A mutation in the human CBP4 ortholog UQCC3 impairs complex III assembly, activity and cytochrome b stability

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Complex III (cytochrome bc1) is a protein complex of the mitochondrial inner membrane that transfers electrons from ubiquinol to cytochrome c. Its assembly requires the coordinated expression of mitochondrial-encoded cytochrome b and nuclear-encoded subunits and assembly factors. Complex III deficiency is a severe multisystem disorder caused by mutations in subunit genes or assembly factors. Sequence-profile-based orthology predicts C11orf83, hereafter named UQCC3, to be the ortholog of the fungal complex III assembly factor CBP4. We describe a homozygous c.59T>A missense mutation in UQCC3 from a consanguineous patient diagnosed with isolated complex III deficiency, displaying lactic acidosis, hypoglycemia, hypotonia and delayed development without dysmorphic features. Patient fibroblasts have reduced complex III activity and lower levels of the holocomplex and its subunits than controls. They have no detectable UQCC3 protein and have lower levels of cytochrome b protein. Furthermore, in patient cells, cytochrome b is absent from a high-molecular-weight complex III. UQCC3 is reduced in cells depleted for the complex III assembly factors UQCC1 and UQCC2. Conversely, absence of UQCC3 in patient cells does not affect UQCC1 and UQCC2. This suggests that UQCC3 functions in the complex III assembly pathway downstream of UQCC1 and UQCC2 and is consistent with what is known about the function of Cbp4 and of the fungal orthologs of UQCC1 and UQCC2, Cbp3 and Cbp6. We conclude that UQCC3 functions in complex III assembly and that the c.59T>A mutation has a causal role in complex III deficiency.

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INTRODUCTION

The majority of cellular ATP is generated by mitochondria in a process called oxidative phosphorylation (OXPHOS). The OXPHOS system is comprised of five multisubunit protein complexes located in the mitochondrial inner membrane (1). The third complex of this system, coenzyme Q: cytochrome c oxidoreductase or complex III, exists as a dimer and couples the oxidation of ubiquinol and reduction of cytochrome c to the translocation of four protons across the inner membrane in a process called the Q-cycle (2). Dimeric complex III in mammals consists of 2 × 11 subunits (3). One of the subunits, cytochrome b, is encoded by the mitochondrial DNA. The other subunits, including the UQCRFS1 presequence that is retained as a structural subunit (4), are encoded by the nuclear genome.

Mitochondrial complex III deficiency (MIM: 124000) is characterized by a wide range of symptoms and disease phenotypes. In most cases, the autosomal recessive forms are severe multisystem disorders. Clinical features observed are as follows: hypotonia, hypoglycemia, failure to thrive, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes, Leigh syndrome, GRACLE and GRACLE-like syndromes (growth retardation, aminoaciduria, choreoatonia, iron overload, lactic acidosis and early death). Exercise intolerance, delayed psychomotor development, hepatopathy and renal tubulopathy are also encountered. Most patients diagnosed with complex III deficiency die in early childhood (6,7). Causal mutations of complex III deficiencies have been traced to cytochrome b (8) and the nuclear-encoded structural subunits UQCRB (9), UQCRQ (10), UQCRC2 (11) and cytochrome c1 (12). In addition, mutations have been found in complex III assembly factors. These include BCS1L, of which >20 mutations have been reported ever since it was first described in 2001 (13), TTC19 (14,15), LYRM7 (16) and UQCC2 (17). In most cases, however, the genetic cause of complex III deficiency remains unknown, partially due to a lack of understanding of complex III assembly. Biogenesis of complex III involves sequentially adding subunits encoded in the nucleus and the mtDNA. Concomitantly, and in a coordinated manner, two heme cofactors are added to these subunits. The assembly process has been mainly studied in the yeast *Saccharomyces cerevisiae* (18). One of the key steps in complex III assembly in yeast is the coordination of cytochrome b synthesis and bc1 complex assembly. Cytochrome b translation takes place on membrane-associated ribosomes bound to the Cbp3–Cbp6 protein complex (19). Sequestration of cytochrome b by Cbp3–Cbp6 inhibits its translation. Upon release of Cbp3–Cbp6 from the ribosome, the complex remains associated with cytochrome b and Cbp4 is recruited to the complex composed of Cbp3, Cbp6 and cytochrome b. Cbp4 is a small protein spanning the inner mitochondrial membrane with a large soluble domain protruding into the inter-membrane space (19,20). The exact function of the Cbp4-containing complex remains unknown, but it could aid in the stabilization the hemylation of cytochrome b, thereby mediating assembly of cytochrome b into a functional bc1 complex. To expand the compendium of human complex III assembly factors and thus aid in the identification of new genes underpinning complex III deficiency, we used sequence-profile-based orthology to detect orthologs of the known *S. cerevisiae* complex III assembly factors (21). With this approach, we successfully identified UQCC1 and UQCC2 as orthologs of Cbp3 and Cbp6, respectively (17), thereby adding two new factors to the list of mammalian complex III assembly chaperones that also includes the recently identified yeast Mzm1p ortholog LYRM7/MZM1L (22). Next to the identification of UQCC1 and UQCC2, this approach also resulted in the identification of C11orf83, hereafter named UQCC3, as the ortholog of Cbp4. Subsequent screening of a patient diagnosed with isolated complex III deficiency with unknown cause resulted in the identification of a mutation in UQCC3. Sequence conservation in animals and biochemical properties of the mutated amino acid suggest that the mutation is deleterious. The UQCC3 protein is not detectable in cells that carry the mutation. In these cells, the complex III activity is decreased, with lower amounts of complex III holocomplex and decreased levels of complex III subunits, including cytochrome b. We establish the mitochondrial localization of UQCC3 and a role in the stabilization of newly synthesized cytochrome b and find that UQCC1 and UQCC2 are necessary for UQCC3 stability.

RESULTS

Identification of a deleterious mutation in UQCC3 in a patient diagnosed with isolated complex III deficiency

There are three known complex III assembly factors in yeast that interact with cytochrome b: Cbp3, Cbp4 and Cbp6. Orthologs of two of these we have recently predicted and validated in human cells, Cbp3 (UQCC1) and Cbp6 (UQCC2) (17). We predicted that the human gene C11orf83/UQCC3 is an ortholog of Cbp4 by comparing the sequence profiles of UQCC3 in metazoa with Cbp4 in fungi (21) using HHpred (23). The alignment shows that the proteins are homologous over most of the length of UQCC3 (Fig. 1A). Note that Cbp4 is considerably longer than UQCC3 (147 versus 93 residues) and Cbp4’s C-terminus is not homologous to UQCC3. This C-terminus may not be essential for its function as it is only conserved in the *Saccharomycotina* phylogenetic branch, representing yeasts closely related to *S. cerevisiae* and not in fungi like *Schizosaccharomyces pombe* (Fig. 1A). Furthermore, the C-terminal 23 residues of Cbp4 have been shown not to be essential for complex III assembly (20). We sequenced the UQCC3 gene in a patient diagnosed with complex III deficiency from which previous genetic analysis did not reveal disease-causing mutations in the mtDNA or the nuclear genes known to be involved in complex III, including UQCC1 and UQCC2. The index patient was born at 37.2 weeks (weight 1930 g) and originated from consanguineous parents (first cousins). The patient presented at 1 day of age with eating difficulties, hypoglycemia and severe lactic acidosis (28 mmol/l). She had no malformations (except, initially, periventricular cysts in the brain), nor dysmorphic features. She displayed slow psychomotor development, hypotonia, hypoglycemia, failure to thrive, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (Leigh syndrome, CSF (2.0 mmol/l). Furthermore, an elevated lactate pyruvate ratio of 37–40 was observed in blood, where the highest reference value of lactate in blood (2.2 mmol/l) and in CSF (2.0 mmol/l). Furthermore, an elevated lactate pyruvate ratio of 37–40 was observed in blood, where the highest reference ratio is 25. In her early years, she had eating and sleeping difficulties that have both improved with age. She has persistent muscular weakness and increased fatigability. Her psychomotor development is significantly delayed, and at 7 years, she was considered to function at 4 years. She now goes to school with special
assistance, and at the age of nine, she can read and write her name. MRI of the brain was normal at 4 years, and there have been no seizures. She has a normal hearing and eye exam (except hypermetropia). There has been no cardiomyopathy or other organ affection. She has had several episodes of more severe lactic acidosis, particularly with intercurrent infections. Growth has been delayed, and at 9 years, she is 4 cm below 3 SD. Investigation of the activities of the respiratory chain enzyme complexes in cultured fibroblasts and frozen muscle tissue of the index patient revealed a severely reduced complex III activity (Table 1), with enzymatic activities of 68 and 13% from the lowest value of the control range in the fibroblasts and muscle tissue, respectively (Table 1). Furthermore, the activity of succinate:cytochrome c oxidoreductase in fibroblasts and in muscle was moderately decreased (75 and 41% of the lowest control value, respectively).

In the UQCC3 genes from the patient, we detected a homozygous c.59T>A mutation (Fig. 1B), for which both parents are heterozygous carriers (Supplementary Material, Fig. S1) and that results in the non-conservative amino acid change p.Val20Glu (Fig. 1A). The mutation is predicted to be damaging by both 2-PholyPhen (25) (score of 0.990) and SIFT (26) (score 0.00). The variant is absent from any of the public genome variation databases like dbSNP (27).

The effects of the mutation in UQCC3 on complex III

To investigate the nature of the complex III deficiency, we solubilized mitoplasts isolated from the patient and control fibroblasts with lauryl maltoside before blue-native polyacrylamide gel electrophoresis (BN-PAGE) analysis. This approach revealed severely reduced levels of holocomplex III in patient cells harboring the mutation in UQCC3 compared with the control (Fig. 2A). This reduction is consistent with the observed complex III enzymatic defect (Table 1). Next to reduced levels of complex III, we found complex I levels to be lowered as well in patient cells, whereas complex IV and complex V were comparable with the control. Complex II appeared to be elevated, also in a repetition of the experiment (Supplementary Material, Fig. S2); this is, however, not reflected in the enzyme activity (Table 1). Lower amount of complex I was also reflected in enzyme measurements (Table 1). A lower amount of complex I is not uncommon in complex III depleted cells, as mature complex I stability depends on assembled complex III residing with it in supercomplexes (28), and a reduced complex I stability and activity has also been observed in patients with complex III deficiency including a patient with a mutation in the complex III assembly factor UQCC2 (17) and in a knockdown of the complex III assembly factor UQCC1 (17). To investigate the effect of the mutation on UQCC3 protein stability, we analyzed mitoplast fractions with sodium dodecyl sulfate (SDS)–PAGE. With this approach, we were not able to detect UQCC3 protein in mitochondrial extracts.

Table 1. Patient mitochondrial respiratory chain enzyme activities in cultured skin fibroblasts and muscle biopsies

<table>
<thead>
<tr>
<th></th>
<th>Fibroblast</th>
<th>Muscle</th>
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<tbody>
<tr>
<td>Complex I</td>
<td>261 (163–599) a</td>
<td>81 (84–273) a</td>
</tr>
<tr>
<td>Complex II</td>
<td>596 (335–888) a</td>
<td>438 (229–593) b</td>
</tr>
<tr>
<td>Complex III</td>
<td>385 (570–1338) a</td>
<td>134 (1020–2530) a</td>
</tr>
<tr>
<td>Complex IV</td>
<td>500 (288–954) a</td>
<td>1336 (520–2080) a</td>
</tr>
<tr>
<td>Complex V</td>
<td>693 (193–819) a</td>
<td>ND a</td>
</tr>
</tbody>
</table>

Control ranges from healthy individuals are indicated between parenthesis.

aValues are given in mU per U citrate synthase.
bValues are given in mU per U cytochrome c oxidase.
cnot determined.
isolated from patient fibroblast indicating the mutation probably destabilizes the protein (Fig. 2B). Additional probing for complex III structural subunits UQCRC1, UQCRC2 and UQCRFS1 revealed lower levels of all these three subunits in patient cells, indicating that their stability or assembly depends on the presence of functional UQCC3 (Fig. 2C). Staining components of other OXPHOS complexes did not reveal significant changes of protein levels in patient cells (Fig. 2B). In S. cerevisiae, the UQCC3 ortholog Cbp4 associates with the Cbp3–Cbp6–cytochrome b complex in an assembly intermediate. A recent study on the human orthologs of Cbp3 and Cbp6 revealed these proteins to be interdependent (17). To examine whether absence of UQCC3 protein affects UQCC1 and UQCC2, the orthologs of Cbp3 and Cbp6, respectively, the blots were additionally probed with UQCC1 and UQCC2 antibodies. This did not reveal alterations in protein levels of these assembly chaperones in UQCC3-deficient cells (Fig. 2B), suggesting the stability of UQCC1 and UQCC2 is not compromised in the absence of UQCC3.

**UQCC3 is a mitochondrial protein**

Mitochondrial targeting prediction programs do not predict the presence of a cleavable, N-terminal targeting sequence for UQCC3 [Mitoprot (not predictable) (30) and TargetP (0.030) (31)], similar to the results of these programs for Cbp4 (not shown). To establish mitochondrial localization of UQCC3, HeLa cells were transfected with UQCC3-GFP and mitochondrial marker mito-dsRed-encoding constructs. Analysis with fluorescence microscopy reveals co-localization of UQCC3-GFP with mito-dsRed (Fig. 3A–D). To further substantiate the mitochondrial localization of UQCC3, western blot analysis of fractionated HEK293 cells was performed showing a strong and specific signal for UQCC3 at the expected height of ~11 kDa in the crude mitochondrial fraction similar to mitochondrial outer membrane localized TOM20 (Fig. 3E). No UQCC3 signal was detected in the cytosolic fraction characterized by the presence of creatine kinase B-type. To address the submitochondrial localization of UQCC3, a proteinase K protection assay was performed using mitochon-deralized outer membranes in the absence or presence of membrane dissolving Triton X-100. This approach showed a reduction in the UQCC3 signal that is furthermore shifted to a lower molecular weight, indicating that UQCC3 is partially degraded. Partial degradation and a shift to a lower molecular weight is also what we observe for OXA1L, whereas TOM20 (a mitochondrial outer membrane protein) already is digested in non-permeabilized mitochondria (Fig. 3F). Only upon dissolving the inner membrane with Triton X-100 are the remainder of UQCC3 and OXA1L and the matrix protein MRPL12 degraded. These results suggest that UQCC3, like OXA1L, is a protein of the inner mitochondrial membrane that extends into the inter-membrane space. UQCC3 has positive charges on the N-terminal side of the predicted transmembrane helix (arginine and lysine at positions 5 and 6). Its N-terminus is concomitantly predicted by TMHMM (32) with a high probability (0.90) to be on the inside (the matrix side of the membrane) whereas its C-terminus would protrude into the IMS. This is consistent with the current model for the transmembrane topology of Cbp4 (19). Taken together, the experimental data and UQCC3’s protein sequence indicate that it is a protein of the inner mitochondrial membrane whose C-terminus extends into the IMS.

**Absence of UQCC3 negatively affects cytochrome b stability**

To assess in more detail the nature of the complex III deficiency caused by the mutation in UQCC3 with respect to cytochrome b, we inhibited cytosolic translation of patient and control fibroblasts and labeled newly synthesized mitochondrial translation products with [35S]-methionine and [35S]-cysteine for 1 h. The labeling was followed by a chase of 0, 6 or 24 h before analysis with SDS–PAGE. Already at 0 h of chase, a specific defect in either cytochrome b synthesis or cytochrome b stability was detected in patient cells (Fig. 4A). This defect became even more apparent after the 24-h chase when compared with the control cells (Fig. 4A). To address whether the remaining cytochrome b protein in patient cells becomes incorporated into
complex III, 2D native PAGE with pulse-chase-radiolabeled mitochondrial-encoded proteins was performed. This approach revealed that, in contrast to the control, no radiolabeled cytochrome \( b \) was observed in high-molecular-weight complexes (i.e. complex III) in patient cells after 6 h (Fig. 4B and C), despite the fact that there was newly synthesized cytochrome \( b \) still present (Fig. 4A). Incorporation of the other mitochondrial translation products into assembly intermediates and complexes are comparable with control cells. Taken together these data suggest a specific complex III assembly defect in fibroblasts lacking functional UQCC3 owing to reduced synthesis, instability and/or failure to incorporate cytochrome \( b \) into complex III.

Stability of UQCC3 depends on the presence of UQCC1 and UQCC2

Studies in yeast have shown Cbp4 to be recruited to and become part of the Cbp3–Cbp6–cytochrome \( b \) complex after completion of cytochrome \( b \) translation and ribosome disassociation (19). To study in more detail a possible functional interaction between the human orthologs, siRNA studies were performed. For this, HEK293 cells were transfected with siRNA targeting UQCC1, UQCC2 and UQCC3 and analyzed with SDS–PAGE. In contrast to the patient cells, depleting HEK293 cells of UQCC3 did not result in detectable changes of holocomplex III (data not shown), or of the structural subunit UQCRC2 (17). Interestingly, depleting cells for either UQCC1 or UQCC2 shows, next to the previously reported interdependency of these two proteins (17), a reduction of UQCC3 protein levels.

DISCUSSION

Here, we provide for the first time evidence that a mutation in UQCC3, the human ortholog of CBP4, leads to complex III deficiency. Cbp4 is one of the cytochrome \( bc_{1} \) complex assembly
factors in *S. cerevisiae* and is recruited to the cytochrome b–Cbp3–Cbp6 complex after the cytochrome b–Cbp3–Cbp6 complex is detached from the mitochondrial ribosome (34). Cbp3 and Cbp6 are orthologs of UQCC1 and UQCC2, respectively. With 2D BN-PAGE analysis and single-step affinity purifications (data not shown) of tandem affinity purification (TAP)-tagged UQCC3, UQCC1 and UQCC2, we were not able to demonstrate an interaction between these proteins. Perhaps the interaction between UQCC3 and UQCC1/UQCC2 is not stable, very transient in nature or depends on intact mitochondrial inner membranes that could be lost in preparing the samples. It is also possible that UQCC3 and UQCC1/UQCC2 do not physically interact in mammalian cells. The observation that depletion of UQCC1 and UQCC2 protein compromises UQCC3 stability (Fig. 5) does, however, point toward a functional interaction between these proteins. Based on the fact that this stability is not interdependent, i.e. cells lacking functional UQCC3 have normal levels of UQCC1 and UQCC2 (Fig. 2C), we conclude that UQCC3, analogous to the yeast assembly model, functions downstream of the UQCC1–UQCC2 complex. Nevertheless, we were not able to complement a *Δcbp4* yeast strain with *UQCC3* (data not shown). This might be explained by the fact that both Cbp4/UQCC3 and the cytochrome b proteins differ significantly between both species, rendering a specific protein–protein contact of heterologous protein pairs unlikely. Fibroblasts lacking UQCC3 have lower levels of cytochrome b as assayed with the pulse-chase radiolabeling of mitochondrial translation products than controls. The effect of a mutation in another complex III assembly factor, UQCC2, appears to be more dramatic as in that patient no newly
expected accumulation of a UQCC1–UQCC2–cytochrome complex at the level of UQCC3 action, one would perhaps UQCC2’s ortholog CBP6 (34). If complex III assembly is compromised in human (35), and methods). Among others due so slow growth of the patient cells (see materials and methods).

In general, OXPHOS assembly factors from yeast that interact with OXPHOS structural proteins are conserved in human (35), in contrast to OXPHOS assembly factors that interact with mitochondrial RNA, likely because splicing is absent from human mitochondria and translation initiation does not involve a 5’ UTR as in yeast. This trend is reflected in the absence of orthologs of the yeast complex III assembly factors that are involved in splicing or translation initiation (Cbp1, Cbp2 and Cbp7) and the conservation in human of the complex III assembly factors that interact with cytochrome b (Cbp3, Cbp4 and Cbp6) (21). It argues for the conservation between yeast and human of the assembly of cytochrome b protein in complex III. In yeast, the IMS-localized assembly factors often have a role in cofactor insertion such as the copper chaperones COX11 and COX17 (36,37) for complex IV. UQCC3’s yeast ortholog Cbp4 plays such a role by directly binding to and stabilizing the semihemylated form of cytochrome b (38). This interaction of the assembly factor with its cytochrome b is necessary for cofactor acquisition that needs to occur at a specific step during the assembly process. It is tempting to speculate that UQCC3 performs a similar role when it comes to insertion of heme in complex III, as has been proposed for Cbp4 (38).

MATERIALS AND METHODS

Generation of UQCC3 expression plasmids

The UQCC3 WT was PCR-amplified with and without stop codon from HEK293 and patient fibroblast cDNA with specific primers adding Attb recombination sites (underlined) at the 5’ and 3’ end. The following primers were used: forward 5’AAAA AGCAGGCTTTGCCACCATGGATTCTTCGGGAAAAT GC 3’ and reversed without stop codon 5’ AGAA AGCTGG GTGTCACGGTGACCTCCCGCCGGCGC 3’ and with stop codon 5’ AGAA AGCTGG GTGTCACGGTGACCTCCCGCCGGCGC C 3’. After the first PCR, the Attb recombination sites were completed with a second PCR using these primers: forward 5’ GG GACAAAAGTTGTACAAAAAGCAGGCT 3’ and reversed 5’ GGGGACCACTTTGTACAAAAAGCAGGCT 3’. The obtained PCR product was then recombined in the pDONR201 vector using the Gateway BP clonase enzyme mix (Invitrogen). Next, the pDONR201 construct with UQCC3 was recombined using the LR clonase enzyme mix (Invitrogen) with mammalian expression vectors under the control of tetracycline-inducible promoter adding a calmodulin–streptavidin TAP tag or GFP tag to the C-terminal part of the protein. All expression constructs were verified by sequence analysis before further use.

Cell culture and generation of stable cell lines

HeLa, Human Embryonic Kidney 293 cells (HEK293) and T-REX™ Flp-In™ HEK293 cells (Invitrogen) were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM; PAA laboratories) supplemented with 10% [v/v] fetal calf serum (PAA Laboratories) and 1% [v/v] penicillin/streptomycin (GIBCO) with additional zeocin (300 μg/ml; Invitrogen) and 5 μg/ml blasticidin (Calbiochem) for the T-REX™ Flp-In™ cells. Generation of stable UQCC3-TAP-expressing cells was achieved by transfecting the corresponding construct into the HEK293 T-REX™ Flp-In™ cells using Superfect transfection reagent (Qiagen). Stable transfected cells were selected by replacing the zeocin in the culture medium for hygromycin (Calbiochem), final concentration 200 μg/ml. Gene expression was induced by adding 1 μg/ml of doxycycline (Sigma) to the culture medium for 24 h.

Fluorescent localization studies

HeLa cells were grown on glass coverslips and transfected with UQCC3-GFP and mito-dsRed-encoding constructs. Twenty-four hours after transfection, cells were fixed with 2%
parafomaldehyde in phosphate-buffered saline (PBS) and imbedded in Prolong Gold Antifade mounting medium (Life technologies). Images were taken with Zeiss Observer.Z1 fluorescence microscope.

Cell culture and biochemical analysis
Skin fibroblasts were cultured in M199 medium (life technologies, breda, the Netherlands) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Measurement of the mitochondrial respiratory chain enzyme activities in muscle tissue and skin fibroblasts was performed according to established procedures (39–43). The values were expressed relative to the activity of cytochrome c oxidase (muscle) and citrate synthase (fibroblasts and muscle).

Cellular fractionation, proteinase K protection assay and isolation of mitoplasts and determination of protein concentrations
Cellular fractionation of cells was done as described earlier (44). Permeabilization of mitochondria with digitonin followed by a proteinase K protection assay was carried out according to the published protocol (45). Protein susceptibility to degradation by proteinase K was assayed with SDS–PAGE followed by western blotting and probing the membranes with specific antibodies. Isolation of mitoplasts from fibroblasts and HEK293 cells was performed as previously described (46). Protein concentrations were determined with the microBCA protein kit (Thermo Scientific).

1D and 2D blue-native PAGE, SDS–PAGE, western blotting and immunodetection
For the analysis of mitoplast isolations, one-dimensional 5–15% gradient and 2D Blue Native polyacrylamide gel electrophoresis (PAGE) were done as described earlier (46). For sodium dodecyl sulfate (SDS)–PAGE proteins, samples were diluted once with Tricine sample buffer (Biorad) supplemented with 2% [v/v] 2-mercaptoethanol and resolved with standard PAGE techniques. Resolved proteins were transferred to nitrocellulose membranes by western blotting, which were blocked with 5% non-fat dry milk in PBS containing 0.1% [v/v] Tween-20 (PBST) prior to incubations with primary antibodies. The following antibodies were used: rabbit anti-UQCC1 (dilution 1 : 1000; Atlas antibodies), anti-UQCC2 (Atlas antibodies, dilution: 1 : 2000; Prim’s Antibody collection), mouse monoclonal anti-SDHA (dilution 1 : 1000), anti-COX1 (dilution 1 : 1000), anti-UQCRCl and UQCRCC2 (dilution 1 : 5000), anti-ATP5a (dilution 1 : 1000) all obtained from MitoSciences, anti-TOM20 (dilution 1 : 5000, BD transduction laboratories), anti-MRPL12 (dilution 1 : 500; Santa Cruz) and anti-CK-B 21E10 (dilution 1 : 5000; (48)). Following the primary incubations, blots were probed with secondary horse radish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (dilution: 1 : 1000; Invitrogen). Immunoreactive bands were visualized using the enhanced chemiluminescence kit (Thermo Scientific) and detected with the Chemidoc XRS+ system (Biorad).

Pulse-chase mitochondrial translation assay
*In vivo* synthesis of mitochondrial proteins in cultured fibroblasts was done as described before (49). Briefly, cells were pretreated for 24 h with chloramphenicol (final concentration 40 μg/ml). Next day patient and control fibroblasts were labeled for 60 min in l-cysteine- and l-methionine-free medium M199 supplemented with dialyzed FCS, emitine (100 μg/ml) and 200 μCi/ml [35S]-cysteine and [35S]-methionine (Tran35S-label, MP biomedicals). After labeling, cells were chased in regular medium for 0, 6 and 24 h, harvested and resuspended in PBS with 2% [w/v] lauryl maltoside. Insoluble material was removed by a 10 min of centrifugation at 10,000 × g. Next, equal amounts of protein were separated by SDS- and 2D BN-PAGE after which the gels were dried and overlayed with a phosphorimager screen. Radiolabeled proteins were visualized with a FLA5100 scanner (Fujimager). Equal protein loading of the SDS gels was confirmed by rehydrating the gel followed by staining with Coomassie Brilliant Blue G-250.

siRNA design and transfection
siRNAs targeting UQCC1 (antisense 5′ UUGUAUCGACACA UGUAC dTdT 3′), UQCC2 (antisense 5′ AGUAGUUGGAG GAGUCG dTdT 3′) and UQCC3 (antisense 5′ UUUAGCAUU UCCUGCUUCC dTdT 3′) were designed using the online available software from the Whitehead Institute for Biomedical Research (50) and synthesized by Biologio. As control, cyclophilin-B-targeting siRNAs (Dharmacon) were used. On Day 1, HEK293 cells were seeded in 6-well plates in antibiotics-free culture medium. The next day cells were transfected with siRNAs (final concentration 10 nm) using Dharmafect 1 transfection reagent (Dharmacon) in OptiMEM (Invitrogen). On Day 4, cells were split 1 : 4 and subjected to a second round of transfection the next day. Cells were harvested on Day 8 and processed for analysis with SDS–PAGE and BN-PAGE.

Complementation of patient cells with UQCC3
To complement UQCC3 in patient cells, we generated lentiviral particles containing C-terminal V5 epitope-tagged UQCC3 or untagged UQCC3 by recombining the corresponding entry vectors with the pLenti6.2/V5 destination vector. For functional complementation of patient fibroblasts, lentiviral particles were produced using the HEK293FT packaging cell line (Invitrogen) and used for transduction of patient and control fibroblasts. Selection for stable UQCC3-V5 and untagged UQCC3 and mito-import-V5 (control) transduced cells was achieved by adding blasticidin to the culture medium. Multiple attempts to achieve functional complementation were unsuccessful, i.e. patient cells did not survive selection whereas control cells were fine or patient cells did survive selection but no expression of UQCC3 was detected. Transgene expression was only observed in stable transduced control fibroblasts, albeit at very low levels. Subsequent transient transductions of patient and control cells neither did result in detectable amounts of UQCC3 and functional complementation. We speculate that adverse effects of UQCC3 transgene expression, in combination with the already growth-compromised patient cells, may prove to be lethal.
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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