A mutation in the FAM36A gene, the human ortholog of COX20, impairs cytochrome c oxidase assembly and is associated with ataxia and muscle hypotonia

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The mitochondrial respiratory chain complex IV (cytochrome c oxidase) is a multi-subunit enzyme that transfers electrons from cytochrome c to molecular oxygen, yielding water. Its biogenesis requires concerted expression of mitochondria- and nuclear-encoded subunits and assembly factors. In this report, we describe a homozygous missense mutation in FAM36A from a patient who displays ataxia and muscle hypotonia. The FAM36A gene is a remote, putative ortholog of the fungal complex IV assembly factor COX20. Messenger RNA (mRNA) and protein co-expression analyses support the involvement of FAM36A in complex IV function in mammals. The c.154A>C mutation in the FAM36A gene, a mutation that is absent in sequenced exomes, leads to a reduced activity and lower levels of complex IV and its protein subunits. The FAM36A protein is nearly absent in patient’s fibroblasts. Cells affected by the mutation accumulate subassemblies of complex IV that contain COX1 but are almost devoid of COX2 protein. We observe co-purification of FAM36A and COX2 proteins, supporting that the FAM36A defect hampers the early step of complex IV assembly at the incorporation of the COX2 subunit. Lentiviral complementation of patient’s fibroblasts with wild-type FAM36A increases the complex IV activity as well as the amount of holocomplex IV and of individual subunits. These results establish the function of the human gene FAM36A/COX20 in complex IV assembly and support a causal role of the gene in complex IV deficiency.

INTRODUCTION

Cytochrome c oxidase (complex IV) is a mitochondrial inner membrane multi-subunit enzyme encoded by both mitochondrial DNA (mtDNA, three subunits) and the nuclear genome (11 subunits, including the subunit formerly considered a constituent of complex I (1)). Complex IV is

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the terminal protein complex of the respiratory chain and couples the oxidation of cytochrome c and the reduction of molecular oxygen to the translocation of two protons across the mitochondrial inner membrane. The sophisticated process of complex IV biogenesis requires coordinated subunit expression between the two genomes, followed by submitochondrial (matrix, inner membrane and intermembrane) localization and assembly of the components of the protein complex. Decreased stability and the failure to assemble the mature complex IV (2,3) lead to severe early-onset neuromuscular disorders in human (mitochondrial complex IV deficiency, OMIM:220110), characterized by a wide range of disease phenotypes that include encephalomyopathies, Leigh syndrome, cardiomyopathy and metabolic acidosis with elevated lactate and pyruvate levels (4,5). Biogenesis of complex IV requires the action of assembly factors facilitating and regulating the synthesis and membrane insertion of the core subunits, the coupling of cofactors and the incorporation of the nuclear-encoded subunits into the complex (reviewed in 6). While only a number of complex IV deficiencies can be traced to mutations in the three mitochondrial (4) and two of the nuclear-encoded subunits (7,8), the majority of disease causing mutations can be found in nine genes encoding assembly factors (9–17). These include the Leucine-rich PPR motif-containing protein (LRPPRC), a mitochondrial messenger RNA (mRNA) binding protein affecting COX1 and COX3 mRNA levels when not functioning properly (15,18); TACO1, a translational activator of COX1 (14) and the human COX14 ortholog C12orf62, a factor involved in the synthesis and assembly of COX1 (17,19). COX10 and COX15 are required for heme synthesis (13,20,21), whereas copper insertion takes place through COX11 (CuB) (22) and Sco1/2 (CuA) (10,12,23,24) for COX1 and COX2, respectively. The exact roles of complex IV assembly factors SURF1 (9,25) and C2orf64/COA5 (16) have not yet been fully established.

The process of the biogenesis of complex IV can be discerned into four discrete steps where subunits and cofactors are added in a sequential manner to subassemblies S1, S2 and S3 (26,27). The assembly process starts with the COX1 protein (subassembly S1) followed by the addition of the nuclear-encoded subunits COX4 and COX5A, forming the S2 subassembly. Next, COX2 and COX3 are added completing the formation of the catalytic core (S3), after which the incorporation of the remaining accessory subunits leads to a fully assembled holocomplex (27). Despite attempts to complete the compendium of human COX assembly factors (15,19), the mammalian COX assembly process, including the genes causal for complex IV deficiency, has yet to be fully elucidated.

RESULTS

This report investigates the role of the mutation encountered in the human FAM36A gene in the child of consanguineous parents born with low weight (2600 g) after a complicated pregnancy (see Materials and Methods). Muscular hypotonia, cerebellar ataxia and delayed speech development were noted. Mild elevation of blood and cerebrospinal fluid (CSF) lactate concentrations were suggestive of a mitochondrial disease. Measurements of the activity of mitochondrial respiratory chain complexes revealed a severe complex IV deficiency in muscle and fibroblast cells of the index patient (Table 1). Sequencing of the mitochondrial DNA (mtDNA) of the index patient as well as nuclear genes known and postulated to be involved in complex IV deficiency (see below) did not reveal any potential disease mutations.

The FAM36A/COX20 gene family

In previous work, we used sequence-profile-based orthology to postulate FAM36A as the likely ortholog of fungal COX20 and prioritized it as the COX assembly candidate in human (19). The fungal Cox20p protein is one of the assembly proteins essential for the biogenesis of complex IV in Saccharomyces cerevisiae (28–30). Cox20p localizes to the inner membrane of mitochondria (28) and participates in presequence processing of Cox2p, the complex IV subunit (28). Cox20p also protects Cox2p from proteolytic degradation and takes part in its C-tail topogenesis (31). The human FAM36A gene contains a Domain of Unknown Function (DUF3767) and is shorter than its fungal ortholog (118 versus 205 residues in fungi). The protein exhibits a similar domain composition in eukaryotes (Fig. 1A) including conserved Cys residues that flank the transmembrane regions.

The gene’s orthologs are present in the major eukaryotic clades, indicating its early evolutionary origin. Supporting a role of COX20 orthologs in complex IV is that they co-occur in genomes, being either both present, like in the unicellular species Phytophtora infestans or both missing like in the related Blastocystis hominis that has complex IV-less mitochondria. An exception to this pattern is the malaria parasite Plasmodium falciparum that misses a COX20 ortholog despite having COX2. Interestingly, in contrast to most eukaryotes Plasmodium encodes COX2 in its nuclear genome and may, therefore, not need the COX20-catalyzed C-tail transport across the inner membrane as observed in fungi (31) (Fig. 1B). FAM36A/COX20 was also lost from Plantae, which present multiple independent relocalizations of the COX2 gene from the mitochondrion to the nucleus (32,33).

Integrative genomics data analysis supports involvement of FAM36A in complex IV

Besides FAM36A’s putative orthology with yeast COX20, also computational analyses of genomics data suggest its functional association with mitochondrial complex IV in mammals. The FAM36A gene highly co-expresses with genes encoding complex IV subunits in mouse tissues (average Pearson’s correlation 0.524, Fig. 1C). The high co-expression with complex IV is significant on the background of all human genes (average expression correlation 0.01, two-sided Mann–Whitney test P-value <10^-6) and 2-fold higher than with genes encoding mitochondrial proteins (average expression correlation 0.25, P-value <4 × 10^-4), revealing mammalian tissue-level co-regulation of FAM36A with complex IV. At the protein level, the analysis
of proteomics data of purified mitochondria from 14 mouse
tissues (34) corroborates the FAM36A co-expression with
COX subunits. Among 1098 mitochondrial proteins,
FAM36A protein coexpresses significantly higher with
COX subunits as measured by the number of detectable pep-
tides in highly purified mitochondria than with other mito-
chondrial proteins (Pearson’s correlation 0.35, two-sided
Mann–Whitney test P-value <0.05, Fig. 1D).

The mutation in the FAM36A gene
We sequenced the FAM36A gene in 40 complex IV-deficient
patients, in whom previous genetic analysis did not reveal
disease-causing mutations in the mtDNA and in the nuclear
genes known to be involved in complex IV deficiency. In one
patient, we found a homozygous c.154A>C mutation in the
second exon of the gene, resulting in the amino acid change
p.Thr52Pro (Fig. 2A). No other homozygous missense
mutations in the coding regions of genes known and postulated
to be involved in complex IV function were detected (including
COX10, COX15, SCO1, SCO2 and SURF1). We also
sequenced additional human complex IV assembly candidates
(AURKAIP1, CHCHD7, C7orf44, C1orf31, PET117 (19) and
CCDC56 (19,35)) in our patients and did not detect any homo-
zygous missense mutations or potentially disease-causing var-
iants. The c.154A>C mutation in FAM36A is predicted to be
pathogenic by PolyPhen-2 (36) (score:1.00, probably dam-
ing) and SIFT (37) (score:0.00, deleterious). The mutation has not been previously reported in the database of single nu-
cleotide polymorphisms (dbSNP build 137 (38)), 1000
Genomes Project (39) or as an Expressed Sequence Tags
available in public databases of GenBank, EMBL or DDBJ
(as of August 2012). The variant is absent in 1051 sequenced
exomes of patients in the internal exome database of Radboud
University Medical Center. The patients (carriers of non-
mitochondrial genetic diseases) as well as healthy individuals
were sequenced at a sufficient depth at this genomic position
(median depth 125×, see Methods). The variant was also
absent among exomes of other 2440 individuals of European
and African ancestry stored in the Exome Variant Server
(40). The analysis of the DNA sequence of consanguineous
parents of the patient revealed that they are heterozygous car-
riers of the variant. Also the patient’s sibling, exhibiting a
normal complex IV activity in prenatal screening as measured
prenatally in chorionic villi (510 mU/U CS, control range
271–922 mU/U CS) is a heterozygous carrier of the variant
(Fig. 2A). The mutation affects the threonine residue located
at the interface between the inner-membrane embedded
region and the predicted mitochondrial matrix-localized loop
fragment of the FAM36A protein (Fig. 2B). As a result of
the mutation, the conserved threonine residue (Fig. 1A) is
replaced with a proline residue. Consistent with the inner-
membrane localization of the fungal ortholog COX20 (28),
FAM36A localizes to mitochondria. Its C-terminus is substi-
tuted protected from proteinase K by the outer mitochondrial
membrane, corroborating the predicted topology of the protein
(Fig. 2C and D).

The effects of mutation on the complex IV assembly
process and molecular subassemblies
To assess the nature of this complex IV deficiency, we ana-
alyzed patient and control fibroblasts with blue native poly-
acylamide gel electrophoresis (BN-PAGE). We observe
reduced in-gel activity (IGA) of complex IV and lower levels of complex IV in subject fibroblasts, whereas complex 1
I GA and levels of other OXPHOS complexes are not com-
prised (Fig. 3A). Subsequent sodium dodecyl sulphate-
polyacrylamide gel electrophoresis (SDS-PAGE) analysis of
these extracts reveals a severe decrease of FAM36A protein
levels in subject fibroblasts compared with controls
(Fig. 3B). Next to lower FAM36A protein levels, patient
cells exhibit reduced levels of COX1 and COX5A subunits
and, to a greater extent, decreased COX2 and COX4
(Fig. 3B). Protein levels of other OXPHOS subunits tested, in-
cluding mitochondrial-encoded complex I subunit ND1, are
not affected (Fig. 3B). These findings demonstrate that
reduced FAM36A protein levels lead to a specific reduction of
holocomplex IV and result in lower levels of individual
complex IV subunits. We carried out 2D BN-PAGE experi-
ments to examine specific complex IV assembly defects with
respect to known complex IV subassemblies S1, S2 and S3
(27). The analysis revealed increased levels of the COX1-
containing subcomplexes S1 and S2 in these cells (Fig. 3C).
The accumulation of S1 and S2 subassemblies in the patient
fibroblasts resembles observations in SURF1 complex IV
assembly-deficient cells (25), and differs from the COA5
mutant assembly profile (16) that more specifically accumu-
lates subcomplex S1. These observations suggest that the as-
sembly is hampered at the transition from subcomplex S2 to
S3, when COX2 becomes incorporated (26,27). To further
support the FAM36A-mediated COX2 incorporation into
complex IV, we assessed a possible interaction between
FAM36A and COX2 by affinity purifications. Using
FAM36A tandem affinity purification (TAP)-tagged protein
from HEK293 cells we performed a purification experiment

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex II+III</th>
<th>Complex IV</th>
<th>Citrate synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>152 (84–273)</td>
<td>n.m.</td>
<td>112 (37–285)</td>
<td>99 (520–2080)</td>
<td>161 (45–187)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>184 (100–310)</td>
<td>528 (520–845)</td>
<td>n.m.</td>
<td>246 (470–860)</td>
<td>206 (144–257)</td>
</tr>
</tbody>
</table>

n.m.: not measured.

Control ranges are provided in parenthesis, activities exceeding control ranges are marked with bold typeface. Activities of complexes are expressed as milliunits per unit citrate synthase (mU/U CS), citrate synthase activity is expressed as milliunits per milligram protein.

Table 1. Respiratory chain enzyme activities in mitochondria-enriched preparations from muscle and fibroblasts of the index patient that is homozygous for the c.154A>C variant in FAM36A

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COX2 transcription and translation are not specifically affected in patient fibroblasts

To investigate whether the mutation in *FAM36A* affects mitochondrial transcription of COX2, we determined the mRNA levels of *MT-CO2*, the gene encoding the COX2 protein, in subject fibroblasts using quantitative RT-PCR. No significant alterations in mRNA levels were observed relative to control fibroblasts for *MT-CO1*, *MT-CO2* and *MT-CO3* (Fig. 5A). Based on this we conclude that *MT-CO2* transcription is not specifically affected compared with the other complex IV genes in FAM36A patient fibroblasts. To evaluate a possible role of FAM36A in mitochondrial translation, we inhibited cytosolic translation and radioactively labeled newly synthesized mitochondrial-encoded proteins with $^{35}$S-methionine and cysteine in patient and control fibroblasts. Although in patient fibroblasts we find an overall decrease in mitochondrial translation compared with the control cell line (Fig. 5B and C), no specific alterations in COX2 translation were observed, suggesting that FAM36A does not act as a translation regulator of COX2.

FAM36A is detected in mitochondria devoid of mitochondrial DNA

Proteins functioning in processes that require the presence of mtDNA such as translation activators or mitochondrial ribosomal subunits are often absent or present at much lower levels in cells lacking mtDNA (47). To substantiate the role of FAM36A in COX2 protein processing and rule out a possible
Figure 2. Molecular genetic analysis of the FAM36A genomic DNA and the inner-membrane localization of the protein. (A) Electropherograms showing the complex IV deficiency patient sequence variant (top panel) as well as parents, sibling and the wild-type alleles (remaining panels). The patient is homozygous for the c.154A>C mutation, while the parents and their healthy siblings are heterozygous carriers. Forward sequence is shown. (B) Submitochondrial localization of the amino acid affected by the mutation is inferred from the predicted transmembrane regions and their topology. The position of the non-synonymous mutation is marked with the arrow. (C) Mitochondrial localization of FAM36A protein. HEK293 cells were fractionated (58) into TC, total cell lysate; CYT, cytosolic fraction; MIT, mitochondrial fraction and analyzed with SDS-PAGE followed by western blotting using indicated antibodies. CK-B (creatine kinase) was used as a cytosolic marker. (D) The C-tail of the protein localizes to the IMS. Proteinase K protection assay (59) of mitochondria and mitochondria with digitonin permeabilized outer membranes (mitoplasts) from HEK293 cells in the absence and presence of membrane-dissolving Triton X-100. The signal from the C-terminus-targeted FAM36A antibody (Sigma Atlas Antibodies, HPA045490) is lost after outer membrane permeabilization. Blots were additionally immunodecorated for TOM20 that is localized in the mitochondrial outer membrane and inner membrane-spanning OXA1L protein that becomes fragmented when accessible to proteinase K. *indicates a-specific band.

Figure 3. Patient fibroblasts with the FAM36A mutation have reduced complex IV activity and lowered levels of holocomplex IV and individual complex IV subunits. (A) Non-denaturating BN-PAGE analysis (60) of subject fibroblasts shows reduced complex IV IGA relative to the control fibroblasts. Subsequent immunoblot analysis after BN-PAGE with anti-COX1 antibody reveals reduced levels of holocomplex IV, whereas no alterations in the other OXPHOS complexes were found after additionally probing the blots for complex I (NDUFA9), complex II (SDHA), complex III (UQCRCl) and complex V (ATP5a). (B) Immunoblot analysis of mitochondrial extracts after SDS-PAGE with indicated antibodies reveals reduced levels of FAM36A, COX1, COX2, COX4 and COX5A protein in subject cells. (C) Two-dimensional BN-PAGE analysis of patient fibroblast and controls with indicated COX-antibodies shows accumulation of COX1-containing subassemblies (indicated with S1 and S2). Holocomplex IV is indicated with CIV. The COX2 and COX4 proteins, almost undetectable in the SDS-PAGE immunoblot (panel B), are visible here owing to higher protein loading (80 µg compared with 40 µg, see Methods) and different gel exposure parameters. (D) COX2 co-purifies with FAM36A-TAP. FAM36A-TAP was expressed in HEK293 cells using a doxycycline-inducible promoter and affinity purified proteins were identified by Western blot analysis. As control non-induced cells were used. Antibodies are indicated at the left, CBP (calmodulin binding protein antibody recognizing the TAP-tag) was used to verify the purification.
function as a COX2 translational activator, we investigated the stability of FAM36A in human cells deprived of mtDNA (Rho0 cells) with SDS-PAGE. In contrast to mitochondrial-encoded COX1 and TACO1, the translational activator of COX1 (14), FAM36A protein levels appear unaffected in Rho0 cells (Fig. 5D) indicating that FAM36A stability does

Figure 4. Functional complementation of patient fibroblasts with lentivirus carrying the wild-type FAM36A gene increases complex IV levels and does not affect other respiratory complexes. Patient fibroblasts were complemented with a viral construct encoding the wild-type gene (FAM36A-V5) and a control vector containing the V5 tag appended to the COX8 targeting signal (mito import-V5). (A) BN-PAGE analysis of complemented cells shows increased levels of holocomplex IV. (B) Increased levels of individual complex IV subunits in patient cells, revealed by SDS-PAGE analysis. Antibodies are indicated at the left, molecular mass marker (kDa) at the right. The FAM36A antibody does not detect FAM36A-V5, possibly due to an altered C-terminal epitope where the V5 tag resides, nevertheless, the V5 antibody recognizes a protein band where FAM36A-V5 is expected (approximately 18 kDa). Wild-type fibroblasts were used as a control. Mito import-V5 signal was not detectable on the blot due to small-molecular weight. (C) Complex IV activity in complemented fibroblasts, measured using previously described methods (45). Complementation of patient’s fibroblasts restores the complex IV activity from the patient’s 36% to 71% of activity observed in a control cell line (P < 0.005, two-sided t-test). (D) Activity of respiratory chain complexes I–V in patient fibroblasts complemented with the control viral construct (mito import-V5) and the wild-type FAM36A gene.

Table 2. Oxidative phosphorylation enzyme activities measured in patient-derived and control fibroblasts complemented with a wild-type FAM36A (FAM36A-V5) and a control viral construct (mito import-V5)

<table>
<thead>
<tr>
<th>Complex</th>
<th>Activity (mU/U CS)</th>
<th>Control + mito import-V5</th>
<th>Control + FAM36A-V5</th>
<th>Patient + mito import-V5</th>
<th>Patient + FAM36A-V5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>Average</td>
<td>369</td>
<td>339</td>
<td>490</td>
<td>465</td>
</tr>
<tr>
<td></td>
<td>Stdev</td>
<td>27</td>
<td>37</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Complex II</td>
<td>Average</td>
<td>415</td>
<td>437</td>
<td>456</td>
<td>467</td>
</tr>
<tr>
<td></td>
<td>Stdev</td>
<td>74</td>
<td>62</td>
<td>30</td>
<td>57</td>
</tr>
<tr>
<td>Complex III</td>
<td>Average</td>
<td>1081</td>
<td>734</td>
<td>1005</td>
<td>1017</td>
</tr>
<tr>
<td></td>
<td>Stdev</td>
<td>14</td>
<td>42</td>
<td>18</td>
<td>32</td>
</tr>
<tr>
<td>Complex IV</td>
<td>Average</td>
<td>744</td>
<td>765</td>
<td>267</td>
<td>530</td>
</tr>
<tr>
<td></td>
<td>Stdev</td>
<td>118</td>
<td>84</td>
<td>48</td>
<td>67</td>
</tr>
<tr>
<td>Complex V</td>
<td>Average</td>
<td>811</td>
<td>677</td>
<td>1149</td>
<td>1268</td>
</tr>
<tr>
<td></td>
<td>Stdev</td>
<td>64</td>
<td>28</td>
<td>82</td>
<td>86</td>
</tr>
</tbody>
</table>

Activities of complex IV in patient cells are marked with bold typeface. Activities of complexes are expressed as milliunits per unit citrate synthase (mU/U CS). BN-PAGE, blue native polyacrylamide gel electrophoresis; ECG, Electrocardiography; EEG, Electroencephalography; HMM, hidden Markov model; IGA, in-gel activity; IMS, inter-membrane space of the mitochondria; MRI, magnetic resonance imaging; mU/U CS, milliunits per unit citrate synthase; SDS, sodium dodecyl sulfate; TAP, tandem affinity purification.
not depend on the presence of mtDNA. This observation argues against a role of FAM36A in COX2 translation or other mtDNA-related tasks and favors its predicted function in COX2 protein processing.

**DISCUSSION**

We provide evidence that the ancient eukaryotic FAM36A protein (COX20) is an essential assembly factor of mitochondrial complex IV. Cox20p is one of the assembly proteins essential for the biogenesis of complex IV in *S. cerevisiae* (28–30). The fungal Cox20p binds to a newly synthesized precursor of the Cox2p and promotes its N-terminal processing by inter-membrane space (IMS) Imp1p/Imp2p proteases (28). However, the N-terminal processing of COX2 is unlikely to be the function of FAM36A in human, as the vertebrate COX2 protein, in contrast to its fungal ortholog, is not proteolytically cleaved. Fungal Cox20p also associates with Cox2p, and protects the unassembled subunit against degradation by i-AAA proteases (31). This function of the protein does not appear to be conserved in all eukaryotes, as orthologs of COX2 subunits are present in species without a COX20/FAM36A ortholog encoded in the genome. Cox20p also carries out the post-translational export of the C-tail of Cox2p from the matrix to the IMS of the mitochondria (31). The phylogenetic co-occurrence of FAM36A/COX20 family with mitochondrial-encoded COX2, but not with nuclear-encoded COX2, suggests a conserved role of the protein in the C-tail topogenesis.

Proteomic analyses have shown that the stability of numerous mitochondrial proteins is negatively affected in Rho0 cells.
that are devoid of mtDNA and RNA. These include proteins functioning in processes that require the presence of mtDNA such as mitochondrial ribosomal subunits and transfer RNA (tRNA)-nucleotidyltransferase CCA (47) or a mitochondrial ribosomal large subunit-binding protein MALSU1 (48). Multiple proteins involved in translation and complex IV function are also affected in Rho0 cells. The LRPPRC protein that is necessary for polyadenylation of mitochondrial mRNA and mitochondrial translation (49) and that is involved in complex IV deficiency (18) is present at lower levels in in Rho0 cells (50). Its interactor, a stem-loop RNA-binding mitochondrial protein SLIRP is also markedly reduced in these cells (50). Likewise, the translational activator of COX1, the TACO1 protein (14), is virtually absent in rho0 cells (Fig. 5D). However, FAM36A levels are not decreased in this cell type, strongly suggesting that FAM36A does not require mtDNA or mRNA for its function, and thus is unlikely to play a role in processes such as translation and/or transcription, for example as a translational activator. Reduced mitochondrial protein expression levels observed in the Figure 5B appear not to be caused by a lower mitochondrial mass in the patient cells (Supplementary Information) and are more likely the result of a different genetic background of patient and control cells and lower rates of growth of patient’s fibroblasts (data not shown). An alternative explanation, a general translation defect, is unlikely to affect complex IV specifically (Figs 4D, 3A and B) without influencing mitochondria-encoded complexes I, III and V.

The residue that is mutated in the patient (p.Thr52Pro) is conserved in animals and is predicted to localize in the mitochondria matrix. The combined data from genomics and proteomics studies together with observed accumulation of complex IV subassemblies, affected activity, protein and holocomplex IV levels as well as physical interaction with COX2 lead us to conclude that FAM36A is an important factor in the early steps of the complex IV assembly. The mutation observed in the patient causes stalling of the assembly process after the formation of S1 and S2 subassemblies of complex IV and before the incorporation of the COX2 subunit, consistent with observed complex IV deficiency in the index patient. The lentiviral complementation with wild-type FAM36A increases complex IV levels (both individual subunits as well as holocomplex) and complex IV activity and has little or no effect on the activity of the other oxidative phosphorylation enzymes (Table 2). The complementation supports the causative role of the mutation FAM36A mutation for the complex IV deficiency in our patient.

The molecular assembly profile of the homozygous FAM36A mutation is reminiscent of the SURF1 defect assembly profile, but the degree of the enzyme deficiency is much milder for the FAM36A patient (30–40% in fibroblasts). Also the clinical phenotype is much less severe compared with other complex IV deficiency patients as magnetic resonance imaging MRI did not reveal any abnormalities and the boy is alive at the present age of 10 years (see Materials and Methods for details). In contrast, patients with a C2orf64/COA5-related defect that hampers earlier stages of complex IV assembly died after 8–10 days of life due to hypertrophic cardiomyopathy (16). It remains to be elucidated how different molecular defects of complex IV assembly affect clinical outcomes and give rise to variable phenotypic presentations of the disorder.

MATERIALS AND METHODS

Case report

The study described here has been carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees of the Radboud University Medical Centre and informed consent. This report describes the first child of consanguineous Turkish parents. The pregnancy was complicated by oligohydramnion and growth retardation. The boy was born at term with low birth weight (2600g) and low length and head circumference. At the age of 12 months, muscular hypotonia was noted. He learned to walk in 16 months but gait remained unsteady, and he developed cerebellar ataxia with intention tremor and pyramidal signs. Speech development was delayed. Mild elevation of blood and CSF lactate concentrations were suggestive of a mitochondrial disease. Respiratory chain complex studies from a skeletal muscle biopsy revealed a reduced activity of mitochondrial complex IV. Echocardiography, electrocardiography (ECG), brain MRI and electroencephalography (EEG) at age 8 or 9 years did not reveal any abnormalities; hearing tests and ophthalmologic investigations gave normal results. There were no episodes of metabolic decompensation or regression. At the present age of 10 years, the boy has small stature (4 cm below the third percentile) and low weight with a normal head circumference.

Identification of FAM36A as the ortholog of fungal COX20 protein

A three-step genomic orthology identification pipeline was developed that makes use of sequence (51), profiles (52) and the profile-derived hidden Markov models (HMMs) (53) to detect orthologs with more sensitivity than the standard sequence-based approaches (19). The pipeline identifies unambiguous reciprocal best hits between fungal and human proteins (19). FAM36A-COX20 orthology, not detectable with the sequence–sequence comparisons, was identified as a putative ortholog of fungal COX20 as the reciprocal best hit at the profile level of the corresponding genes in human and fungi, fulfilling therefore the operational definition of orthology. Also for the profile-based HMMs representing FAM36A and COX20 genes, the E-value at the genome-wide search with HHsearch 1.5.1.1 (53) is statistically significant (E < 6.1×10−25). The identified orthology was submitted to the HUGO Gene Nomenclature Committee consortium (genenames.org) in January 2012.

mRNA and protein expression

Pearson’s correlation of mRNA expression was calculated for 91 murine cell types and tissues for 182 microarray sample measurements with Affymetrix Mouse Genome 430 2.0 Array (54). The GNF Mouse GeneAtlas V3 data were downloaded from Gene Expression Omnibus, record GSE10246 (54). The negative expression values were removed and the
expression data were log2-transformed. All-against-all genes Pearson’s correlation matrix was calculated with the transformed data (average co-expression $-0.27$). The co-expression data between FAM36A (NCBI entrez gene identifier 66359) and other murine genes were selected for Figure 1C. The genes encoding mitochondrial murine proteins were taken from Mitocarta compendium (34), as well as from ref. (19). For the protein co-expression, the number of peptides identified per protein per each of 14 tissues was calculated using data from ref. (34). The Pearson correlation was calculated between a vector representing peptide counts for FAM36A and all mitochondrial proteins present in the proteomics data.

Genetic analysis of the FAM36A gene

Genomic DNA from cultured patient fibroblasts was isolated as described before (55). Primers were designed from the Genbank accession number of the FAM36A gene (NM_198076). The primer sequences are available on request. The size of the PCR products were checked on a 1.5% agarose gel before being directly sequenced using the BigDye terminator cycle on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Generation of FAM36A expression plasmids

The entry vector containing FAM36A complementary DNA (cDNA) without a stop codon was obtained from Harvard Medical School (clone ID: HsCD00372325) (56) and recombined with the use of LR clonase enzyme mix (Invitrogen) into a tetracycline inducible mammalian expression vector adding a calmodulin–streptavidin TAP tag to the C-terminus. For the generation of Lentiviral particles containing FAM36A-V5, the corresponding entry vector was recombined with the pLenti6.2/V5 destination vector (Invitrogen). A pLenti6.2/V5 with the V5-tag fused to the COX8 mitochondrial import signal was constructed as control (mito-import V5).

Cell culture OXPHOS enzyme measurements

Human skin fibroblasts were cultured in M199 (Gibco) supplemented with 10% [v/v] fetal calf serum (FCS) and 1% [v/v] penicillin/streptomycin (Gibco). T-REX™ Flp-In™ Human embryonic kidney 293 cells (HEK293; Invitrogen) were grown and maintained in Dulbecco’s Modified Eagles Medium (DMEM, Lonza) supplemented with 10% FCS, 1% [v/v] penicillin/streptomycin, zeocin (300 μg/ml, Invitrogen) and 5 μg/ml blasticidin (Calbiochem). To generate stable FAM36A-TAP HEK293 cells were transfected with the corresponding construct using Superfect transfection reagent (Qiagen) and selected for stable transfectants by replacing the zeocine in the culture medium with hygromycin (final concentration 200 μg/ml, Calbiochem). Gene expression was induced by adding doxycycline (Sigma) to the culture medium with a final concentration of 1 μg/ml for a minimum of 24 h. Rh0 cells were derived from 143B osteosarcoma cells and cultured, including a Cy73 fibroblast control as described in (57).

Lentiviral transduction of human skin fibroblasts

Lentiviral particles were produced using the HEK293FT packaging cell line (Invitrogen) according to the manufacturer’s specifications. For transduction, fibroblasts were incubated for 24 h with virus-containing medium mixed in a 1:1 ratio with growth culture medium in the presence of 4 μg/ml polybrene. Selection for stable FAM36A-V5 and mito-import-V5 transduced cells was achieved by adding blasticidin (2.5 μg/ml) to the culture medium resulting in the complete loss of untransduced cells after 2 weeks of selection, in contrast to the transduced cells shown in Figure 4.

Cellular fractionation, proteinase K protection assay and isolation of mitoplasts and determination of protein concentrations

Cellular fractionation was carried out as described before (58). Permeabilization of mitochondria with digitonin followed by a proteinase K protection assay was performed according to the published protocol (59). Susceptibility of proteins 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 done as previously described (60). Protein concentrations were determined using the microBCA protein kit (Thermo Scientific).

1D and 2D Blue-Native PAGE, complex I and IV in-gel activity, SDS-PAGE, western blotting and immunodetection

One-dimensional (1D) 5–15% gradient and 2D BN-PAGE as well as measuring the IGA of complex I and IV were done as described in ref. (60). 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 before transferred to nitrocellulose membranes by western blotting. Membranes were blocked with 5% non-fat dry milk in Phosphate buffered saline (PBS) containing 0.1% [v/v] Tween-20 (PBST) prior to incubations with primary antibodies. The following antibodies were used: rabbit polyclonal anti-FAM36A HPA045490 (dilution 1:500; Atlas Antibodies), anti-CBP for detection of the TAP-tag (dilution 1:1000; GenScript), anti-ND1 (dilution 1:1,000; kindly provided by A. Lombe`s, (61)), mouse monoclonal anti-SDHA (dilution 1:1000), anti-COX1 (dilution 1:1000), anti-COX2 (dilution 1:1000), anti-COX4 (dilution 1000), anti-COX5A (dilution 1:1000), anti-UQRC1 and UQRC2 (dilution 1:5000), anti-ATP5a (dilution 1,1000) all obtained from MitoSciences, anti-TOM20 (dilution 1:5000, BD transduction laboratories) anti-CK-B 21E10 (dilution 1:5000, (62)) and anti-TACO1 antibody (dilution 1:1000; Abnova). 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 using the Chemidoc XRS+ system (Biorad).
Quantitative RT-PCR of mitochondrial genes

Total RNA was extracted from patient and control fibroblasts by using Trizol (Invitrogen). Subsequent cDNA synthesis was performed with M-MLV reverse transcriptase using the standard RT-PCR protocol. Relative amounts of MT-CO1, MT-CO2, MT-CO3 and MT-CYB translation products were assayed with specific 6-carboxyfluorescein-aminohexyl amide (6-FAM) labeled probes (Applied Biosystems) using AmpliTaq Gold (Roche) on a Biorad C1000 Thermal Cycler with CFX96 Real-Time System.

Mitochondrial translation assay

*In vivo* mitochondrial protein synthesis in cultured fibroblasts was analyzed as described previously (63). Briefly, patient and control fibroblasts were labeled for 60 min in *l*-methionine- and *l*-cysteine-free M199 supplemented with 10% dialyzed FCS, etimine (100 µg/ml) to block cytosolic translation and 200 µCi/ml [35S]-methionine and [35S]-cysteine (Tras35S-Label; MP Biomedicals). After labeling, the cells were chased for 10 min in regular M199 with 10% FCS, harvested and resuspended in PBS containing 2% [w/v] lauryl maltoside. Next insoluble material was removed from the cell lysate by centrifugation for 10 min at 10,000 × g after which equal amounts of protein were separated by SDS–PAGE on a 16% gel after which the gel was dried and exposed to a Phosphorimager screen. Labeled proteins were visualized by scanning the screen with a FLA5100 scanner (Fujiimager). Equal protein loading was confirmed by staining the gel with Coomassie Brilliant Blue G-250 after rehydration.

Validation of the novelty of the variant with the exome sequencing data

The presence of the variant at the genomic position chr1:245,005,357 (NCBI human genome hg19) was validated in the internal database of sequenced exomes at the Human Genetics Department of Radboud University Medical Centre. The position was typically very well covered by sequence reads (average 135, median 125). The presence of the variant at the genomic position chr1:245,005,357 (NCBI human genome hg19) was validated

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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