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C6ORF66 Is an Assembly Factor of Mitochondrial Complex I

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Homozygosity mapping was performed in five patients from a consanguineous family who presented with infantile mitochondrial encephalomyopathy attributed to isolated NADH:ubiquinone oxidoreductase (complex I) deficiency. This resulted in the identification of a missense mutation in a conserved residue of the *C6ORF66* gene, which encodes a 20.2 kDa mitochondrial protein. The mutation was also detected in a patient who presented with antenatal cardiomyopathy. In muscle of two patients, the levels of the C6ORF66 protein and of the fully assembled complex I were markedly reduced. Transfection of the patients' fibroblasts with wild-type *C6ORF66* cDNA restored complex I activity. These data suggest that C6ORF66 is an assembly factor of complex I. Interestingly, the *C6ORF66* gene product was previously shown to promote breast cancer cell invasiveness.

Introduction

Congenital disorders of the mitochondrial respiratory chain are common inborn errors of metabolism with an incidence of 1:5000–8000 live births.[1,2] Among these, isolated NADH:ubiquinone oxidoreductase (complex I) deficiency (MIM 252010) is the most frequently diagnosed, accounting for one-third of all cases of respiratory-chain deficiency.[3] Complex I is the largest and most intricate of the five mitochondrial respiratory-chain complexes. It comprises 45 proteins, of which seven are encoded by the mitochondrial DNA (mtDNA) and the rest by the nuclear genome.[4] Mutations in mtDNA genes were detected in only 20% of the children with complex I deficiency,[5,6] suggesting that the vast majority of patients with isolated complex I deficiency suffer from mutations in nuclear genes encoding complex I structural subunits or assembly factors. Heretofore extensive sequence determination of candidate genes revealed mutations in ten structural-subunits genes (reviewed in[7]) and in two assembly factors of complex I, *NDUFA12L* and the human *NDUFAF1*.[8,9] Nonetheless, molecular diagnosis is still lacking for more than 50% of the patients.[10] It is believed that their primary molecular defects reside in as-yet-unknown assembly factors. The present report is the result of our endeavor to identify new players in complex I biogenesis by using homozygosity mapping in 20 consanguineous families of patients with isolated complex I deficiency.

Material and Methods

Subjects

Five patients originating from a consanguineous family of Arab-Muslim origin (Figure 1) were the subjects of this study. An additional four patients had a similar course, but tissues for enzymatic and molecular studies were unavailable.

All the patients were born at term, and their birth weights were appropriate for their ages; fetal movements were invariably reported to be normal. All presented soon after birth with severe metabolic acidosis and very high plasma-lactate levels (peak level: 38 mM, normal values < 2.2 mM). Patients F528, UA3, and UA4 died at 2–5 days of intractable acidosis. Patients who survived longer were repeatedly admitted because of exacerbation of the acidosis during intercurrent infections. Generalized muscle hypotonia was noted soon after birth, and failure to thrive, irritability, paucity of spontaneous movements, dystonic posturing during crying, nystagmus, failure to interact with the surroundings and lack of eye contact were evident at 6 months. Patient F511, who was still alive at 16 months of age, had spastic tone with quadriplegic involvement, thoracic kyphosis, and reduced range of motion at the knees and hips. The pupillary response to light was sluggish, and the patient failed to follow moving objects. Fundoscopy was normal. No reaction to auditory stimuli could be elicited, and the auditory-brain-stem-evoked response was bilaterally extinguished except for a normal first wave. The physical examination of the oldest surviving patient, F334, at 7 years, was similar, with kyphosis and multiple contractures. The resting tone was increased with a windswept appearance; the tendon reflexes were brisk. There was no reaction to external stimuli, and intermittent irritability was present. Reflex eye movements were normal, the pupil reaction to light was sluggish, and examination of the fundi revealed bilateral optic atrophy.

Generalized tonic-clonic convulsions occurred in two patients during a decompensation episode, but persistent seizure disorder was not part of the clinical picture. Echocardiography was normal throughout infancy and early childhood in all but patient F528, in whom severe cardiomyopathy was detected at 1 day of age. No evidence of liver, renal, or hematological involvement was detected in any of the patients.

Patients F359 and F511, who were treated with dichloroacetate, thiamine, riboflavine, and carnitine and fed via nasogastric tube,

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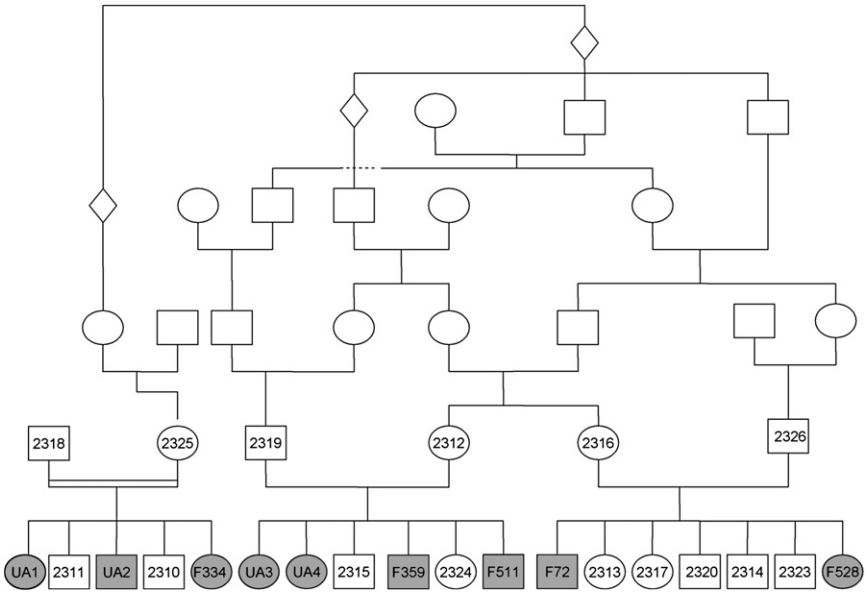


Figure 1. The Family Pedigree
Patients' symbols are filled. Numbered symbols represent individuals whose DNA samples were available for analysis. UA1–UA4 refer to patients whose DNA was unavailable but who were mentioned in the text.

expired at 9–18 months of age of exacerbation of their persistent acidosis. Patients F72, UA1, and UA2, who were not treated, died at the same age.

Brain MRI of patient F511 at 16 months of age revealed severe atrophy of both gray and white matter, with demyelination, most prominent at the anterior aspects of the brain, leaving a cortical ribbon. At the occipito-parietal region there were subventricular cysts, emphasizing the ventricular walls. The cerebellum, basal ganglia, pons, and medulla were severely atrophic (Figure 2).

Methods

Fibroblast cultures were obtained from forearm skin biopsies. Tissue cultures were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, supplemented with 10% fetal calf serum, 50 μg/ml uridine, and 110 μg/ml pyruvate at 37°C in an atmosphere of 5% CO_2.

The Enzymatic Activities

The enzymatic activities of the mitochondrial respiratory-chain complexes in mitochondria isolated from muscle and fibroblasts were determined as previously described.[11]

Complex I Assembly

Complex I assembly was studied in lauryl-maltoside-solubilized muscle mitochondria by blue-native 5%–15% gradient acrylamide gel electrophoresis.[12] Gel-density analysis was performed by ImageJ software.

Homozygosity Mapping

Genomic DNA was extracted from muscle or fibroblasts of patients who suffered infantile encephalomyopathy with variable cardiomyopathy associated with isolated complex I deficiency. These patients originated from 20 consanguineous families, and DNA was also extracted from the blood samples of their parents and healthy sibs. The patients' DNA was analyzed with Affymetrix Human Mapping 50K SNP Array Xba240 as previously described,[13] whereas their relatives' DNA was only genotyped for relevant polymorphic microsatellite markers. All experiments involving DNA of the patients, their relatives, healthy controls, and patients' cells were approved by the Hadassah Ethical Review Committee (#26-160905) and by the Supreme Helsinki committee of the Israeli Ministry of Health (#920050420).

Wild-Type cDNA Transfection

C6ORF66 cDNA was cloned into the pLenti6/V5-D-TOPO 6969 bp expression vector by directional TOPO cloning (primers available upon request). The recombinant vector was propagated in One Shot Stbl3 *E. coli* and introduced into 293FT cells after cotransfection with pLP1, pLP2, and pLP/VSVG plasmids according to the manufacturer's instructions (ViraPower, Invitrogen). Patient cells were infected with the lentiviral construct, and stably transduced cells were established by blasticin selection.

C6ORF66 mRNA Quantification

Total RNA, isolated from the patients' fibroblasts, was reversed transcribed and quantified by real-time PCR. Calibration curves were established by serial dilutions of two plasmids containing a cDNA insert of either *beta-actin* or *C6ORF66* and used to determine the ratio of the two transcripts. TaqMan detection reagent was used for the detection of *beta-actin* and SYBR green for the *C6ORF66* (primers available upon request).

Isolation of Subcellular Fractions

Mitochondrial and cytosolic fractions were obtained through a differential centrifugation protocol according to the method described by Palacino et al.[14] The purity of the subcellular fractions was confirmed by using the specific marker antibodies alpha-Tubulin (cytosol) and pyruvate dehydrogenase (PDH) E1-alpha (mitochondria).

Western-Blot Analysis

Subcellular fractions (12–50 μg total protein/lane) were resolved on 12% SDS-PAGE gels and transferred onto an Immobilon-P Transfer membrane (Millipore, Bradford, Pennsylvania). Western-blot analysis was performed with anti-C6ORF66 (raised against a highly purified C6ORF66 protein that was cloned in our lab; the antibodies were prepared by Sigma-Aldrich, Israel), anti-alpha-Tubulin (Serotec, Oxford, UK), and anti-PDH-E1-alpha (Molecular Probes, Eugene, Oregon) antibodies at 1:2000, 1:20,000, 1:5,000 dilution, respectively, to identify the proteins. Western-blot analysis of the mitochondrial fractions from muscle of two control individuals and two patients (F434 and F359) were studied in triplicates. Density analysis was done with ImageJ software.

Results

All nine patients in this pedigree presented immediately after birth with severe metabolic acidosis and very high plasma-lactate levels (peak level: 38 mM, normal values < 2.2 mM). Three patients died during the first week of intractable acidosis, and the rest suffered from recurrent exacerbations of the acidosis. Patients who survived developed severe encephalopathy, which was already evident at

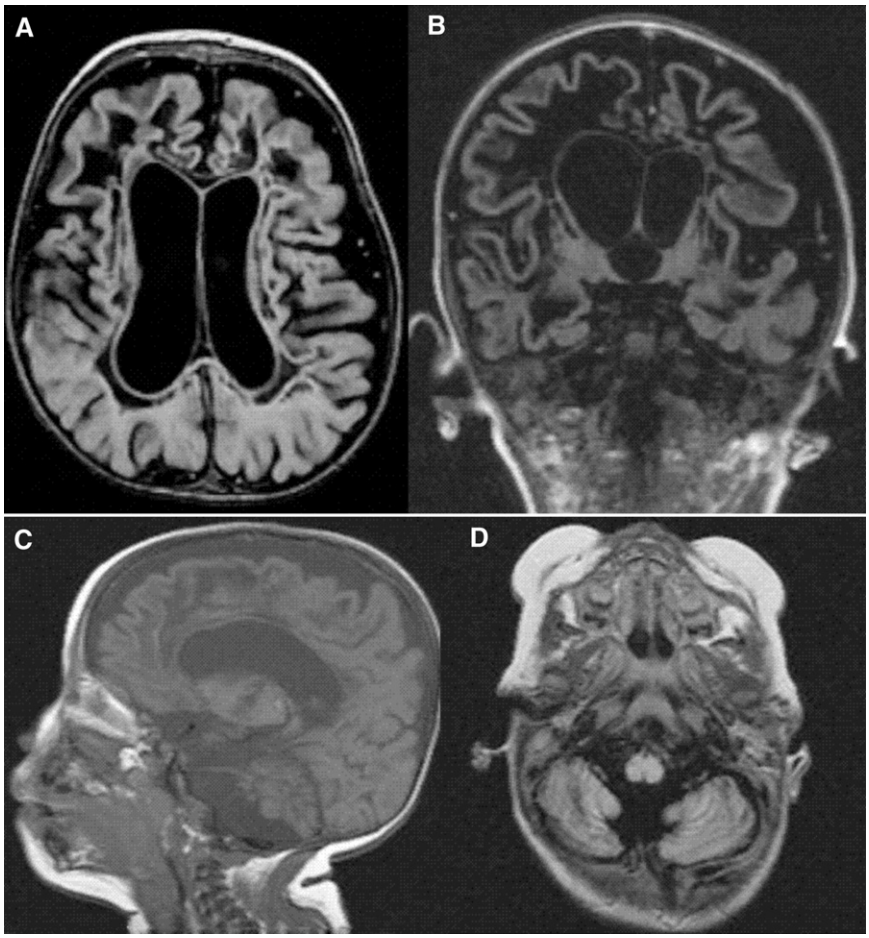


Figure 2. Brain MRI of Patient F511 at 16 Months of Age

Axial (A) and coronal (B) T1 weighted image showing severe atrophy of both gray and white matter, with demyelination, most prominent at the anterior aspects of the brain, leaving a cortical ribbon. Subventricular cysts at the occipito-parietal region emphasize the ventricular walls. Mid-sagittal and axial images (C and D) show severe atrophy of the cerebellum, pons, and medulla.

4–6 months of age. Severe cardiomyopathy was present in only one patient, but other extracerebral systems were unaffected. The oldest surviving patient was 7 years of age at the time of the study.

The enzymatic activity of complex I in isolated mitochondria from muscle of the five patients who were analyzed in our laboratory was reduced to an average of 12.15% of the control mean (range: undetectable to 21%), whereas in fibroblasts of four patients, complex I activity was reduced to an average of 46% (range: 32%–67%). The other complex activities were normal in muscle and fibroblasts, with the exception of elevation of complex II activity in muscles of three patients and mild reduction of complex V in muscle of two patients (Table 1).

The blue-native sodium gel electrophoresis disclosed reduced abundance of the mature complex I to 30% of the control. Two smaller intermediates, which were not present in the control lane, were additionally observed (Figure 3).

To identify the defective gene in this family, we have first determined the sequence of the seven mitochondrial-encoded genes and the ten genes that were previously reported to be mutated in patients with isolated complex I deficiency (NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFV1, NDUFV2, and NDUFA12L). No mutations were identified in these genes in any of the patients.

Homozygosity mapping revealed four stretches of homozygous SNPs larger than 3.0 Mb in the DNA of patient F334 (chromosome 6: 92.12–102.27 Mb, chromosome 8: 134.3–143.9 Mb, chromosome 4: 86.69–90.5 Mb, and chromosome 5: 109.01–112.02 Mb). In the DNA of patient F511, 12 homozygous regions larger than 3 Mb were detected, including chromosome 6: 90.02–106.85 Mb. The two patients shared an identical haplotype over a 10 Mb region of chromosome 6: 92.12–102.27 Mb. All the available DNA samples were examined for the haplotype of four markers that spanned the region: D6S300, rs9373985, D6S1606, and D6S1717. Because patient F72 was heterozygous for D6S1717 at 99.77 Mb and the healthy individual 2313 had an identical genotype to that of the patients for rs9373985 at 97.17 Mb, the critical region could be narrowed down to a 2.6 Mb on chromosome 6 (97.17–99.77 Mb).

We initially prioritized the seven open reading frames within this interval according to the presence of their orthologs in species that are known to express complex I activity. Only KLHL32 complied with this criteria; its orthologs were present in the genomes of the aerobic yeasts *Y. lipolytica* and *D. Hansenii*, which express complex I and were absent from the genomes of the nonaerobic yeasts *S. cerevisiae*, *C. Glabrata*, and *K. Lactis*. However, the sequence of the ten coding exons of the *KLHL32* gene was normal in the patients' DNA. We next examined the three exons of the *C6ORF66* gene, which has orthologs in insects but not in fungi (primers available upon request). In the second exon of this gene, at nucleotide 194 a T→C substitution that predicts a Leu65Pro was detected (Figure 4). All of the patients in the family whose DNA samples were available were homozygous for the mutation. The six parents and five healthy sibs were heterozygous for the mutation, and four healthy sibs were homozygous for the normal allele. None of the healthy sibs was homozygous for

Table 1. Enzymatic Activities in Muscle and Fibroblasts of the Patients

Tissue	Assay	Sample						
		Control (n = 50)	F359	F434	F511[c]	F334	F72	F528
Muscle								
	Citrate synthase[a]	2120 ± 370	1250	1700		710	1750	820
	Complex I[a]	274 ± 127	26 (17%)	37 (17%)		5 (5.5%)	UL	14 (14%)
	Complex I+III[a]	508 ± 164	40 (14%)	48 (12%)	(47%)[c]	8 (16%)	90 (21%)	40 (21%)
	Complex II+III[a]	340 ± 84	194 (97%)	408 (150%)		73 (65%)	378 (135%)	156 (119%)
	Complex II[a]	327 ± 52	-	298 (114%)		80 (74%)	-	209 (170%)
	Complex IV[b]	14.8 ± 3.6	7.9 (91%)	12.3 (104%)		3.7	14.0 (115%)	6.5 (113%)
	Complex V[a]	720 ± 203	507 (123%)	276 (48%)		262 (110%)	421 (71%)	154 (57%)
Fibroblasts								
	Citrate synthase[a]	249 ± 97	327	473	250	-	-	328
	Complex I[a]	31 ± 11	13 (32%)	28 (42%)	21 (67%)	-	-	18 (43%)
	Complex II[a]	80 ± 30	84 (82%)	123 (100%)	82 (103%)	-	-	72 (68%)
	Complex IV[a]	412 ± 140	492 (92%)	877 (105%)	547 (132%)	-	-	489 (86%)

Enzymatic activities in muscle and fibroblasts of the patients. Enzymatic activities are also presented in the brackets as a percentage of the control mean, normalized for citrate synthase activity. UL denotes under the detection limit.

[a] nmol/min/mg.

[b] Velocity constant.

[c] Measured by another laboratory.

the mutation. A BLAST search showed that the mutation was not present in any reported human expressed-sequence tag (EST), nor was it found in 122 ethnic-matched controls.

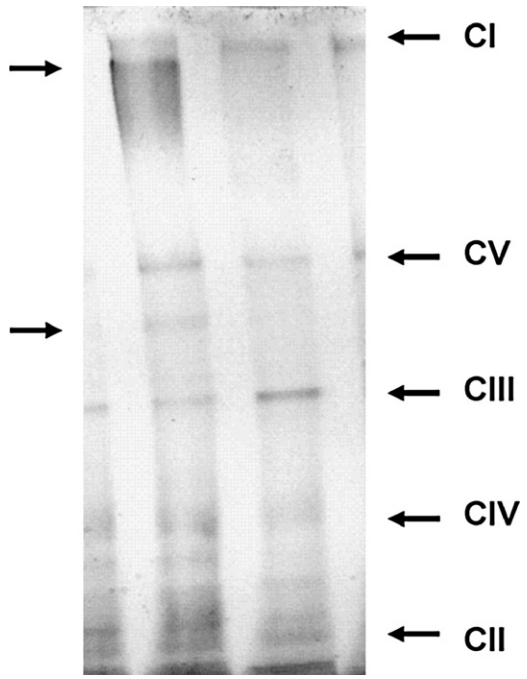
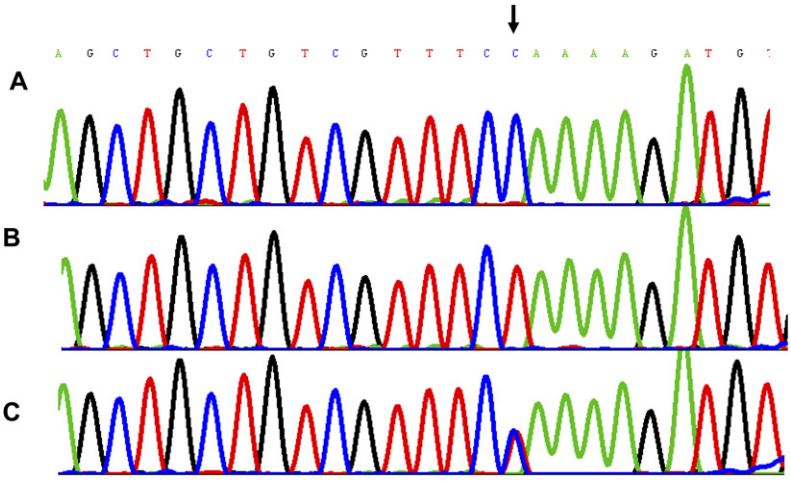


Figure 3. Blue Native Acrylamide-Gel Electrophoresis of Muscle Mitochondria

Equal amount of citrate synthase activity (12 mU) was loaded in each lane. Left lane shows the patient, and right lane shows the control. The five enzymatic complexes, identified by *Rf*, are indicated by the arrows on the right side. The two smaller intermediates, which appear only in the patient sample, are indicated by the arrows on the left side.

Of the 20 consanguineous families of patients with isolated complex I deficiency who were subjected to homozygosity mapping, we identified the same mutation in the *C6ORF66* gene in one family. The patient (F434) was the second born to first cousins of Arab-Muslim origin. Fetal echocardiography at the 33rd gestational week revealed dilated cardiomyopathy, more prominent on the right, with reduced contractility of the two ventricles (shortening fraction [SF] = 28%). The patient was delivered at 37 weeks but had low birth weight (1970 g); the physical examination was unremarkable apart from a grade 2/6 systolic murmur, which was heard all over the precordium. Echocardiography revealed right-ventricular hypertrophy of moderate severity with moderate tricuspid regurgitation. At 14 hr of age, he developed bradycardia and hypotension due to poor myocardial contractility accompanied by severe metabolic acidosis and hyperammonemia. There was no response to vasopressors, and the patient expired at 24 hr of age. Pathological examination of the liver and myocardium revealed microvesicular fatty infiltration. In mitochondria isolated from the skeletal muscle and fibroblasts, isolated complex I deficiency was detected (Table 1). In repeated interview, the parents' common grandmother recalled that her grandmother was born in the same village where members of the large pedigree still reside.

C6ORF66 was initially cloned from the rat Nb2 T lymphoma cell line.[15] The gene encodes 175 amino acids, which form a 20.2 kDa protein. The Leu65 is conserved from *D. melanogaster* to humans (Figure 4) and resides within a putative calmodulin-binding domain that spans residues 54–74; nonetheless, only a small fraction of the protein was previously shown to bind calmodulin in a Ca^{2+}-dependent manner.[16]



Human (61-74)	L	L	S	F	L	K	D	V	Y	V	D	S	K	D
Mouse	L	L	S	L	L	R	D	V	Y	V	D	S	K	D
Zebrafish	L	L	S	M	L	K	D	V	Y	V	E	S	K	D
D. Melanogaster	L	D	G	R	L	K	D	V	Y	V	T	S	Q	D

Figure 4. The T194C Mutation Is Shown in the Patient DNA, in Control DNA, and in an Obligate Heterozygote DNA

The T194C mutation is shown (arrow) in the DNA samples of a patient (A), a healthy control (B), and an obligate heterozygote (C). Conservation from *D. melanogaster* to humans is shown in the table where the mutated codon Leu65 is bordered by bold lines.

To test whether the mutated *C6ORF66* gene is the cause of complex I deficiency in our patients, we transfected their cells with the wild-type cDNA of *C6ORF66*. Following transfection, the mRNA ratio of C6ORF66:beta-actin was doubled (Figure 5A). The C6ORF66:beta-actin mRNA ratio was unchanged in patients' cells transfected with the empty vector. Concomitantly, NADH:ubiquinone reductase activity in isolated mitochondria from the patient cells had normalized, whereas cells transfected with the empty vector remained deficient (Figure 5B). Complex IV activity was not altered by the transfection (Figure 5C). The results clearly establish the importance of C6ORF66 for complex I activity.

Finally, we have produced antibodies to the C6ORF66 protein and studied its subcellular localization. Cytosolic and mitochondrial fractions from control fibroblasts were prepared and analyzed by western blot. This analysis revealed that the C6ORF66 protein is localized to the mitochondria (Figure 6A). In isolated mitochondria from the muscle of patients F434 and F359, the ratio of the C6ORF66:PDH-E1-alpha proteins was 0.12 ± 0.006 and 0.39 ± 0.02 (control 0.67–0.72). The difference between patients and controls was significant: $p < 0.005$ (Figure 6B and 6C). Although the different protein levels in the two patients could be the result of a variability at the level of the regulatory factors responsible for the steady state of the mutant protein, this finding nevertheless indicates that the mutation affects the level of the C6ORF66 protein.

Discussion

We describe six patients who presented in infancy with devastating encephalomyopathy or antenatal cardiomyopathy, attributed to deficiency of complex I of the mitochondrial respiratory chain. Using homozygosity mapping, we identified in the *C6ORF66* gene a missense mutation that affected a conserved residue and was associated with a reduction of the mRNA level in fibroblasts and a significant decrease of the C6ORF66 protein in muscle. The dysfunction of complex I was the direct consequence

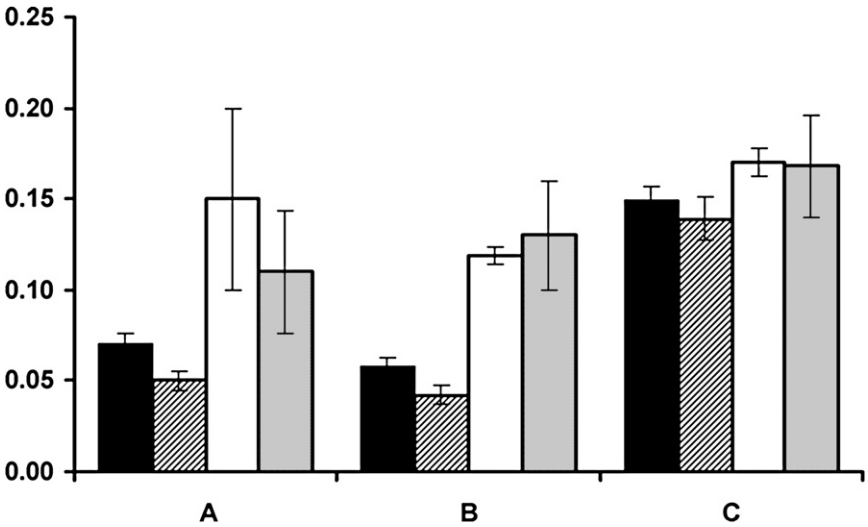


Figure 5. C6ORF66 cDNA Transfection in Fibroblasts

(A) The ratio of C6ORF66:beta-actin mRNA.
(B) The ratio of complex I:citrate synthase activity.
(C) The ratio of complex IV:citrate synthase activity (divided by 10).
Black bars represent the patient, hatched bars represent patient cells transfected with an empty vector, white bars represent patient cells transfected with the C6ORF66 cDNA-containing vector, and gray bars represent control cells. Results are presented as mean of quadruplicate determinations ± standard deviation, analyzed by Student's t test.

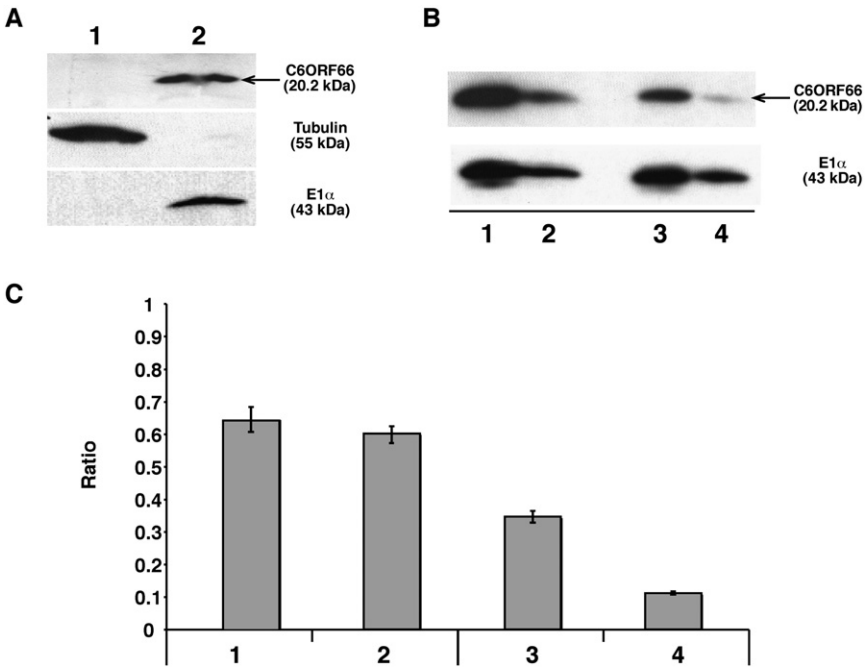


Figure 6. The C6ORF66 Subcellular Localization in Fibroblasts and Its Content in Muscle Mitochondria

(A) Western-blot analysis of the cytosolic (lane 1) and mitochondrial (lane 2) fractions of control fibroblasts with antibodies against C6ORF66, tubulin, and PDH-E1-alpha proteins.

(B) Western-blot analysis of the mitochondrial fractions from muscle of control (lanes 1 and 2) and patients (lane 3, patient F434; lane 4, patient F359) with antibodies against C6ORF66 and PDH-E1-alpha proteins.

(C) Anti-C6ORF66:anti-E1-alpha ratio calculated with density analysis of the western blot shown in (B). Numbers of bars are as in (B).

Results are presented as mean ± standard deviation.

of the mutation in the *C6ORF66* gene, as demonstrated by the functional restoration of complex I activity upon transfection of the patients' cells with the wild-type cDNA.

Complex I has an L-shaped structure that consists of a hydrophobic arm, which contains the seven mitochondrial encoded subunits and is embedded in the lipid membrane, and a hydrophilic, peripheral arm, which contains the iron-sulfor clusters and protrudes into the matrix. The assembly process of this 900 kDa complex and the factors that participate in it are largely unknown. Clearly, this is a stepwise process with seven intermediary subcomplexes shared by two assembly pathways: de novo synthesis headed by the mitochondrial encoded subunits, and regeneration of existing complexes by exchange of already integrated subunits with newly imported ones.[17,18]

Heretofore, three assembly factors have been identified in humans: NDUFA12L, which plays a role at a late stage of complex I biogenesis;[8] NDUFAF1, which is essential for the integration of complex I subunits into early assembly intermediates;[9] and Ecsit, which interacts with the NDUFAF1 protein.[19]

Using C6ORF66 antibodies, we have shown that the C6ORF66 protein is found within the mitochondria. The first 34 residues of the protein are predicted by the TargetP software to form the mitochondrial-targeting sequence. The fact that a mutation in the gene is associated with reduction of the mature complex I form in muscle of the patients yet the protein is not part of the final complex structure is indicative of its importance for complex I biogenesis, suggesting that C6ORF66 is a novel complex I assembly factor.

The *C6ORF66* gene product was previously demonstrated to promote breast cell cancer invasiveness by inducing the excretion of the extracellular-matrix-degrading enzyme MMP-9.[16] The involvement of a complex I assembly factor in tumorigenesis is unexpected because it is generally accepted that cancer cells preferentially use "aerobic glycolysis," i.e., they convert glucose to lactate in the presence of oxygen (Warburg effect).[20] This is mainly done by enhancing glucose supply, increasing the expression of glycolysis genes, and inhibiting the pyruvate dehydrogenase complex.[21] Although there is considerable evidence to support the Warburg effect, there are no data to suggest that mitochondrial respiration is less active in cancer cells than in normal cells. Furthermore, the Warburg effect is inconsistent with the fact that activation of Myc oncogene is associated with increased mitochondrial biogenesis and respiration.[22] The finding that the pro-oncogenic C6ORF66 is essential for the normal function of the mitochondrial respiratory chain may therefore support a role for mitochondrial respiration in tumorigenesis. Interestingly, the assembly factor NDUFA12L was identified in a screen of transcriptional targets of c-*myc*. Furthermore, knocking down *NDUFA12L* by RNAi inhibited tumorigenesis.[23] Whether the pro-oncogenic effect is mediated via the cellular requirement for complex I activity or is an entirely separate process is a matter of conjecture. Dual roles have been described for the complex I assembly factor Ecsit, which was primarily identified in the Toll signaling pathway and in the BMP pathway.[24]

In summary, we have identified C6ORF66 as a novel complex I assembly factor and described the clinical phenotypes associated with a mutation in the gene. Our data open the way for revisiting the involvement of mitochondrial function in tumorigenesis.

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for NADH:ubiquinone oxidoreductase [complex I] deficiency)
TargetP, http://www.cbs.dtu.dk/services/TargetP/
ImageJ, http://rsb.info.nih.gov/ij/

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