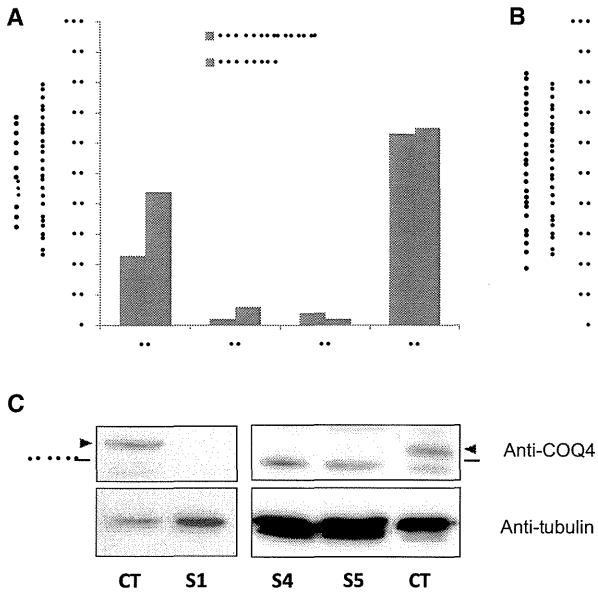

Figure 2. Biochemical Studies in *COQ4* Mutant Muscle and Fibroblasts
(A) CoQ_{10} in muscle from affected subjects S1 and S3–S5 is reported as a percentage of the mean of control values (the analyses were performed in different laboratories, and the reference values are diverse; see text). Data are reported after normalization to protein content or CS activity.
(B) Maximal respiration rate (MRR) measured in fibroblasts from subjects S1, S4, and S5; MRR values are expressed as percentages of MRR values obtained in control fibroblasts. The graphs represent the mean values from two independent experiments, each with six to eight replicates. Error bars represent the SD.
(C) Immunoblot analysis of COQ4 in fibroblasts from subjects S1, S4, and S5 and control individuals (Ct). Arrowheads indicate the band corresponding to COQ4. An antibody against tubulin was used as a loading control.

(88.9 μg CoQ_{10}/g protein; n.v. = 101–183; 1.70 μg nmol CoQ_{10}/CS; n.v. = 1.75–3.46) (Figure 2A). No residual sample from the muscle biopsy of S2 was available. Together, these findings are consistent with a deleterious role of the mutations identified in *COQ4*.

By Seahorse micro-oxygraphy,[16] we detected that maximal respiratory rates were lower in S1, S4, and S5 fibroblasts than in control cells (Figure 2B). Moreover, a drastic decrease in the amount of COQ4 was detected by immunoblot analysis in S1, S4, and S5 fibroblasts (Figure 2C), confirming that the identified *COQ4* nucleotide variants are deleterious.

The *Saccharomyces cerevisiae* ortholog of human *COQ4* is *yCOQ4*; *yCOQ4*-null strains have been reported to be effectively complemented by human *COQ4*.[10] In order to functionally test the effect of all the mutations found in our cohort, we transformed a *COQ4*-null strain (*Δcoq4*) by inserting the following *hCOQ4* variants into the multicopy pYES2.1 vector: pYES:$hCOQ4^{WT}$ (human wild-type [WT]), pYES2.1 (empty vector), pYES:$yCOQ4^{WT}$ (positive control), pYES:hcoq4$^{p.Arg145Gly}$ (mutation c.433C>G), pYES:$hcoq4^{p.Arg141*}$ (c.421C>T), pYES:$hcoq4^{p.Arg240Cys}$ (c.718C>T), pYES:$hcoq4^{p.Leu52Ser}$ (c.155T>C), pYES:$hcoq4^{p.Thr174del}$ (c.521_523delCCA), and pYES:$hcoq4^{p.Pro64Ser}$ (c.190C>T). In addition, to replicate the compound-heterozygous condition found in probands of families 2 and 3, we transformed the *Δcoq4* strain via a pYES construct harboring both the c.155T>C and the c.521_523delCCA mutations (pYES:hcoq4$^{p.Leu52Ser/p.Thr174del}$) and a pYES construct expressing the c.421C>T and c.718C>T mutations (pYES:$hcoq4^{p.Arg141*/p.Arg240Cys}$). A WT strain transformed with the pYES2.1 empty vector was also included as an additional control. In order to reveal a possible respiratory defect, we compared the growth of our transformant strains cultured in either glucose (a fermentable carbon source) or glycerol (a non-fermentable carbon source) after inducing gene expression with galactose for 4 hr. Notably, whereas the growth of the pYES2.1:$hCOQ4^{WT}$ transformant strain was comparable to that of the pYES2.1:$yCOQ4^{WT}$ transformant strain, the strains transformed with the *hCOQ4* mutant vectors grew as slowly as that transformed with pYES2.1 (Figure 3A). This result clearly indicates that each mutation reported in our probands leads to a virtually complete loss of function of the corresponding protein, COQ4. Next, we found that the CoQ_6 content in one *Δcoq4* mutant strain, $hcoq4^{p.Arg145Gly}$, was markedly decreased, whereas *Δcoq4* strains transformed with either pYES2.1:y*COQ4* or pYES2.1:*hCOQ4* had CoQ_6 levels similar to those in the WT strain (Figure 3B). This result indicates that mutant $hcoq4^{p.Arg145Gly}$ impairs CoQ biosynthesis.

Primary CoQ_{10} deficiency, caused by genetic defects in CoQ_{10} biosynthesis, is a clinically heterogeneous condition associated with a spectrum of different phenotypes, including encephalomyopathic forms with seizures and/or ataxia,[17–19] multisystem infantile forms with encephalomyopathy and renal failure,[20] nephrotic syndrome with sensorineural deafness,[21,22] adult Leigh syndrome,[23] and isolated myopathic forms.[24] Mutations in seven genes encoding proteins involved in CoQ_{10} biosynthesis have been reported in single families or in a few singleton cases;[25] the genetic defect has not been determined in most of the cases of CoQ_{10} deficiency, and only a few data are available regarding specific genotype-phenotype correlations. Secondary CoQ_{10} deficiency has been reported in association with glutaric aciduria type IIC (MIM 231680), caused by mutations in *ETFDH* (MIM 231675; encoding electron-transfer dehydrogenase); ataxia-oculomotor apraxia syndrome (MIM 208920), caused by mutations in *APTX* (MIM 606350; encoding aprataxin); a cardiofacio-cutaneous syndrome caused by a mutation in *BRAF* (MIM 115150; encoding serine/threonine-protein kinase B-Raf)[26]; and glucose transporter GLUT1 deficiency.[27]

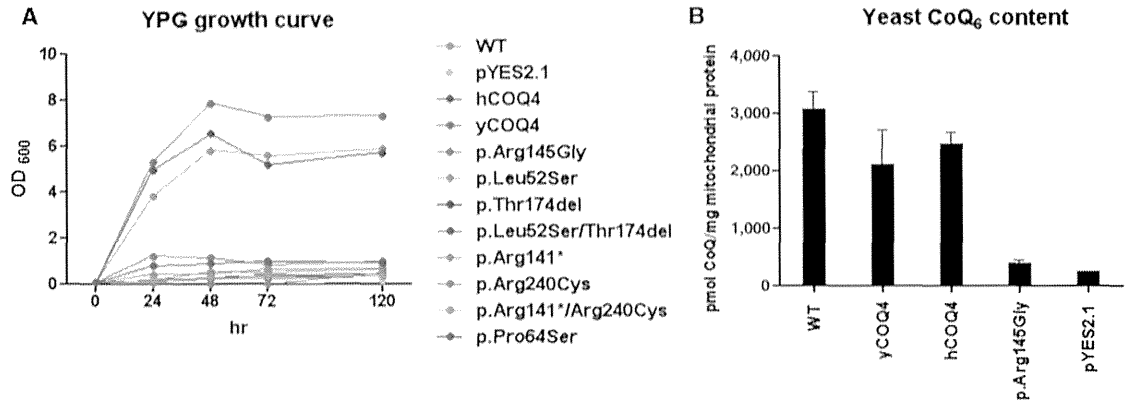

Figure 3. Yeast Studies

(A) Glycerol (YPG) growth of transformed *ΔCOQ4* yeast with the different mutated versions of human *COQ4* (pYES2.1, empty vector; hCOQ4, pYES:*hCOQ4*^{WT}; yCOQ4, pYES:*yCOQ4*^{WT}; c.433C>G, pYES:*hcoq4*$^{p.Arg145Gly}$; c.421C>T, pYES:*hcoq4*$^{p.Arg141*}$; c.718C>T, pYES:*hcoq4*$^{p.Arg240Cys}$; c.155T>C, pYES:*hcoq4*$^{p.Leu52Ser}$; c.521_523delCCA, pYES:*hcoq4*$^{p.Thr174del}$; c.190C>T, pYES:*hcoq4*$^{p.Pro64Ser}$; c.155T>C and c.521_523delCCA, pYES:*hcoq4*$^{p.Leu52Ser/p.Thr174del}$; and c.421C>T and c.718C>T, pYES:*hcoq4*$^{p.Arg141*/p.Arg240Cys}$). WT indicates the wild-type yeast transformed with the YES2.1 empty vector. Cells were grown in selective medium for 16 hr, induced in galactose for 4 hr, and inoculated in YPG at 0.1 U of optical density (OD) at 600 nm. Growth at 30°C was monitored over 5 days by measurement of OD cultures at 600 nm.

(B) Yeast mitochondrial CoQ_6 levels. Purified mitochondria lipid extraction and high-performance-liquid-chromatography quantification of CoQ_6 was performed in the *ΔCOQ4* strain transformed with the empty vector (pYES2.1), WT yeast (yCOQ4), or human (hCOQ4) or *hcoq4*$^{p.Arg145Gly}$ (c.433C>G) *COQ4* genes. A WT strain transformed with the empty vector was included as a positive control. Error bars represent the SD.

Interestingly, although the mechanisms linking these heterogeneous genetic conditions to a decrease in CoQ_{10} remain obscure, most of these individuals benefitted from CoQ_{10} supplementation.[28,29]

We found six *COQ4* mutations in five affected subjects from four unrelated families. All these individuals carried homozygous or compound-heterozygous mutations, clearly indicating that the resulting disease is an autosomal-recessive trait. Two alleles carried nonsense mutations, which are both transmitted by descent in combination with missense *COQ4* mutations to different individuals (S2 and sisters S3 and S4) and are predicted to lead to a truncated and aberrant COQ4. Given that the heterozygous parents carrying the nonsense mutations are alive and well, it is unlikely that COQ4 haploinsufficiency is pathogenic, even though a previous study reported on a boy carrying a de novo heterozygous deletion, including *COQ4*, in chromosomal region 9q34.[30] Because the biosynthetic pathway of CoQ is conserved throughout evolution from human to *Saccharomyces cerevisiae*, we modeled in yeast the mutations found in our subjects. Using this system, we demonstrated that each mutation, or the allelic combinations found in S2 and siblings S3 and S4, was associated with a severe defect of oxidative growth. In parallel, we also showed that COQ4 was strongly reduced in mutant fibroblast cell lines from S1, S4, and S5. In the skeletal muscle of S1 and S3–S5, the CoQ_{10} content was reduced as well. Taken together, these results demonstrate the pathogenic role of the *COQ4* mutations found in our cohort.

In keeping with the essential role of COQ4, four of our five subjects had a prenatal or perinatal onset with a fatal outcome in the first days of life. S1 and S2 presented with severe hypotonia, bradycardia, and respiratory insufficiency at birth; in S2, hypertrophic cardiomyopathy had been evident since fetal development. A markedly different, albeit equally severe, clinical presentation dominated by premature delivery, antenatal cerebellar hypoplasia, neonatal respiratory-distress syndrome, and epileptic encephalopathy characterized sisters S3 and S4. Rapidly progressive, severe lactic acidosis was a common feature in all four affected newborn subjects and is likely to have determined their fatal outcome. Involvement of the heart has been very rarely documented in CoQ_{10}-deficient subjects, often as part of multisystem phenotypes, where cardiomyopathy develops later than brain, muscle, or kidney impairment.[20] For instance, a homozygous nonsense mutation in *COQ9* was described in a baby who presented with neonatal lactic acidosis and later developed hypertrophic cardiomyopathy as part of a multisystem disease including intractable seizures, global developmental delay, and renal tubular dysfunction.[9] In spite of his early onset, the clinical course of S5 was slowly progressive and dominated by neurological deterioration with hardly any involvement of other organs, including the heart and kidneys.

Although the link between specific genetic defects and phenotypes is often unclear in mitochondrial disorders, organs with the highest energy requirements, such as the heart, kidneys, and brain, have the highest CoQ_{10} concentrations[31] and are the most frequently affected by CoQ_{10} deficiency. The level of expression of *COQ* genes in different cells seems to correlate poorly with the primarily affected tissue or organ; for instance, *COQ2*, mutations of which typically cause renal impairment, has expression

levels that are relatively higher in skeletal muscle and the heart than in other organs,[32] whereas *COQ4*, mutated in our subjects with cardiac or brain failure, is ubiquitously expressed and has relatively higher levels in the liver, lungs, and pancreas.

Because cardiomyocytes have a remarkably high energy requirement, and cardiomyopathy is quite common in individuals with various inherited mitochondrial disorders, the cardiac involvement in subjects with mutations in *COQ* genes can be overlooked. Indeed, the crucial role of CoQ_{10} in cardiomyocyte function has been recognized for a very long time; for instance, myocardial biopsies from individuals with congestive heart failure[33] or cardiomyopathy[34,35] show low CoQ_{10} levels, which correlate with the severity of heart damage.[36] Moreover, statins, cholesterol-lowering drugs that inhibit HMG-CoA reductase (the key enzyme common to the biosynthesis of both cholesterol and CoQ_{10}) can cause CoQ_{10} deficiency, ultimately leading to cardiomyopathy;[37] interestingly, this harmful side effect can be overcome by oral CoQ_{10} supplementation.[38] Moreover, long-term CoQ_{10} treatment of individuals with chronic heart failure is safe, improves symptoms, and reduces major adverse cardiovascular events.[39] These observations all converge on a strict association between CoQ_{10} deficiency and cardiomyopathy.

Notably, S3–S5 showed no sign of heart involvement, whereas the clinical phenotype was dominated by encephalopathy with seizures and a more progressive, but mainly neurological, syndrome is the clinical hallmark of S5, indicating the heterogeneity of the clinical presentations associated with *COQ4* defects. The variable specificity of organ failure (e.g., heart versus brain) in the neonatal cases of our cohort could be due to the fulminant course of the disease, which prevented the deployment of multisystem involvement. In support of this view, although cardiomyopathy dominated the clinical picture, the presence of severe hypotonia and hyporeflexia suggests concomitant involvement of the nervous system in S1 and S2 as well. Clinical heterogeneity was accompanied by an equally striking variability of the biochemical findings, which ranged from multiple (S1 and S5) to isolated (S2 and S3) ETC defects in muscle and fibroblasts to hardly any detectable defect at all (S4). This biochemical diversity could be due to differences in individual adaptive responses to reduced CoQ_{10} availability or could reflect the striking tissue specificity observed in the clinical presentations, but at the moment, a mechanistic explanation for these observations is lacking. Poor correlation with the clinical and biochemical phenotypes has also been reported for other genes related to CoQ_{10} biosynthesis. For instance, mutations in *COQ2*, the first mutated gene identified in affected individuals with primary CoQ_{10} deficiency, have been associated with a wide range of clinical presentations, often including nephrotic syndrome but also including fatal neonatal multisystemic disorder, Leigh syndrome, myoclonic epilepsy, hypertrophic cardiomyopathy, deafness, and adult-onset multisystem atrophy.[25,40] In any case, the identification of *COQ4* mutations in subjects with such a wide spectrum of clinical and biochemical abnormalities is a further indication of the advantage of unbiased screening such as WES for the identification of genes newly associated with mitochondrial disorders.

Unfortunately, the fulminant fatal outcome in S1–S4 was so rapid that it prevented both the diagnosis of CoQ_{10} deficiency and the start of CoQ_{10} supplementation. Prompt diagnosis is a main challenge for syndromes of primary CoQ10 deficiency but is very important given that co-factor deficiencies are virtually the only group of mitochondrial disorders for which beneficial pharmacological treatment is currently available. Treatment of the long-surviving subject, S5, has now started and will hopefully provide some useful indication of its efficacy in the near future.

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2014.12.023.

Acknowledgments

We would like to thank the families for their collaboration. We thank Roberto Bellavia, Yoshihito Kishita, Yoshimi Tokuzawa, and Ana Sánchez-Cuesta for their technical support; Consolato Sergi for his help; and K. Muroya and M. Adachi for referral of subject materials. This work was supported by Fondazione Telethon (GGP11011), the Italian Ministry of Health (GR2010–2316392 [D.G.]; "Ricerca corrente" [E.B.]), Fondazione CARIPLO (2011/0526), the Mariani Foundation, the Italian Association of Mitochondrial Disease Patients and Families (Mitocon), the European Research Council Advanced Grant FP7-322424, the German Ministry of Education and Research through the E-Rare project GENOMIT (01GM1207 [T.M.; H.P.], FWF I 920-B13 [J.A.M.], J41J11000420001 [D.G.]), the German Network for mitochondrial disorders (mitoNET; 01GM1113C [T.M.; H.P.]), the German Center for Heart Research (Z76010017300; Z56010015300 [T.M.]) by the German Research Foundation within the Munich Cluster for Systems Neurology (EXC-1010-SyNergy), the UK Medical Research Council, the Spanish Instituto de Salud Carlos III (FIS-PI11-00078), the Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology [Y.O.], Grants-in-Aid for the Research on Intractable Diseases (Mitochondrial Disease) from the Ministry of Health, Labour, and Welfare of Japan [A.O.; K.M.], and the Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics [K.M.]. We also acknowledge the Cell Lines and DNA Bank of Paediatric Movement Disorders and Mitochondrial Diseases and the Bank of Muscle Tissue, Peripheral Nerve, DNA, and Cell Culture of the Telethon Network of Genetic Biobanks (grant GTB12001J) and the EurobiobanK Network.

Received: November 20, 2014
Accepted: December 18, 2014
Published: February 5, 2015

Web Resources

The URLs for data presented herein are as follows:

Exome Aggregation Consortium (ExAC) Browser, http://exac.broadinstitute.org/

NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/

OMIM, http://www.omim.org

UCSC Genome Browser, http://genome.ucsc.edu

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The American Journal of Human Genetics
Supplemental Data

COQ4 Mutations Cause a Broad Spectrum of Mitochondrial Disorders Associated with CoQ_{10} Deficiency

Gloria Brea-Calvo, Tobias B. Haack, Daniela Karall, Akira Ohtake, Federica Invernizzi, Rosalba Carrozzo, Laura Kremer, Sabrina Dusi, Christine Fauth, Sabine Scholl-Bürgi, Elisabeth Graf, Uwe Ahting, Nicoletta Resta, Nicola Laforgia, Daniela Verrigni, Yasushi Okazaki, Masakazu Kohda, Diego Martinelli, Peter Freisinger, Tim M. Strom, Thomas Meitinger, Costanza Lamperti, Atilano Lacson, Placido Navas, Johannes A. Mayr, Enrico Bertini Kei Murayama, Massimo Zeviani, Holger Prokisch, and Daniele Ghezzi

SUPPLEMENTAL DATA

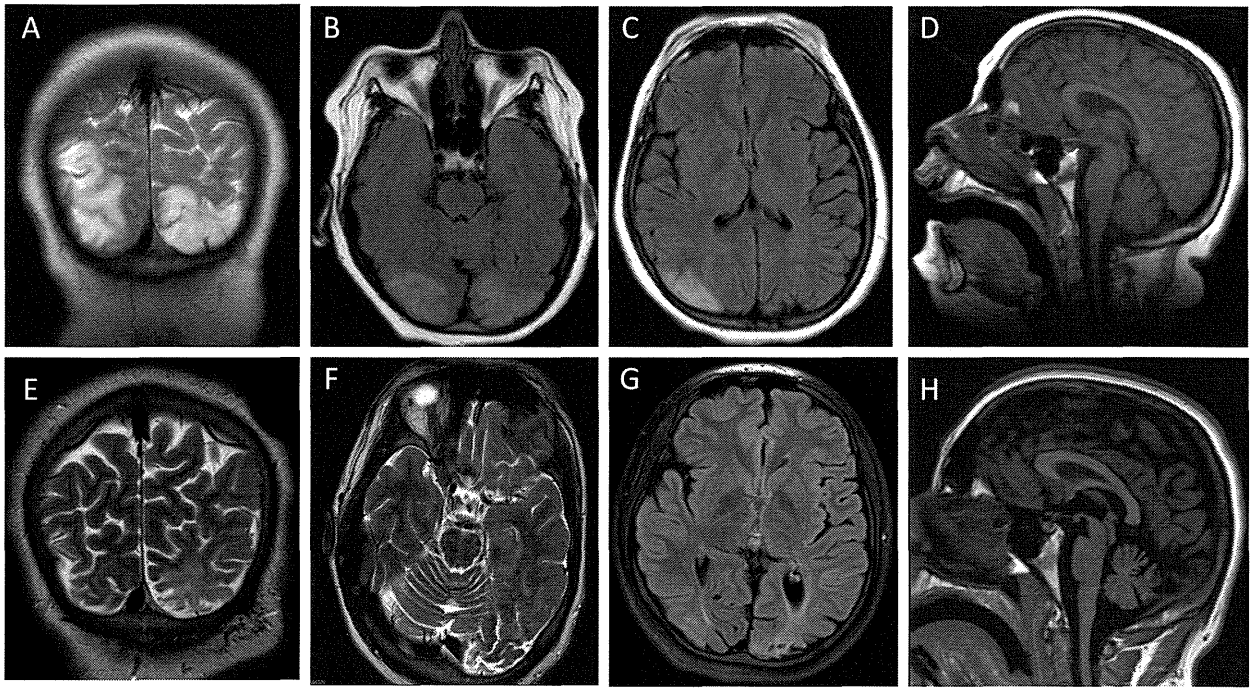

Figure S1. Brain MRI of subject 5.

Two serial brain MRI images of subject S5 obtained at age 12 years (A-D) and at age 17 years (E-H). A and E are coronal T2 weighted sections to show abnormal hyperintense cortical areas corresponding to infarct-like lesions of the occipital lobes at two different stages of progression to cortical atrophy with scars (E-G); The same occipital abnormal hyperintense cortical areas are shown in axial sections B and C (FLAIR weighted), F (T2 weighted) and G (FLAIR weighted). Notice that no abnormality is present in basal ganglia and brainstem. Finally, figures D and H are sagittal T1 sections showing progressive cerebellar atrophy of the vermis.

Id	Type	Reads	Mapped	Percent	Seq (Gb)	on bait	Avg cov	Cov 1x	Cov 4x	Cov 8x	Cov 20x
S1	SureSelect50Mbv5	106627996	105933934	99.35	10.77	78.48	132.44	99.92	99.72	99.35	97.38
S2	SeqCapEZ V1	130889538	125611825	95.97	13.81	62.71	155.83	98.36	97.32	96.41	93.80
S4	SureSelect50Mbv5	90821087	90214603	99.33	9.17	78.99	113.62	99.80	99.52	99.05	96.55
S5	SureSelect50Mbv4	125822167	124979728	99.33	12.71	73.98	146.50	99.95	99.80	99.55	98.22

Table S1: Exome Sequencing Statistics

Avg: average; Cov: coverage

Nucleotide Change	Amino acid Change	Subject	Status	Polyphen2	SIFT	PMUT	Mutation taster
c.[433C>G]	p.[Arg145Gly]	S1	Homozygous	Probably damaging	Deleterious	Pathological	Disease causing
c.[718C>T]	p.[Arg240Cys]	S2	CE with a nonsense mutation p.[Arg141*]	Probably damaging	Deleterious	Pathological	Disease causing
c.[155T>C]	p.[Leu52Ser]	S3, S4	CE with a deletion p.[Thr174del]	Probably damaging	Deleterious	Neutral	Disease causing
c.[190C>T]	p.[Pro64Ser]	S5	Homozygous	Probably damaging	Deleterious	Neutral	Disease causing

Table S2: In silico prediction of pathogenicity for *COQ4* mutations

CE: compound heterozygous.

REPORT

Mutations in *GTPBP3* Cause a Mitochondrial Translation Defect Associated with Hypertrophic Cardiomyopathy, Lactic Acidosis, and Encephalopathy

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Respiratory chain deficiencies exhibit a wide variety of clinical phenotypes resulting from defective mitochondrial energy production through oxidative phosphorylation. These defects can be caused by either mutations in the mtDNA or mutations in nuclear genes coding for mitochondrial proteins. The underlying pathomechanisms can affect numerous pathways involved in mitochondrial physiology. By whole-exome and candidate gene sequencing, we identified 11 individuals from 9 families carrying compound heterozygous or homozygous mutations in *GTPBP3*, encoding the mitochondrial GTP-binding protein 3. Affected individuals from eight out of nine families presented with combined respiratory chain complex deficiencies in skeletal muscle. Mutations in *GTPBP3* are associated with a severe mitochondrial translation defect, consistent with the predicted function of the protein in catalyzing the formation of 5-taurinomethyluridine (τm^5U) in the anticodon wobble position of five mitochondrial tRNAs. All case subjects presented with lactic acidosis and nine developed hypertrophic cardiomyopathy. In contrast to individuals with mutations in *MTO1*, the protein product of which is predicted to participate in the generation of the same modification, most individuals with *GTPBP3* mutations developed neurological symptoms and MRI involvement of thalamus, putamen, and brainstem resembling Leigh syndrome. Our study of a mitochondrial translation disorder points toward the importance of posttranscriptional modification of mitochondrial tRNAs for proper mitochondrial function.

Defects of the mitochondrial respiratory chain underlie a diverse group of human disorders characterized by impaired oxidative phosphorylation (OXPHOS). The generation of a functional respiratory chain requires the coordinated expression of both the nuclear genome and mitochondrial DNA (mtDNA). Defective translation of mtDNA-encoded proteins, caused by mutations in either the mitochondrial or nuclear genomes, represents a rapidly expanding group of human disorders, which often manifest as severe infantile combined OXPHOS deficiencies.[1]

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http://dx.doi.org/10.1016/j.ajhg.2014.10.017.

The mitochondrial genome contains a total of 37 genes, 13 of which encode protein subunits of the respiratory chain complexes and the ATP synthase. Translation of these genes is achieved by the organelle's own protein synthesis machinery, of which only the RNA components (rRNAs and tRNAs) are encoded by mtDNA. All protein factors required for mitochondrial translation are encoded in the nucleus and must be imported after their synthesis in the cytoplasm. Mitochondrial (mt-) tRNAs require extensive posttranscriptional modifications before achieving translation competency. Modifications to tRNAs might contribute to their proper folding, stability, or decoding capacity. In mitochondria a minimal set of 22 different tRNAs is used to translate all codons.[2] Modifications to the wobble position of the anticodon loop of mt-tRNAs play an important role in ensuring correct mRNA-tRNA interactions. In ten mt-tRNA species, all of which correspond to two codon sets, four different types of modified nucleotides have been identified at the wobble position.[3,4] One of these modifications is 5-taurinomethyluridine (τm^5U), found at position 34 (U34) of mt-tRNAsLeu^{UUR}, Trp, Gln, Lys, and Glu, which has been suggested to be synthesized cooperatively by GTPBP3 and MTO1.[5] In addition to τm^5 U, mt-tRNAs Gln, Lys, and Glu also contain a 2-thiouridine modification at U34 (s^2U), introduced by TRMU (also known as MTU1). This results in a 5-taurinomethyl-2-thiouridine ($\tau m^5 s^2 U$) modification in these mt-tRNA molecules. Modifications of U34 have been proposed to modulate either the accuracy or the efficiency of translation.[6,7] Three types of mutations affecting U34 have been associated with human mitochondrial disease: (1) mutations in the mt-tRNAs;[8] (2) mutations in *TRMU* (MIM 610230) affecting U34 2-thiouridylation and leading to acute infantile liver failure resulting from combined OXPHOS deficiency;[9] and (3) more recently, mutations in *MTO1* (MIM 614667) found to underlie cases of hypertrophic cardiomyopathy and lactic acidosis, associated with impaired mitochondrial translation rate and reduced respiratory chain activities.[10,11]

Whole-exome sequencing (WES) of 790 individuals with suspected mitochondriopathy in five centers identified eight index case subjects (plus two affected siblings) with homozygous or two heterozygous rare variants (minor allele frequency < 0.1%) in *GTPBP3* (MIM 608536), with no such case being found in 11,295 control subjects. This presents a genome-wide significant enrichment in *GTPBP3* (RefSeq accession number NM_032620.3) mutation load in samples from individuals with the clinical diagnosis "mitochondrial disease" ($p < 3.2 \times 10^{-10}$, Fisher exact test) in comparison to nonmitochondrial disorder samples. In addition, when filtering for genes coding for mitochondrial proteins,[12] in several individuals *GTPBP3* was the only gene with two mutations. Further evidence for the pathogenic role of *GTPBP3* mutations was derived from follow-up candidate gene sequencing of 18 individuals with similar phenotypes, which identified two more index cases. Collectively, mutations in *GTPBP3* were detected in 12 individuals from 10 families. However, segregation analysis of a single affected individual (#66654) revealed that the two identified heterozygous mutations in *GTPBP3* affected the same allele, leaving genetic evidence about 11 individuals from 9 families (Figure 1).

Written informed consent was obtained from all individuals investigated or their guardians, and the ethics committee of the Technische Universität München approved the study.

Individual #49665 (family F1, Figure 1A) is a boy born to consanguineous parents from the UAE. He presented at the age of 10 years with mild intellectual disability, fatigability, mild hypertrophic cardiomyopathy, and visual impairment. At presentation he measured 134 cm with a body weight of 25 kg. Clinical examination revealed slight dyspnea when climbing stairs and mild intellectual disability. Plasma lactate was consistently elevated (3.0 to 7.2 mmol/l, reference < 2.1 mmol/l). Electroencephalogram, hearing test, and visual-evoked potentials showed no abnormalities. Electrocardiography (ECG) revealed signs of left ventricular hypertrophy confirmed by echocardiography. There was no obstruction of the left ventricular outflow tract. He had a pale optic disc on both sides but visual acuity and visual field could not be examined. Brain MRI was normal, but MR spectroscopy revealed lactate peaks in the parietal and precentral cortex. Respiratory chain (RC) measurement in muscle revealed a significant reduction of complex I and IV activities. He was substituted with CoQ_{10} (200 mg/day), riboflavin (400 mg/day), carnitine (1 g/day), and a fat-rich diet (60% of daily caloric intake). A follow-up examination 1 year after the initial presentation showed no significant changes of his clinical signs/symptoms.

His 17-year-old elder brother, individual #36349 (family F1, Figure 1A), had a very similar clinical picture.

Individual #66143 (family F2, Figure 1A), a boy, is the second child of healthy unrelated parents of Arab-Moslem origin from Israel. He presented at the age of 2 years with sudden respiratory failure. Heart ultrasonography indicated a hypertrophic cardiomyopathy and congestive heart failure. His cardiac symptoms improved on treatment with furosemide, spironolactone, carvedilole, and digoxin. In addition, a high-dose vitamin treatment (100 mg/day riboflavin, 100 mg/day vitamin B1, and 60 mg/day CoQ_{10}) was initiated. RC enzyme measurement in muscle revealed a significant reduction of complex I and IV activities. On follow-up examinations (over 3 years), the child's psychomotor development is normal and his parents reported that he is active like his peers. Digoxin and spironolacton treatment was stopped and his recent echocardiography revealed a stable condition of the heart including normal global function of left ventricle with no further hypertrophy of interventricular septum and no pulmonary hypertension.

Individual #72425 (family F3, Figure 1A) was a girl born to unrelated parents. At 3 months of age, she had feeding difficulties and failure to thrive. At the age of 7 months,

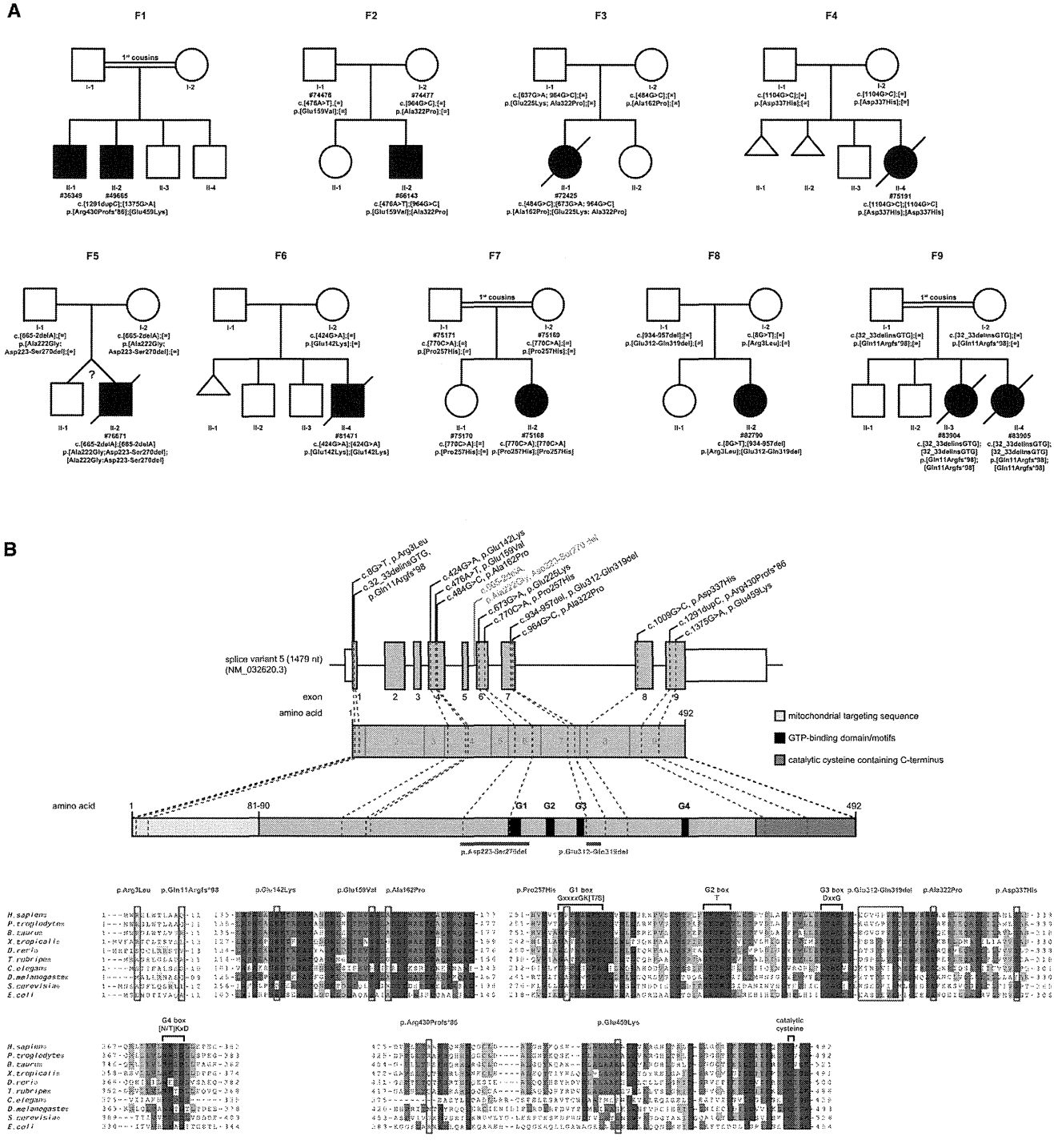

Figure 1. *GTPBP3* Mutation Status and Gene Structure

(A) Pedigrees of nine families with mutations in *GTPBP3*.

(B) Gene structure of *GTPBP3* with known protein domains of the gene product and localization and conservation of amino acid residues affected by mutations. Black and orange text indicate exonic and intronic variants. Intronic regions are not drawn to scale. Coloring in the sequence alignment represents the identity of amino acid residues.

she developed recurrent cough and fever and was admitted to the emergency room with severe fatigue, pallor, and progressive malaise. Blood exams showed leukocytosis, and 2 days later her general condition worsened, showing cyanosis and hyporeactivity. Echocardiography showed severe dilated cardiomyopathy with an ejection fraction of 20% that was unresponsive to therapy. She had severe refractory hyperlactatemia (23.3 mmol/l, reference range 0.5–2.3 mmol/l). Histochemical and spectrophotometric analysis of the muscle biopsy showed a severe complex IV deficiency. She died 10 days after admission from cardiac failure.

Individual #75191 (family F4, Figure 1A), a girl, was born to nonconsanguineous parents after an uneventful pregnancy of 40 weeks. The mother had had two miscarriages at 6 and 8 weeks and had a healthy son aged 16 months. In the first hours after birth, individual #75191 developed mild stridor and dyspnea which rapidly worsened. She fed poorly and became less responsive, and a Kussmaul breathing pattern was seen. She was transferred to a specialist center and was found to be severely hypotonic, moving very little, either spontaneously or after stimulation. She had hyperlactatemia (23 mmol/l), hypoglycemia (18 mg/dl), hyperammonemia (135 μmol/l, control value 11–48 μmol/l), and hyperlactaturia. She progressively developed respiratory insufficiency and bradycardia. Cardiac ultrasound showed apical right ventricular hypertrophy and an open duct of Botalli with minor shunting. Fractional shortening was 28% (mildly decreased). Cerebral ultrasound showed a minimal grade I bleeding, and the cerebral matter appeared mildly hyperechogenic. She died of asystolia at day 1. A muscle biopsy performed immediately after death showed decreased activities of RC complexes I and IV.

Individual #76671 (family F5, Figure 1A) was the second boy of nonconsanguineous parents. The infant was born at 41 weeks of gestation from a twin pregnancy. Generalized hypotonia and difficulty in suction was noted since birth and he rapidly developed failure to thrive. He acquired head control at the age of 7 months but parents reported normal cognitive skills. At the age of 9 months he was admitted to the intensive care unit for acute aspiration pneumonia that required intubation. Laboratory test revealed a metabolic acidosis with hyperlactatemia (5.2 mmol/l) and brain MRI showed bilateral thalamic T2-weighted hyperintense abnormalities with low diffusion. Analysis of a muscle biopsy revaled a clear reduction in histochemical cytochrome *c* oxidase activity and decreased complex I and IV enzyme activities. The cardiological examination disclosed hypertrophic cardiomyopathy and a Wolff-Parkinson-White pre-excitation syndrome (MIM 194200). The baby died after 15 days of hospitalization with clinical signs of heart failure.

Individual #81471 (family F6, Figure 1A) was a boy born to nonconsanguineous Romanian parents at 34 weeks gestation (birth weight 2.18 kg). His mother had premature and prolonged (85 hr) rupture of membranes before delivery, and the baby was treated with i.v. antibiotics before being discharged home on day 7. He was readmitted to hospital on day 25 with weight loss (2.23 kg). He was hypothermic and jaundiced and initial blood analysis showed profound metabolic acidosis. He was treated with i.v. antibiotics for presumed sepsis. The acidosis did not resolve, and serum lactate was elevated (11.0 mmol/l). ECG was abnormal and echocardiography showed concentric left ventricular hypertrophy. CSF lactate was 12.4 mmol/l (normal range 0.9–2.4 mmol/l) prompting bicarbonate treatment. Brain MRI showed abnormal diffusion of the subthalamic nuclei extending down to the brain stem. There was abnormal T2 signal in the midbrain and basal ganglia bilaterally. On examination he was thin but not dysmorphic. He was mildly jaundiced and had puffy feet. There was little spontaneous movement but normal muscle bulk and he was distinctly hypotonic. Feeding through a nasogastric tube was established but he did not become responsive despite high caloric intake. He developed recurrent apnea and died aged 5 weeks. Biochemical analysis performed in muscle revealed a significant decrease of RC complexes I and IV.

Individual #75168 (family F7, Figure 1A) is the second girl of first-cousin parents from India. She was first seen at the age of 2 years with development delay. She was able to walk but she couldn't speak. She received occupational and speech therapy. During a febrile illness when she was 3 years old, she had an acute metabolic failure with hyperlactatemia and hyperlactatorachia. She recovered but had epileptic seizures and more severe intellectual disability. Brain MRI showed pronounced bilateral hyperintensities affecting the whole thalamus and extending to the mesencephalon. Hyperlactatemia (>10 mmol/l) and hyperlactatorachia (6 mmol/l) were noticed. RC activity in muscle was normal as well as PDH complex tested by immunoblot. The girl was treated with qa carnitine 3 × 350 mg/day, CoQ_{10} 3 × 50 mg/day, vitamins B1 3 × 50 mg/day and B6 3 × 50 mg/day, and bicarbonate 4 × 1 g. Epilepsy was in good control with levetiracetame 40 mg/kg/day and a high-fat diet. The girl is in a special school for children with developmental delay. Her general condition is good. She is always in a good temper. Development is delayed about 1.5 years. She has continual hyperlactatemia (8–10 mmol/l).

Individual #82790 (family F8, Figure 1A) is a girl born at 40 weeks of gestation with normal birth weight to nonconsanguineous Japanese parents. At the age of 1 year, she developed frequent epileptic seizures, and she was medicated with phenobarbital. Severe developmental delay was noted and at the age of 15 months she was admitted to children's hospital. Her weight gain (9.25 kg, −0.06 SD) is within the normal range, but she developed severe muscle hypotonia. There is no cardiac involvement by ECG and echocardiogram. Hyperlactatemia was noted (5.72–6.49 mmol/l) whereas metabolic profiling of amino acids, urinary organic acids, and acylcarnitine was normal. RC analysis in muscle showed a significant decrease in complexes I and IV activities. Brain MRI showed bilateral hyperintensities in the putamen and weakly also in the anterior thalamus. A lactate peak was detected on $[H^+]$-MR spectroscopy. She is now 2 years of age and still presents with a severe global developmental delay.

Individual #83904 (family F9, Figure 1A) was the second child of consanguineous, healthy parents of Turkish origin. She was born at 39 weeks of gestational age (birth weight 2,740 g, length 49 cm, head circumference 32 cm). Shortly after birth, she presented with Wolff-Parkinson-White syndrome. Cardiac ultrasound was normal. Treatment was started with amiodarone and she

Table 1. Genetic and Clinical Findings in Individuals with *GTPBP3* Mutations

		***GTPBP3* Mutations**	**OXPHOS Activities in Skeletal Muscle**				**Clinical Features**				
ID	**Sex**	**cDNA (NM_032620.3) and Protein (NP_116009.2)**	**RCC**	**% of Lower Control Range**	**Absolute Values**	**Reference Range**	**AO**	**Course**	**HCM**	**Histochemical COX Defect**	**Other Features**
#49665[a,b]	male	c.[1291dupC; 1375G>A], p.[Pro430Argfs*86; Glu459Lys]	I	15%	0.025	0.17–0.56	10 years	alive 14 years	yes	ND	consanguineous parents (1st cousins), mild intellectual disability, fatigability, limited vision, lactic acidosis
			II	ND	ND	ND					
			II+III	normal	0.201	0.08–0.48					
			IV	24%	0.267	1.1–5.0					
#36349[b]	male	c.[1291dupC; 1375G>A], p.[Pro430Argfs*86; Glu459Lys]	I	no data	no data	no data	no data	alive 17 years	no data	no data	sibling of #49665 with similar clinical symptoms
			II								
			II+III								
			IV								
#66143[a]	male	c.[476A>T; 964G>C], p.[Glu159Val; Ala322Pro]	I	7%	0.01	0.19–0.48	2 years	alive 5 y ears	yes	ND	unrelated parents, sudden respiratory failure, lactic acidosis
			II	normal	0.10	0.07–0.12					
			II+III	normal	0.12	0.09–0.22					
			IV	28%	0.12	0.44–0.92					
#72425[a]	female	c.[484G>C; 673G>A; 964G>C], p.[Ala162Pro; Glu225Lys; Ala322Pro]	I	14%	0.015	0.11–0.30	3.5 months	died 8 months	DCM	yes	unrelated parents, cyanosis, hyporeactivity, DCM with residual ejection fraction of 20%, lactic acidosis
			II	normal	0.21	0.12–0.25					
			II+III	normal	0.06	0.006–0.14					
			IV	45%	0.76	1.7–4.0					
#75191[a]	female	c.[1009G>C; 1009G>C], p.[Asp337His; Asp337His]	I	31%	0.03	0.10–0.25	birth	died 1 day	yes	yes	unrelated parents, Kussmaul breathing, stridor, hypotonic, hyporeactivity, RVH, lactid acidosis
			II	normal	0.16	0.14–0.25					
			II+III	normal	0.12	0.13–0.25					
			IV	15%	0.09	0.60–1.48					
#76671	male	c.[665−2delA; 665−2delA], p.[Ala222Gly; Asp223_Ser270del; Ala222Gly; Asp223_Ser270del]	I	45%	0.05	0.11–0.30	birth	died 10 months	yes	yes	unrelated parents, hypotonia from birth, RVH, WPW, lactic acidosis
			II	normal	0.16	0.12–0.25					
			II+III	ND	ND	0.06–0.14					
			IV	17%	0.29	1.7–4.0					
#81471[a]	male	c.[424G>A; 424G>A], p.[Glu142Lys; Glu142Lys]	I	12%	0.012	0.104 ± 0.036	4 weeks	died 5 weeks	yes	yes	consanguineous parents, two healthy siblings, one miscarriage, FTT, poor weight gain and feeding, concentric LVH, lactic acidosis
			II	normal	0.098	0.145 ± 0.047					
			II+III	normal	0.850	0.544 ± 0.345					
			IV	17%	0.127	1.124 ± 0.511					

(*Continued on next page*)

Table 1. *Continued*

ID	Sex	*GTPBP3* Mutations: cDNA (NM_032620.3) and Protein (NP_116009.2)	OXPHOS Activities in Skeletal Muscle: RCC	% of Lower Control Range	Absolute Values	Reference Range	Clinical Features: AO	Course	HCM	Histochemical COX Defect	Other Features
#75168[a]	female	c.[770C>A; 770C>A], p.[Pro257His; Pro257His]	I	normal	no data	no data	2 years	alive 5 years	no	ND	consanguineous parents (1st cousins), developmental delay, epileptic seizures, intellectual disability, MRI hyperintense lesions of basal ganglia typical to Leigh syndrome, lactic acidosis
			II	normal							
			II+III	normal							
			IV	normal							
#82790[a]	female	c.[8G>T; 934_957del], p.[Arg3Leu; Gly312_Val319del]	I	36%	0.107	0.301 ± 0.05	1 year	alive 2 years	no	ND	unrelated parents, seizures, severe hypotonia, developmental delay, lactic acidosis
			II	normal	0.424	0.272 ± 0.05					
			II+III	normal	0.21	0.25 ± 0.093					
			IV	21%	0.008	0.035 ± 0.011					
#83904[a,c]	female	c.[32_33delinsGTG; 32_33delinsGTG], p.[Gln11Argfs*98; Gln11Argfs*98]	I	64%	4.2	6.5–17	1 week	died 9 months	yes	ND	consanguineous parents (1st cousins), lactic acidosis, WPW
			II	normal	16.1	13.6–45.7					
			II+III	normal	5.8	4.3–13.2					
			IV	25%	9.9	74–294					
#83905[a,c]	female	c.[32_33delinsGTG; 32_33delinsGTG], p.[Gln11Argfs*98; Gln11Argfs*98]	I	no data	no data	no data	birth	died 10 days	yes	ND	consanguineous parents (1st cousins), lactic acidosis, WPW
			II								
			II+III								
			IV								
#66654[a]	female	c.[673G>A; 964G>A]; [=] p.[Glu255Lys; Ala322Pro]; [=]	I	64%	0.09	0.14–0.35	1.5 months	alive	no	ND	intrauterine growth retardation, lactic acidosis, leukodystrophy, generalized hypotonia
			II	normal	0.19	0.18–0.41					
			II+III	90%	0.27	0.30–0.67					
			IV	normal	1.42	0.42–1.26					

Abbreviations are as follows: AO, age of onset; HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; FTT, failure to thrive; LVH/RVH, left/right ventricular hypertrophy; ND, not determined; WPW, Wolff-Parkinson-White syndrome.
Mitochondrial respiratory chain complexes (RCC) in muscle: I, NADH:CoQ-oxidoreductase; II, succinate:CoQ-oxidoreductase; II+III, succinate:cytochrome c reductase; IV, cytochrome c oxidase (COX).
Enzyme activities were determined in muscle biopsies and normalized to citrate synthase (CS). Absolute values and reference ranges are given in [mU / mU CS].
[a]Investigated by exome sequencing.
[b,c]These individuals are siblings.

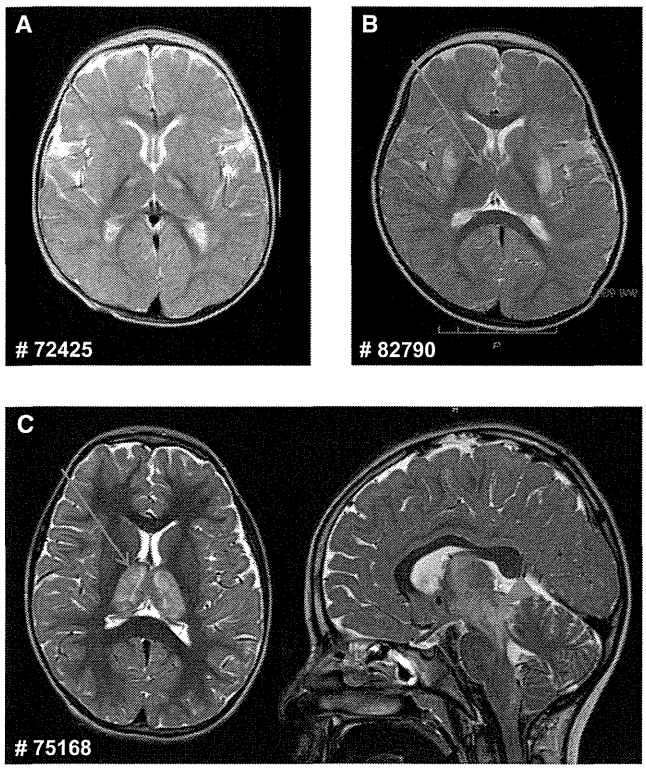

Figure 2. Brain MRI of Affected Individuals #72425, #82790, and #75168

(A) MRI of individual #72425 shows small T2 hyperintensities in the anterior thalamus bilaterally (arrow).

(B) In individual #82790, T2-weighted MRI shows bilateral hyperintensities in the putamen (arrowhead) and weakly also in the anterior thalamus (arrow).

(C) T2-weighted MRI of individual #75168 shows pronounced bilateral hyperintensities affecting the whole thalamus (arrow, axial view at the left) and extending to the mesencephalon (arrowhead, sagittal view at the right).

remained stable, without cardiac symptoms or arrhythmia. At 7 months of age, she had cardiogenic shock and metabolic acidosis. Heart ultrasound detected dilated cardiomyopathy and decreased contractility (ejection fraction 35%). She presented hyperlactatemia (20 mmol/l), hyperalaninemia (1,175 μmol/l; normal range, 190–450 μmol/l), and an increased lactate-to-pyruvate ratio (47; normal range, 10–20). Her disorder progressed despite intensive medication for heart failure. She died at the age of 9 months of cardiac insufficiency with arrhythmia.

Her younger sister, individual #83905 (family F9, Figure 1A), had a very similar clinical picture. She died at 6 months of age of cardiac insufficiency unresponsive to resuscitation procedures.

Genetic, biochemical, and clinical findings are summarized in Table 1. Pedigrees of the families we studied are shown in Figure 1A. The location of the identified mutations within the gene and the conservation of the affected amino acid (aa) residues are shown in Figure 1B. Individual #49665 (F1: II-2) was found to carry a frame shift and one missense variant. The next generation sequencing (NGS) data demonstrated a compound heterozygous status of the two variants (Figure S1 available online). Individual #76671 (F5: II-2) was homozygous for an intronic single base pair deletion, c.665−2delA, which is predicted to cause the loss of a splice acceptor site. Analysis of cDNA from fibroblasts revealed a shorter transcript, and sequencing found that in more than 95% of transcripts, the downstream acceptor of exon 7 was used for splicing, resulting in the skipping of exon 6 including the conserved G1-box guanine nucleotide-binding signature motif (Figure S2). Individual #82790 (F8: II-2) was found to be compound heterozygous for a missense mutation c.8G>T (p.Arg3Leu) and a 24 bp deletion c.934_957del (p.Gly312_Val319del). The 24 bp deletion is predicted to cause the deletion of 8 amino acids containing conserved residues. The p.Arg3Leu substitution at the very N terminus of the protein is scored as a predicted polymorphism but causes a loss of a positively charged residue, which is predicted to interfere with mitochondrial targeting (Predotar, PsortII). The two missense variants found in individual #66654, c.[673G>A; 964G>C], p.[Glu225Lys; Ala322Pro], were identical to the variants found on the paternal allele of individual #72425 (F3: II-1). Analysis of parental DNA revealed that both variants were also located on the same allele in individual #66654, meaning that only one allele is affected. Because of this observation, combined with the absence of a heart phenotype and because this individual is the only one exhibiting an isolated complex I defect, we consider the mutations found in *GTPBP3* not to be causative in subject #66654.

In summary, the identification of 13 different alleles in 11 individuals with suspected mitochondrial disease from 9 families provides strong evidence for the pathological role of mutant *GTPBP3* in the investigated families. It links *GTPBP3* mutations to combined respiratory chain complex deficiency (9/11), cardiomyopathy (9/11), lactic acidosis (11/11), and encephalopathy (4/11).

Brain MRI was performed in three individuals (Figure 2). It showed bilateral T2 hyperintensities in the thalamus, ranging from weak (#82790) or small (#72425) changes in the anterior thalamus to very pronounced hyperintensities affecting the whole thalamus in individual #75168. In addition, T2 hyperintensities affected the putamen bilaterally in individual #82790 and extended markedly to the mesencephalon in individual #75168. Taken together, the MRI involvement of basal ganglia and brainstem resembles the (MRI) findings in Leigh syndrome (which is, however, an ill-defined entity).

Skin fibroblast cell lines were available from seven individuals for functional studies. We first analyzed the cellular oxygen consumption rate (OCR^{13}) by microscale respirometry with the XF96 extracellular flux analyzer (Seahorse Bioscience). When cells of individuals from families F1 to F5 were cultured in glucose-containing medium, only cell lines from individuals #75191 (F4: II-4) and #76671 (F5: II-2) showed a decreased OCR (of 59% and 58%, respectively) indicating defective oxidative phosphorylation

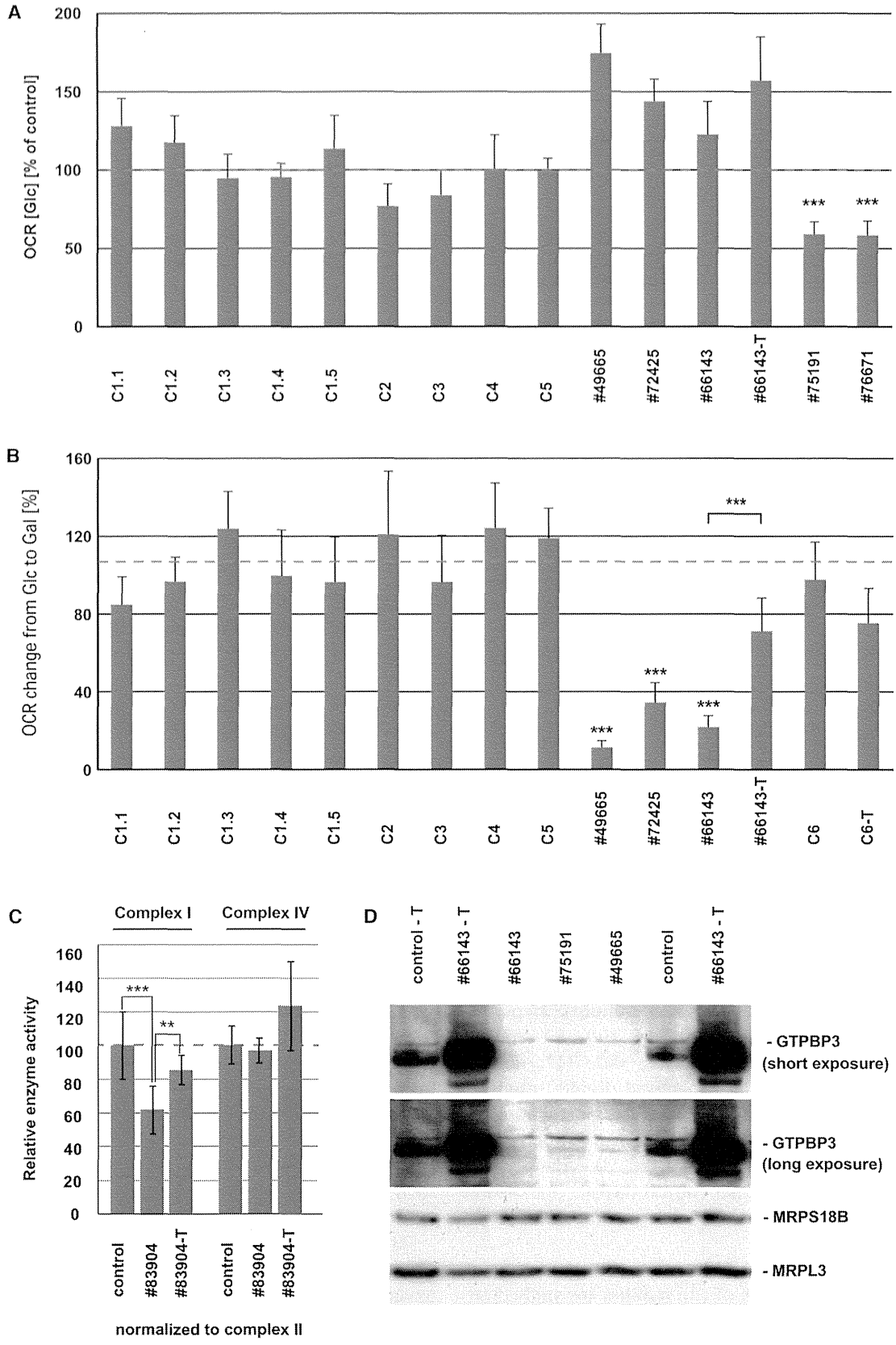

Figure 3. Analysis of Respiration Defects and GTPBP3 Protein Levels in Fibroblast Cell Lines
(A) Oxygen consumption rate (OCR) of fibroblast cell lines from five affected individuals and five control subjects cultured in high-glucose (Glc) medium. Each analysis was performed in more than 15 replicates. Control one (C1) was measured five time at different passage numbers (C1.1–1.5, NHDFneo, Lonza). OCR was expressed as percentage relative to the average of all controls. Cells from

(legend continued on next page)

(Figure 3A). When cells were cultured with galactose as the primary carbon source, rather than glucose, cells are forced to rely on oxidative phosphorylation rather than glycolysis in order to meet the energy demand.[14,15] Accordingly, in control cells an increase in OCR of approximately 2-fold was observed when galactose was substituted as the primary carbon source. This increase in OCR was impaired in fibroblasts from affected individuals #49665 (F1: II-2), #66143 (F2: II-2), and #72425 (F3: II-1), which showed OCR increases of only 11%, 35%, and 22%, respectively (Figure 3B). In order to confirm that defects in GTPBP3 are the cause of this defect, we transduced three cell lines with a wild-type copy of *GTPBP3* cDNA (RefSeq NM_32620.3) by using a lentiviral vector (pLenti 6.3/V5 TOPO, Life Technologies) as previously described.[16,17] Fibroblasts from individuals #49665 and #66143 were used for the rescue experiment, with fibroblasts from #66654 (subject with only one affected allele) being included as a control (C6). Unfortunately, we were unable to recover any viable cells from subject #49665 after the transduction procedure. Although the transduction had no noticeable effect on the control cell line (C6-T), transduced fibroblasts from #66143 (#66143-T) displayed a significant improvement of OCR in galactose-containing medium (Figure 3B). Furthermore, we detected an isolated respiratory chain complex (RCC) I deficiency in a fibroblast cell line from family 9. Cotransfection of individual #83904 fibroblasts with two putative *GTPBP3* isoforms amplified by RT-PCR, RefSeq NM_32620.3 and NM_0128855.2 (missing 63 base pairs of exon 8), significantly improved enzyme activities of RCC I (pIRES2-EGFP, Clontech) (Figure 3C). Analysis of the protein levels of GTPBP3 in five fibroblast cell lines demonstrated reduced or undetectable amounts in individuals #49665, #75191, #66143, #83904, and #83905, although they showed a clear increase after transduction or transfection (Figures S4 and 3D). In conclusion, our data demonstrate a causal role for *GTPBP3* mutations in the oxidative metabolism deficiency in these individuals.

Given that homologs of GTPBP3 in other systems have been implicated in protein synthesis, we next concentrated on the analysis of GTPBP3 in mitochondrial translation. The synthesis of mtDNA-encoded polypeptides, investigated by pulse-labeling of mitochondrial translation products via [^{35}S]methionine in fibroblasts of affected individuals (for methods see Haack et al.[18]) was severely and uniformly decreased to 20%–30% of control levels in individuals #49665, #66143, and #75191 (Figures 4A and 4B). There was no detectable defect in fibroblasts from individual #72425, which might be explained by the relatively low conservation of the mutated residue in this individual (Figure 1B). In order to exclude possible defects of mitochondrial transcription or precursor RNA processing, we analyzed all mitochondrially encoded rRNAs and mRNAs in fibroblasts of individuals #49665, #66143, #72425, and #75191 by RNA blotting and by RNA-seq in fibroblasts of individual #49665. We found no differences in the expression levels of the mt-RNAs between case and control subjects. On average, the mt-RNA expression levels were only 6% lower in individual #49665 as compared to control individuals (data not shown). We did not observe any appreciable reduction in steady-state levels of mature RNAs, nor was there any accumulation of precursor RNAs (Figure S3A). Next, we analyzed the steady-state levels of mt-tRNAs, including those five species for which the τm^5 U modification has been reported in mammals (Gln, Glu, Lys, Leu^{UUR}, and Trp).[4] We again observed no appreciable changes in their steady-state levels (Figure S3B). In order to further corroborate a direct role of GTPBP3 in mitochondrial translation, we downregulated its expression via RNA interference in HeLa cells (Figure 4C). Reduction of GTPBP3 protein levels upon RNAi treatment of HeLa cells was comparable to the reduction of its level in *GTPBP3* mutant fibroblasts (Figure 4D). Downregulation of *GTPBP3* expression resulted in a general mitochondrial translation defect, similarly to the reduction observed in subject fibroblasts (Figure 4D). In conclusion, the reduced translation efficiency observed in three out of four *GTPBP3* mutant cell lines, as well as in human cells treated with *GTPBP3* RNAi, confirmed an important function for GTPBP3 in efficient mitochondrial protein synthesis.

In order to test the consequences of this reduced translation rate upon the protein levels of OXPHOS complexes in mutant fibroblast cell lines, we analyzed the steady-state levels of several nuclear-encoded subunits of the OXPHOS system by immunoblotting. In fibroblasts from individuals #72425, #75191, and #76671 (F3: II-1, F4: II-4, and F5: II-2), we observed strongly reduced amounts of RCC IV. Fibroblasts from subjects #72425, #75191, and #49665 also showed reduced levels of RCC I, whereas the levels

individuals #75191 and #76671 showed a significant reduction of oxygen consumption whereas cells from individuals #49665, #72425, and #66143 presented no defective respiration. Error bar indicates 1 SD; ***$p < 0.001$.

(B) Oxygen consumption rate of fibroblast cells cultured in galactose (Gal) growth medium. The average increase of OCR from five control cells cultured in galactose-containing medium compared to glucose-containing medium was 107%. Cell lines from individuals #49665, #72425, and #66143 show significant lower increase in OCR. Lentiviral expression of wt*GTPBP3* in cells from individual #66143 significantly increases the change in OCR although it has only little effect in control cells (C6-T). Error bar indicates 1 SD; ***$p < 0.001$.

(C) Activities of respiratory chain complexes I and IV (expressed as ratio to CII activity) are decreased in individual #83904 cells transfected by electroporation with empty vector (pIRES2-EGFP) according to the manufacturer's protocol (LONZA) but are improved upon expression of *GTPBP3* cDNAs from the same plasmid. Measurements were performed as previously described.[29,30] Error bar indicates 1 SD. Activity in controls was set as 100%. **$p < 0.01$, **$p < 0.001$.

(D) Levels of GTPBP3 were reduced in cells from individuals #49665, #75191, and #66143 and elevated after transduction with wt*GTPBP3* cDNA. MRPS18B and MRPL3 served as mitochondrial loading controls.

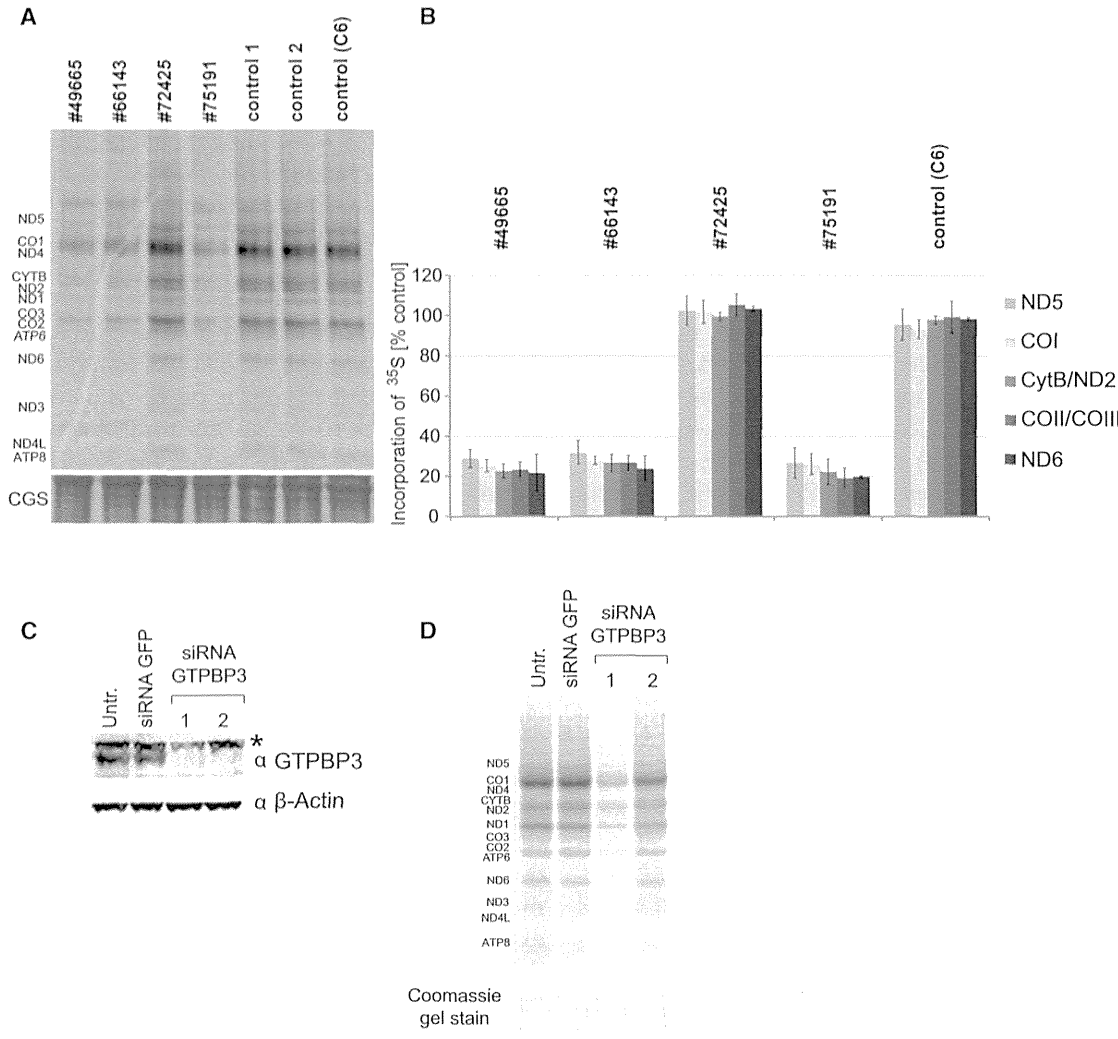

Figure 4. Analysis of Mitochondrial Protein Synthesis in Primary Fibroblasts and in HeLa Cells Treated with RNAi against GTPBP3
(A) [^{35}S]methionine metabolic labeling of mitochondrial proteins in fibroblasts. Products of mitochondrial translation were labeled with [^{35}S]methionine for 30 min, separated by a 4%–12% gradient SDS-PAGE, and visualized by autoradiography. To validate equal protein loading, a small section of the gel was stained with Coomassie (CGS). Fibroblasts from individuals #49665, #66143, and #75191 demonstrate significant inhibition of mitochondrial protein synthesis although translation in cells from individual #72425 is not affected.
(B) Quantification of radiolabelled products of mitochondrial translation. Incorporation of [^{35}S] as in (A) was quantified by ImageQuant software after exposure to a PhosphorImager screen from three independent experiments. Error bar indicates 1 SD.
(C) Downregulation of GTPBP3 in HeLa cells via RNA interference. Immunoblot analysis of total HeLa cell lysate transfected with two different siRNA to GTPBP3 show decreased level of GTPBP3 upon RNAi treatment for 6 days. siRNA to GFP was used as transfection control. Asterisk indicates nonspecific band recognized by anti-GTPBP3 antibody in HeLa cells. β-actin serves as a loading control. Two different siRNA duplexes targeting GTPBP3 were used, 1 and 2.
(D) Mitochondrial translation in HeLa cells upon GTPBP3 downregulation. HeLa cells were transfected for 6 days with siRNA against GTPBP3 and subjected to [^{35}S]methionine metabolic labeling. Inactivation of GTPBP3 leads to the reduced efficiency of mitochondrial translation. Two different siRNA duplexes targeting GTPBP3 were used, 1 and 2.

of RCC II and V remained normal in all cell lines (Figure 5). The diminished steady-state levels of respiratory chain complexes I and IV in fibroblast cell lines are in agreement with the impaired mitochondrial de novo translation rates in these cells and match the enzymatic defects identified in muscle biopsies of the same individuals.

Within an international cooperation between European (Germany, UK, Italy, France, and Belgium), Israeli, and Japanese Centers for mitochondrial disorders, we provide statistically convincing evidence for *GTPBP3* mutations leading to mitochondrial disease. To further support collaborative studies, the global mitochondrial disease community has established a Mitochondrial Disease Sequence Data Resource (MSeqDR) for common genomic data deposition and mining.

The genotype-driven analysis performed here was independent from the clinical presentation. Nevertheless, we identified common clinical features of the affected individuals that include lactic acidosis (11/11), cardiomyopathy (9/11), and neurological symptoms (6/11). The latter

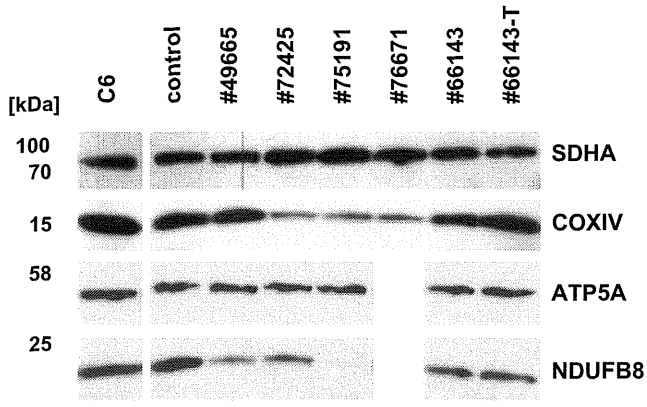

Figure 5. Immunoblot Analysis of OXPHOS Proteins in Fibroblasts

10 μg of detergent-solubilized total cell extract was subjected to immunoblot analysis of OXPHOS components. Amounts of SDHA (complex II) and ATP5A (ATPase) were unchanged in all individuals. In cells from individuals #72425, #75191, and #76671, a reduction of COXIV (complex IV) was observed. Cells from individuals #49665, #72425, and #75191 showed decreased levels of NDUFB8 (complex I). Antibodies used: mouse antibodies against SDHA (ab14715), NDUFB8 (ab110242), ATP5A (ab14748), and rabbit antibodies against COXIV (ab16056) from Abcam and rabbit anti GTPBP3 (HPA042158) from SIGMA Aldrich.

comprised symptoms such as development delay, intellectual disability, feeding difficulties, muscle hypotonia, fatigue, visual impairment, and epileptic seizures. Severity of the disease ranged from neonatal onset and death to late-infantile onset and survival into the second decade of life. Most affected individuals, however, manifested clinical symptoms before their first birthday. This is consistent with the normal cellular respiration, in organello translation, and normal levels of respiratory chain complexes reported in individuals less severely affected and the significantly reduced mitochondrial translation, respiration, and low levels of complex I and IV in those severely affected.

Modifications of the tRNA "wobble-base" in the anticodon loop are required for accurate and efficient codon recognition. The modification of position 5 (xm^5) of the U34 wobble-base of certain tRNAs is evolutionarily well conserved, although different modified side chains have been identified in different species. In mammals, mitochondria 5-taurinomethyluridine (τm^5U34) is found at the wobble-base position.[19] Based upon studies in bacteria and yeast mitochondria, GTPBP3 and MTO1 have been proposed to generate this modification in mammalian mitochondria. Although this prediction awaits direct biochemical validation, the proposed functional conservation of GTPBP3 and MTO1 has been supported by the mitochondrial localization of these proteins in human cells and by complementation of the respiratory-deficient phenotype in yeast by their mammalian homolog cDNAs.[20,21] Functional deficiency of homologs of GTPBP3 and MTO1 in bacteria and yeast mitochondria has been associated with abnormal U34 modification and consequently a reduced efficiency of translation.[21–23] Our data support an analogous activity of GTPBP3 in human mitochondria since we identified a reduced efficiency of translation in three cell lines with *GTPBP3* mutations and in cells with RNAi-mediated downregulation of GTPBP3 expression. Other groups have also reported impaired protein synthesis and reduced mitochondrial function in GTPBP3-depleted cells.[24] The defect in mitochondrial translation was a likely cause of the combined respiratory chain complex deficiency detected in muscle tissues of all but one affected individual.

Like *GTPBP3* mutations, *MTO1* mutations are also associated with hypertrophic cardiomyopathy (HCM), lactic acidosis, and combined respiratory chain deficiency. An association of *MTO1* mutations with impaired mitochondrial translation has yet to be shown for human mitochondria, but the common clinical presentation provides support for a common pathomechanism in the U34 modification for both diseases. So far, all individuals with *MTO1* mutations presented a HCM. However, nearly all of them have been specifically screened for *MTO1* mutations based on the clinical presentation of a HCM. Clinical and MRI signs of brain involvement are found for both *GTPBP3* and *MTO1* cases. The genotype-driven investigation presented here identified individuals with lactic acidosis, developmental delay, and MRI involvement of thalamus, putamen, and brainstem but without HCM. It can be expected that the clinical spectrum associated with MTO1 deficiency will also broaden, with more subjects being genome-wide investigated. In a very recent study, Taylor et al. indeed reported a case subject with *MTO1* mutations and central neurological features who did not have a cardiomyopathy.[25]

Our study highlights that defects in mitochondrial translation, probably owing to incorrect posttranscriptional modification of mt-tRNAs, are an important contributory factor to the spectrum of human mitochondrial disease. Recent data have suggested that more than 7% of all mt-tRNA residues undergo posttranscriptional modification, with close to 30 different modifications so far described.[4] Therefore, it is expected that future WES analyses of individuals clinically diagnosed with mitochondriopathy will reveal further mutations within genes coding for mt-tRNA modifiers. Indeed, in addition to the aforementioned mutations in *MTO1* and *TRMU*, mutations in *PUS1* (MIM 608109) (which introduces pseudouridine [Ψ] at base positions 27, 28, and 29 in several mt-tRNAs) have been reported in subjects affected with mitochondrial myopathy and sideroblastic anemia (MLASA)[26] (MIM 600462) and very recent studies have identified mutations in *TRIT1* (which is responsible for i^6A37 modification of a subset of mt-tRNAs) in individuals with severe combined mitochondrial respiratory chain defects.[27] Furthermore, mtDNA mutations in mt-tRNA genes, which are a very frequent cause of human respiratory chain deficiencies (MITOMAP), might also affect mt-tRNA modification. Related to the present study, it has been reported that τm^5U34 is not present in mt-tRNALeu^{UUR} harboring the m.3243A>G mutation (or other pathological mutations) responsible for mitochondrial encephalopathy, lactic

acidosis, and stroke-like episodes (MELAS) (MIM 540000). The absence of τm^5U34 has been suggested to be responsible for the mitochondrial translation defect in these subjects.[28] These results imply that deficiency of mt-tRNA modification plays a critical role in the molecular pathogenesis of human respiratory chain disease. Further studies of these pathways, such as analysis of tissue-specific regulation of mt-tRNA-modifying enzymes, might help to explain the clinical heterogeneity observed for mitochondrial diseases caused by mutations in mt-tRNA genes.

In conclusion, this study shows a mitochondrial translation disorder with a broad spectrum of clinical presentations, which emphasizes the importance of post-transcriptional modification of mitochondrial tRNAs for proper mitochondrial function.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2014.10.017.

Acknowledgments

We thank C. Terrile, M. Borzes, and C. Fischer for technical support and F. Miyake and T. Wada for referral of sample materials. This work was supported by the Deutsche Forschungsgemeinschaft within the framework of the Munich Cluster for Systems Neurology (EXC 1010 SyNergy), the German Bundesministerium für Bildung und Forschung (BMBF) through funding of the E-Rare project GENOMIT (01GM1207 for T.M. and H.P., 2011-RARE-005-03 for A.R. and M.D.M., J41J11000420001 for M.Z., and FWF I 920-B13 for J.A.M.), German Network for Mitochondrial Disorders (mitoNET 01GM1113C for T.M., H.P., and P.F. and 01GM1113A for T.K.), the German Center for Heart Research (Z76010017300 and Z56010015300 for T.M.), European Commission 7th Framework Program (Project N261123 GEUVADIS), Medical Research Council, UK (MC_U105697135 for T.J.N., J.R., S.F.P., C.A.P., and M.M.), Wellcome Trust Strategic Award (096919/Z/11/Z for R.W.T. and P.F.C.), MRC Centre for Neuromuscular Diseases (G0601943), UK NHS Highly Specialised "Rare Mitochondrial Disorders of Adults and Children" Service for R.W.T. and P.F.C., Fund for Scientific Research Belgium (FWO, contract number G.0200.10 for A.V., J.S., and R.V.C.), Fondazione Telethon (GGP11011 and GPP10005), Italian Ministry of Health (GR2010-2316392), CARIPLO (2011/0526), Pierfranco and Luisa Mariani Foundation, and Italian Association of Mitochondrial Disease Patients and Families (Mitocon) to D.G., F.I., E.L., and M.Z., Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation), Grant-in-Aid for the Development of New Technology from The Promotion and Mutual Aid Corporation for Private Schools of Japan from MEXT for Y.O., Grants-in-Aid for the Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan for A.O., Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics for K. Murayama, Association Française contre les Myopathies (AFM) for A.R. and M.D.M., and Fellowship from the AFM (16615 for M.D.M.).

Received: July 30, 2014
Accepted: October 29, 2014
Published: November 26, 2014

Web Resources

The URLs for data presented herein are as follows:

MITOMAP, http://www.mitomap.org/MITOMAP
MSeqDR, https://mseqdr.org/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
Predotar, https://urgi.versailles.inra.fr/predotar/predotar.html
PSORTII Prediction, http://psort.hgc.jp/form2.html
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq

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