

A Mutation in *C2orf64* Causes Impaired Cytochrome *c* Oxidase Assembly and Mitochondrial Cardiomyopathy

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The assembly of mitochondrial respiratory chain complex IV (cytochrome *c* oxidase) involves the coordinated action of several assembly chaperones. In *Saccharomyces cerevisiae*, at least 30 different assembly chaperones have been identified. To date, pathogenic mutations leading to a mitochondrial disorder have been identified in only seven of the corresponding human genes. One of the genes for which the relevance to human pathology is unknown is *C2orf64*, an ortholog of the *S. cerevisiae* gene *PET191*. This gene has previously been shown to be a complex IV assembly factor in yeast, although its exact role is still unknown. Previous research in a large cohort of complex IV deficient patients did not support an etiological role of *C2orf64* in complex IV deficiency. In this report, a homozygous mutation in *C2orf64* is described in two siblings affected by fatal neonatal cardiomyopathy. Pathogenicity of the mutation is supported by the results of a complementation experiment, showing that complex IV activity can be fully restored by retroviral transduction of wild-type *C2orf64* in patient-derived fibroblasts. Detailed analysis of complex IV assembly intermediates in patient fibroblasts by 2D-BN PAGE revealed the accumulation of a small assembly intermediate containing subunit COX1 but not the COX2, COX4, or COX5b subunits, indicating that *C2orf64* is involved in an early step of the complex IV assembly process. The results of this study demonstrate that *C2orf64* is essential for human complex IV assembly and that *C2orf64* mutational analysis should be considered for complex IV deficient patients, in particular those with hypertrophic cardiomyopathy.

Identification of the disease-causing mutation in patients with a mitochondrial disorder due to cytochrome *c* oxidase (complex IV) deficiency (MIM 220110) is complicated by the sheer number of candidate genes. Mutations in mtDNA-encoded genes *MT-CO1*, *MT-CO2*, and *MT-CO3*, as well as *MT-TS1* and *MT-TL1*, have been identified, although these are relatively rare.¹ Remarkably, mutations in the ten nuclear-encoded structural complex IV subunits appear to be even rarer because only one case has been described to date.² The majority of deficiencies are caused by mutations in nuclear genes encoding proteins involved in the synthesis and assembly of complex IV. These include TACO1 (MIM 612958), a translational activator of COX1;³ LRPPRC (MIM 607544) that stabilizes COX1 and 3 mRNA;^{4,5} COX10 (MIM 602125) and COX15 (MIM 603646) that are involved in heme A biosynthesis;^{6,7} SCO1 (MIM 603644) and SCO2 (MIM 604272) that catalyze copper insertion into COX2;^{8–10} and SURF1 (MIM 185620) that is involved in early maturation of COX1.^{11,12} Despite the progress that has been made in understanding the mechanism of complex IV assembly, many complex IV deficient cases remain unsolved at the molecular genetic level. Thus, in yeast, many more assembly factors that have human orthologs have been identified, including Cox11p, Cox16p, Cox17p, Cox19p, and Pet191p. Some of the human orthologs of the genes encoding these proteins have been screened for the

presence of mutations in complex IV deficient patients. However, no mutations have been found thus far, suggesting that these genes might not be relevant to human pathology.^{13,14}

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 Nijmegen Medical Center and informed consent. This report describes two patients from a consanguineous family of Turkish origin. The first child was born by Caesarian section because of fetal stress after 39 weeks of an uneventful pregnancy. The patient died on day 8 after birth because of hypertrophic cardiomyopathy of the left and right ventricles. During the next pregnancy, an ultrasound examination performed at week 34 revealed signs of fetal cardiomyopathy. The girl was born at week 36 by caesarean section because of fetal stress. On day 10 after birth, this patient also died because of hypertrophic restrictive cardiomyopathy. For both patients, functional impairment of brain or skeletal muscle, often observed in complex IV deficiency,¹⁵ were not documented. Postmortem microscopic investigations showed accumulation of lipid droplets in cardiomyocytes and mitochondrial proliferation. Measurements of the mitochondrial respiratory chain enzyme activities were performed by previously described methods^{16,17} in cultured fibroblasts of patient P1 revealed a severe

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Table 1. Activity of Respiratory Chain Enzymes in Fibroblasts from Patient P1

Enzyme	Enzyme Activity ^a	Control Range ^a
Complex I	150	100–310
Complex II	528	520–845
Complex III	1523	1320–2610
Complex IV	150	680–1190
Citrate synthase	206	144–257

Patient P1 is homozygous for the c.157G>C mutation in *C2orf64*.

^a Activities of complex I, complex II, complex III, and complex IV are expressed as milliunits per unit citrate synthase (CS); activity of citrate synthase is expressed as milliunits per milligram protein.

complex IV deficiency (Table 1). The activity of complex IV in cultured fibroblasts and in heart muscle was reduced as well (Table 2). In the same family, two subsequent pregnancies were tested for the presence of complex IV deficiency by measuring enzyme activities in native chorionic villi.¹⁸ The activities in both the chorionic villi and in cultured fibroblasts obtained from skin biopsies collected after birth revealed (near-) normal complex IV activities (Table 2). Genetic analysis did not reveal mutations in the mtDNA and in the nuclear genes known to cause complex IV deficiency, including *SCO1*, *SCO2*, *COX10*, and *COX15*. Subsequently, homozygosity mapping was performed on the index case patient P1 (VI-1 in Figure 1A), patient P2 (VI-2 in Figure 1A), and the two healthy siblings (VI-3 and VI-4 in Figure 1A), revealing a large candidate region on chromosome 2 (73.28–145.07 Mb). This region, containing a total of 710 genes, was screened for known human complex IV genes as well as for human orthologs of yeast genes encoding known complex IV assembly and maintenance proteins. Only one candidate gene is present in this region, *C2orf64*, the human ortholog of *PET191*. It has been shown that complex IV activity is deficient in yeast *pet191Δ* mutant strain, whereas the activity of succinate:cytochrome c oxidoreductase (complex II+III) is elevated, and complex

Table 2. Activity of Complex IV in Tissues and Cultured Fibroblasts from Patients and Healthy Sibling

Patient or Healthy Sibling	Genotype ^a	Tissue	Complex IV Activity ^b	Control Range ^b
Patient P2	mut/mut	heart	4	114–424
		fibroblasts	16	680–1190
Healthy sibling S3	wt/mut	chorionic villi	269	271–922
		fibroblasts	623	680–1190
Healthy sibling S4	wt/wt	chorionic villi	557	271–922
		fibroblasts	714	680–1190

^a Genotype refers to the presence of wild-type (wt) or c.157G>C mutated (mut) *C2orf64* alleles.

^b The activity of complex IV is expressed as milliunits per unit citrate synthase.

III and V protein levels are unaffected.¹⁹ This indicates that mitochondrial translation in *pet191Δ* cells is normal, yet levels of Cox1p, Cox2p, and Cox3p protein were found to be reduced. From these findings, it was concluded that Pet191p is a complex IV assembly protein in yeast.¹⁹ In a previously published study of this gene in a large cohort of complex IV deficient patients, no mutations were observed.¹⁴ We found a homozygous mutation at c.157G>C (p.Ala53Pro) in *C2orf64* (NM_001008215.1) in the two affected children, whereas healthy sibling S3 was heterozygous for this mutation, and healthy sibling S4 carried two wild-type alleles (Figure 1B). This was in agreement with the homozygosity mapping data (Table S1). Both parents were heterozygous for the mutation. This mutation was not detected in 216 alleles of healthy control individuals of Turkish origin, nor is it present in EST databases, consistent with pathogenicity of the mutation. In order to assess whether the *C2orf64* p.Ala53Pro mutation had an effect on complex IV assembly, we performed 1D and 2D blue native PAGE (BN-PAGE) analysis on fibroblasts of the two patients and their healthy siblings. One-dimensional BN-PAGE showed that both the activity and amount of holocomplex complex IV was strongly reduced in both patients compared to the siblings (Figure 2A). Two-dimensional BN-PAGE analysis subsequently confirmed the near absence of holocomplex IV and showed COX1 accumulation in subcomplexes (Figure 2C). Complex IV assembly is a stepwise process with three milestones in the form of subassemblies S1, S2, and S3 that are formed by the sequential addition of subunits and cofactors.^{20,21} The predominant subcomplex in the *C2orf64* patients was similar in size to the smallest subcomplex observed in control cells (Figure 2C). This subcomplex appears to be similar to the previously described subcomplex S1²² that has also been observed in complex IV deficiency due to mutations in the gene-encoding assembly factor SURF1,^{23,24} although these patients also show a varying degree of accumulation of subcomplex S2, the next subassembly in the complex IV assembly pathway. The levels of individual complex IV subunits COX1, COX2, COX4, and COX5a were also reduced (Figure 2B), which suggests that the very low levels of holocomplex IV and absence of higher order assembly intermediates beyond subcomplex S1 results in downregulation or destabilization of individual complex IV subunits. The reduced levels of COX1 and COX2 are compatible with the reduced levels of the yeast orthologs in *Pet191p* deficient yeast cells.¹⁹ Taken together, these observations suggest a role for *C2orf64* in an early stage of the complex IV assembly process.

To confirm that the mutation in *C2orf64* caused the strong reduction of complex IV holocomplex amount and activity, we retrovirally transduced both index patient and healthy sibling fibroblasts with human full-length *C2orf64*. Complementation of patient fibroblasts with wild-type *C2orf64* resulted in normalization of fully assembled complex IV (Figure 3).

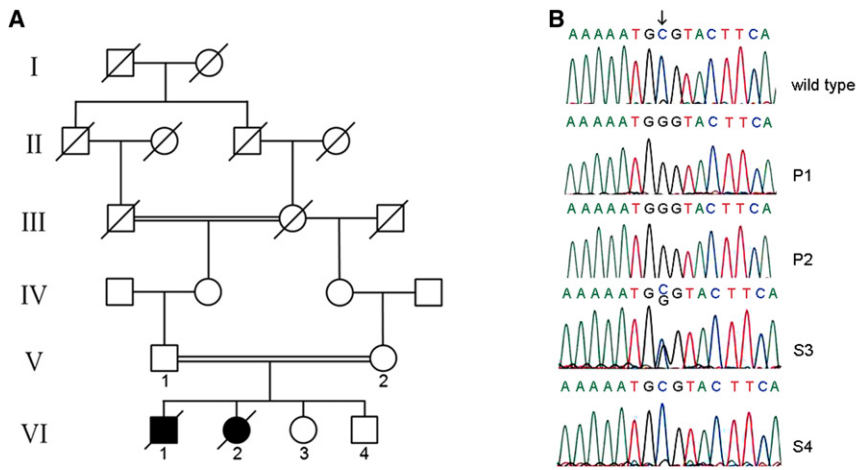


Figure 1. Family Pedigree and Molecular Genetic Analysis of the *C2orf64* cDNA

(A) Pedigree of the family of the two patients described in this report. (B) Electropherograms showing the wild-type sequence of *C2orf64* (top panel) and the nucleotide changes in the complex IV deficient patients P1 (VI-1 in A) and P2 (VI-2) and the healthy siblings S3 (VI-3) and S4 (VI-4). The arrow indicates the mutated nucleotide c.157G>C. P1 and P2 are homozygous for the c.157G>C mutation, whereas S3 is a heterozygous carrier and S4 carries the wild-type sequence. Please note that the reverse sequence is shown.

Moreover, normal in-gel activity of complex IV was present in fibroblasts from the healthy siblings and in the *C2orf64*-complemented patient cell line (Figure 3). Overexpression of wild-type *C2orf64* in healthy siblings had no effect on complex IV levels or activity (Figure 3). These observations support the notion that the mutation

in *C2orf64* is responsible for the complex IV deficiency in our patients.

Further support for the pathogenic role of the p.Ala53Pro mutation came from protein sequence analysis. The *C2orf64* protein contains two Cx₉C motifs that are highly conserved among all eukaryotes with complex

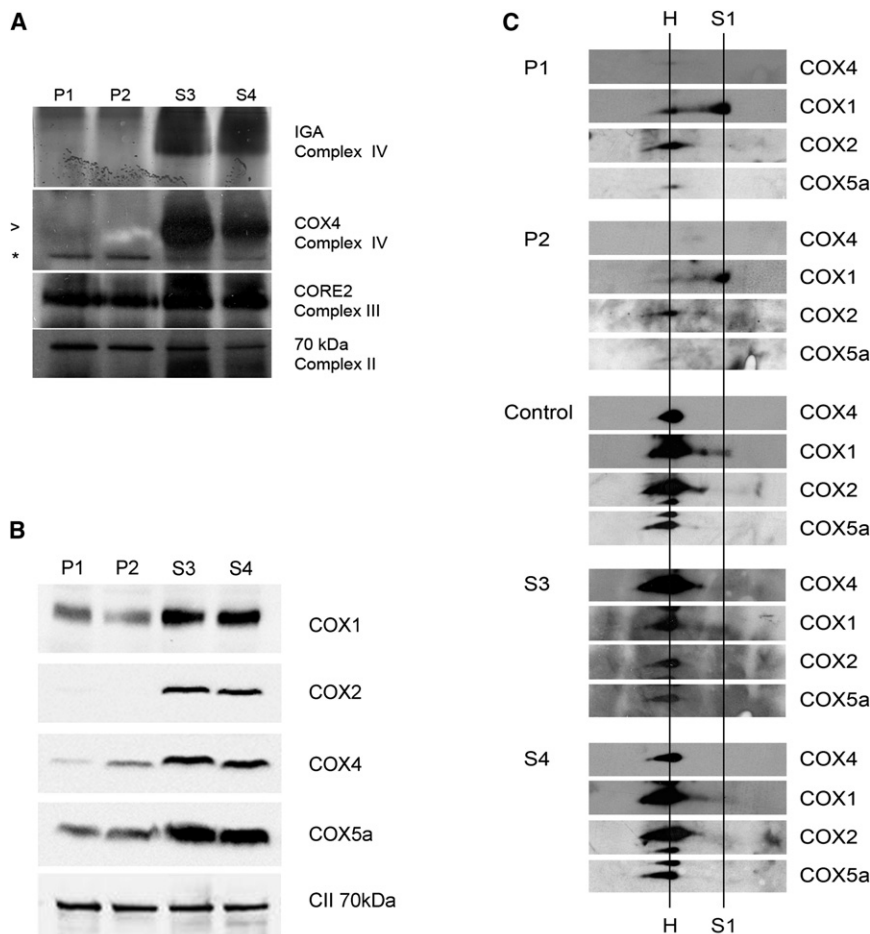


Figure 2. Reduced Complex IV Activity and Amount in Fibroblasts from the Patients with a *C2orf64* Mutation

(A) Fibroblasts from patients P1 and P2 show a severely reduced in-gel activity of complex IV³² compared to the unaffected siblings S3 and S4 (top panel). The lower three panels show the results of immunoblots after nondenaturing BN-PAGE,³³ revealing a severely reduced complex IV amount in patients P1 and P2. Complex IV was stained with anti-COX4 antibodies. Equal loading of the gel was tested by staining for complex III (by using anti-CORE2) and complex II (by using anti-70 kDa subunit). Holocomplex IV is indicated by the arrowhead; the asterisk indicates a nonspecific band. (B) Immunoblot after SDS-PAGE of fibroblast extracts of patients P1 and P2 and healthy siblings S3 and S4 showing reduced expression levels of complex IV subunits COX1, COX2, COX4, and COX5a in fibroblasts of both patients. The complex II 70 kDa subunit (CII 70 kDa) was used as a loading control. (C) Two dimensional BN-PAGE analysis of fibroblasts from patients P1 and P2 and the healthy siblings S3 and S4 was performed in accordance with standard procedures. Blots were stained by using antibodies against different complex IV subunits as indicated. Holocomplex IV is indicated (H). Patients P1 and P2 show accumulation of a small subcomplex (indicated by S1) that contains COX1 but not COX2, COX4, and COX5a. This indicates that mutation of *C2orf64* results in accumulation

of a COX1 containing early complex IV assembly intermediate. Note that blots of different fibroblasts and blots stained with different antibodies can only be compared qualitatively because exposure times are not the same.

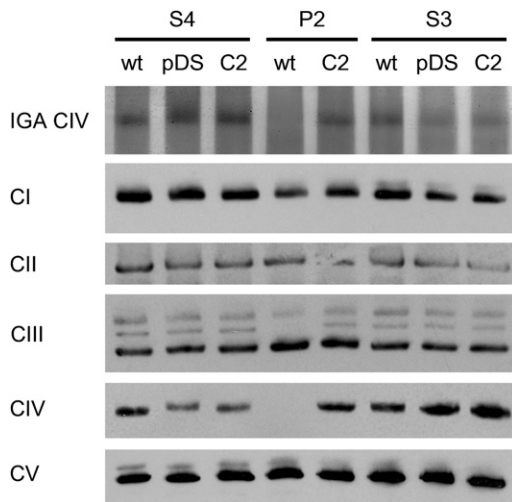


Figure 3. Complementation with Wild-Type *C2orf64* Restores Complex IV

Fibroblasts from patient P2 and healthy siblings S3 and S4 were infected with retrovirus containing no insert (pDS) or *C2orf64* (C2). The *C2orf64* complementation was done by cloning full length human *C2orf64* cDNA clone IOH26651 (GeneID: 493753; Imagenes, Berlin, Germany) into the Gateway retroviral destination vector pDS_FBneo (# MBA-295, LGC, Middlesex, UK) with the Gateway LR Clonase II enzyme mix (Invitrogen). Recombinant viruses were produced by using the amphotropic packaging cell line PA317 (#CRL-9078, LGC, Middlesex, UK) according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). P2, S3, and S4 fibroblasts were incubated for 24 hr with a 1:1 mixture of growth medium and virus-containing medium in the presence of 4 μ g/ml polybrene,³⁴ followed by 14 days selection with 500 μ g/ml geneticin (G418, PAA, Pasching, Austria). G418-resistant cells were used for biochemical analyses within 5 passages after transduction. P2 fibroblasts grew very slowly and all cells failed to survive the selection procedure upon retroviral transduction of the empty vector. Mitochondrion-enriched fractions from all other transduced cell lines were tested for in-gel activity of complex IV after BN-PAGE. In addition, amounts of OXPHOS complexes were visualized with anti-NDUFA9 (complex I; CI), anti-70 kDa subunit of complex II (CII), anti-CORE2 for complex III (CIII), anti-COX1 for complex IV (CIV) and anti-complex V α for complex V (CV). The results show a clear complex IV deficiency, consistent with the results in Figure 2A, which is rescued by complementation with wild-type *C2orf64* (indicated by C2). Lanes indicated with wt are nontransduced fibroblasts, lanes with pDS indicate fibroblasts transduced with empty virus. Note that the complex III panel shows three bands, the lowest of which represents holo-complex III, whereas the two more slowly migrating bands most likely represent supercomplexes containing complex III. The middle band is absent in patient P2 and returns after complementation with wild-type *C2orf64*. This middle band therefore most likely represents a supercomplex containing complex III and complex IV.

IV (Figure 4A). In twin Cx₉C motif proteins with a known 3D structure, COX17 and COX6b, the cysteines in these motifs form intramolecular disulfide bridges that stabilize two antiparallel α helices in a hairpin conformation.^{25,26} If *C2orf64* adopts such a helical hairpin structure, the p.Ala53Pro mutation will have a significant influence on protein structure, because proline residues lack an amide proton, precluding the hydrogen bond required for the

formation of α -helices.²⁷ Additionally, steric interference of the proline sidechain with the α -helix backbone²⁸ restricts the presence of prolines to the first four positions of a helix.²⁷ Indeed, analysis of eukaryotic orthologs of not only Pet191p (Figure 4A) but also all other 23 human mitochondrial twin Cx₉C proteins shows that prolines appear exclusively within the first four residues of α helices (data not shown). In contrast, the p.Ala53Pro mutation is present at the seventh position and is likely to interfere with its conformation (Figure 4B). Consistent with the predicted structure of Pet191p, the first cysteine (C14) of the first Cx₉C motif and the second cysteine (C57) of the second Cx₉C motif, which are predicted to interact with each other, have been shown to be essential for respiration in yeast.¹⁹ Thus, the p.Ala53Pro mutation in *C2orf64* is likely to interfere with the oxidative folding process of this twin Cx₉C domain-containing protein that is linked to its mitochondrial inter membrane space localization.^{29–31}

Supplemental Data

Supplemental Data include one table and can be found with this article online at <http://www.cell.com/AJHG/>.

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Web Resources

The URLs for data presented herein are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank>

NCBI database, <http://www.ncbi.nlm.nih.gov>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim>

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