

Biallelic variants in *CHCHD4* are associated with combined OXPHOS defect leading to mitochondrial disease

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Summary

Mitochondrial disorders show remarkable clinical and genetic heterogeneity and result from variants in either mitochondrion- or nucleus-encoded genes. *CHCHD4* is a component of the mitochondrial import and assembly pathway that imports small cysteine-containing substrates. We report a pediatric patient with biallelic *CHCHD4* variants who presented with severe neurological regression and early death. Western blot analysis showed decreased levels of *CHCHD4* and diminished assembly of complexes I and IV in his fibroblasts. To demonstrate that *CHCHD4* variants were responsible for the observed biochemical phenotype, we overexpressed wild-type *CHCHD4* in control and subject fibroblasts, restoring levels of complex I and IV proteins and the associated assembly defects. Proteomic studies pointed to electron transport and complex I biogenesis as the main dysregulated pathways and showed a severe loss of several complex I and IV proteins and/or assembly factors rescued by overexpression of wild-type *CHCHD4*. *CHCHD4* has numerous targets and interacting factors and is involved in the export of iron-sulfur clusters synthesized inside mitochondria. Surprisingly, few of these interacting factors or non-mitochondrial functions were impacted by the observed *CHCHD4* defect. In conclusion, our work establishes *CHCHD4* deficiency as a cause of dysregulated mitochondrial protein import resulting in a severe neurological condition.

Introduction

Most mitochondrial proteins are encoded by nuclear genes and subsequently imported into the organelle through the outer-membrane translocase (TOM), the sorting and assembly machinery (SAM), and the inner-membrane translocase (TIM).¹ A subgroup of small cysteine-containing mitochondrial proteins carrying (CX₉C)₂ motifs is imported by the evolutionary conserved mitochondrial import and assembly (MIA) pathway, located in the inter-membrane space (IMS). The MIA pathway, which catalyzes the oxidative folding of incoming mitochondrial proteins through a disulfide relay system (DRS), is composed of *CHCHD4* and growth factor ERV1-like (GFER).² *CHCHD4* imports small cysteine-containing substrates, such as TIM proteins (TIMM8A and TIM13), complex I subunits (NDUFA8, NDUFB7, NDUFB10, NDUFS5, and NDUFS8), complex IV subunits and assembly factors (COX6B1, COX17, COX19, COA4, COA5, and COA6), or the regulator of mitochondrial Ca²⁺ uniporter, expanding the diversity of *CHCHD4* substrates.³ The *CHCHD*-containing proteins are (CX₉C)₂ motif-containing proteins involved

in several mitochondrial functions. *CHCHD1* is a subunit of the mitoribosome, *CHCHD3* and *CHCHD6* are components of the mitochondrial contact site and crista-organizing system (MICOS), *CHCHD10* is involved in MICOS stability, and *CHCHD8* is an assembly factor of complex IV.⁴ *CHCHD4* is the only known *CHCHD*-containing protein involved in protein import into the IMS. It also interacts with the apoptosis-inducing factor AIFM1, which acts as an effector of *CHCHD4* activity.⁵ *CHCHD4* contains an iron-sulfur cluster (ISC) and is also a component of the mitochondrial ISC export machinery, modulating cellular iron homeostasis.⁶ Finally, *CHCHD4* can regulate mitochondrial DNA (mtDNA) release into the cytosol, triggering the cGAS-STING-nuclear factor κ B (NF- κ B) pathway.⁷

Pathogenic variants in genes encoding *CHCHD* proteins such as *CHCHD2* (MIM: 616244; coiled-coil-helix-coiled-coil-helix domain-containing protein 2) and *CHCHD10* (MIM: 615903; coiled-coil-helix-coiled-coil-helix domain-containing protein 10) have been reported in Parkinson disease⁸ and in frontotemporal dementia and/or amyotrophic lateral sclerosis-2,⁹ respectively. Moreover, *GFER* (MIM: 600924) variants result in mitochondrial myopathy

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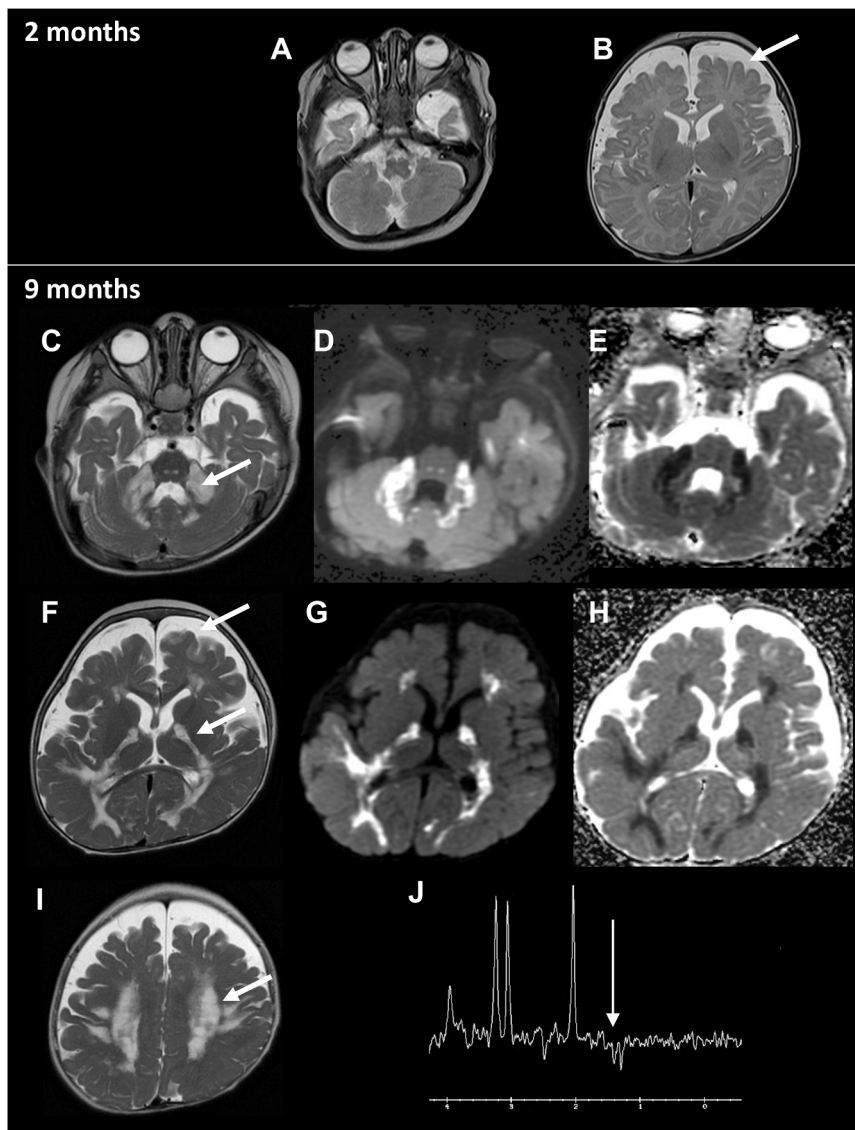


Figure 1. Brain MRI of the subject at 2 and 9 months of age

(A and B) Brain MRI axial slices in T2. The arrow indicates the enlargement of the periventricular spaces in the supratentorial region.

(C–I) Illustration of brain images at 9 months of age. Shown are brain MRI axial slices on T2 (C, F, and I) and diffusion weighted images (D and G) and apparent diffusion coefficients (E and H). The arrows indicate marked hypersignal in middle cerebellar peduncles (C), semi-oval centers (I), pyramidal tracts (F), and marked enlargement of the periventricular spaces in the supratentorial region (F). (J) MR spectroscopy showed a lactate peak (arrow).

gestational hypertension diagnosed at 37+2 weeks. The infant was born at 37+3 weeks following induction for abnormal fetal heart rate tracing. Apgar scores were 9/10/10. Intrauterine growth restriction was noted (birth weight: 1,860 g, <third percentile; head circumference: 31.5 cm, third percentile; Table S1). Birth length was not documented. At 9 h of life, the infant presented with persistent hypoglycemia refractory to oral glucose supplementation, requiring continuous intravenous glucose infusion. Metabolic workup revealed severe lactic acidosis (pH 7.00, lactate 26 mmol/L), hepatocellular cytolysis (AST, 176 U/L; ALT, 42 U/L), cholestasis, and moderate hyperammonemia (135 μ mol/L), consistent with acute liver dysfunction. Further investigations showed massive lactic aciduria. Rapid normalization of hepatic function and acid-base balance occurred within the first

days of life. However, recurrent short-fasting hypoglycemia persisted, requiring enriched feeding regimens and nocturnal enteral nutrition. Chronic hyperlactatemia (2–6.5 mmol/L) persisted, with an elevated lactate/pyruvate ratio (blood lactate/pyruvate ratio [L/P] = 33.5). From 6 months of age, glycemic control improved progressively. However, a delay in developmental milestones was noted, with increased peripheral hypertonia. At 9 months, the patient developed psychomotor development regression with loss of previously acquired motor skills and impaired social interaction. Neurological deterioration progressed rapidly, with episodes of inconsolable crying, marked hypertonia, and major discomfort. He had worsened hyperlactatemia (7.5 mmol/L), elevated cerebrospinal fluid lactate (4.5 mmol/L), and increased L/P (cerebrospinal fluid [CSF] L/P = 27.7). The neurological phenotype evolved toward a severe encephalopathic state with recurrent dystonic episodes and major discomfort. The patient died at 11 months of age.

Subjects and methods

The subject was the first child of non-consanguineous healthy parents, with no family history. Pregnancy was uneventful except for

A first brain MRI performed at 2 months of age showed enlargement of the periventricular spaces in the supratentorial region (Figure 1). At 9 months of age, brain MRI detected hypersignal in the white matter of the semi-oval centers, the pyramidal tracts, the brain stem, and the middle cerebellar peduncles. Diffusion

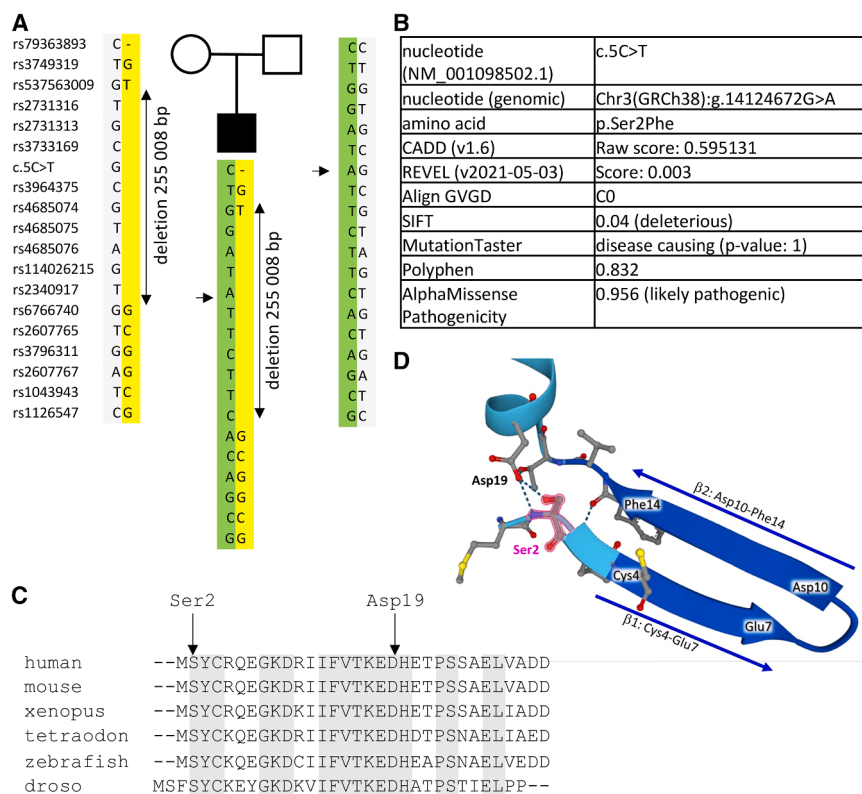


Figure 2. CHCHD4 variants

(A) Pedigree of the subject's family and haplotypes at the *CHCHD4* locus. The mutant paternal and maternal alleles are indicated in green and yellow, respectively. The arrow indicates the c.5C>T variant. The maximum size of the deletion is 255,753 bp (between heterozygous rs537563009 and rs6766740 SNPs), and the minimum size is 22,215 bp (between homozygous rs2731316 and rs2340917 SNPs).

(B) Evidence of pathogenicity associated with the c.5C>T *CHCHD4* variant.

(C) Multisequence alignment of *CHCHD4* proteins.

(D) Three-dimensional representation of the human *CHCHD4* structure predicted by AlphaFold, showing the location and the prediction of hydrogen-bonding (H-bond, dotted lines) of Ser2 (pink). The two arrows indicate the β strands of the N-terminal part of the protein. The colors of the 3D structure correspond to the confidence scores of the different parts of the protein: dark blue, very high (pLDDT > 90); light blue, confident (90 > pLDDT > 70); yellow, low (70 > pLDDT > 50); and orange, very low (pLDDT < 50).

weighted imaging showed apparent diffusion coefficient restriction in the pathological areas. Spectroscopy revealed a lactate peak. No abnormality in the basal ganglia could be observed.

Informed consent was obtained for the subject in accordance with the Declaration of Helsinki protocols and approved by local institutional review boards in Paris.

Details of cell culture, whole-exome sequencing, protein extraction, SDS-PAGE and immunoblotting, BN-PAGE, *CHCHD4* overexpression, and proteomics analysis are presented as [supplemental information](#).

Results

Molecular genetic investigations were undertaken using EDTA-blood DNA samples. mtDNA sequencing did not detect any pathological variant. WES was performed on the subject as a family trio. A first WES analysis in the trio did not allow identification of any gene with biallelic heterozygous variations predicted to be deleterious. Nevertheless, analysis of WES data of the subject alone resulted in the identification of an apparently homozygous variant in *CHCHD4* (NM_001098502.1, *CHCHD4.1*), also known as MIA40. This c.5C>T variation (p.Ser2Phe) was inherited from the father, but the mother was apparently homozygous for the WT allele, which prompted us to hypothesize that instead she carried a deletion at the *CHCHD4* locus. Indeed, analysis of the WES data at *CHCHD4* locus showed loss of heterozygosity of several successive SNPs in the mother and her son, confirming

the presence of a large deletion of 255 kb maximum, encompassing the complete *CHCHD4* gene and exons 1–7 of the adjacent *TMEM43* gene (Figure 2A). The *CHCHD4* Ser2Phe change replaces a hydrophilic with a hydrophobic amino acid; this change affects a highly evolutionary conserved amino acid and is predicted to be deleterious (Figures 2B and 2C). *TMEM43* is a nuclear envelope protein involved in the organization of protein complexes at the inner nuclear membrane.¹² Heterozygous *TMEM43* missense or nonsense variations result in adult-onset autosomal-dominant arrhythmogenic right ventricular dysplasia,¹³ Emery-Dreifuss muscular dystrophy,¹⁴ or auditory neuropathy,¹⁵ but no large deletion has been previously reported. Considering that the mother is healthy, we hypothesize that the *TMEM43* deletion has no clinical consequences.

To visualize the location of mutant amino acid, we used the AlphaFold machine learning algorithm that predicts protein structures and that has high confidence in the location of the identified missense variant.¹⁶ Ser2 is located just before the β 1 strand (Cys4-Glu7), forming a β hairpin with the β 2 strand.¹⁷ Ser2 is predicted by AlphaFold to be involved in two hydrogen-bonding (H bonds) with Asp19. Both Ser2 and Asp19 are highly conserved residues among species, suggesting they are important residues for *CHCHD4* protein function and/or structure (Figure 2D).

SDS-PAGE and western blot analysis revealed a decreased steady-state level of *CHCHD4* protein in cultured skin

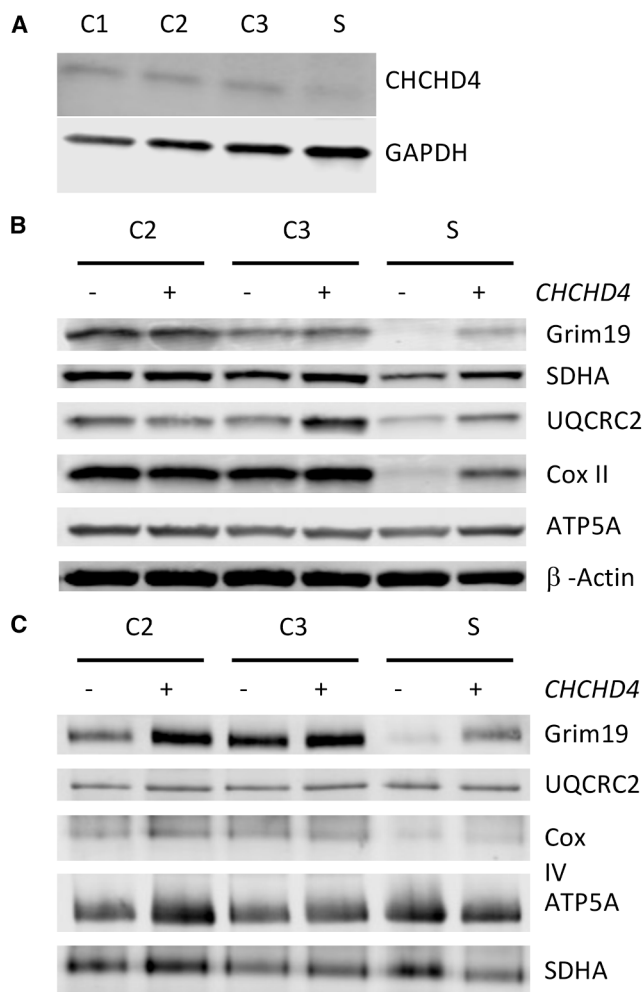


Figure 3. Biochemical investigation of fibroblasts

(A) Western blot analysis of CHCHD4 performed on total protein of fibroblasts of the subject (S) compared to 3 controls (C1, C2, and C3). GAPDH was used as a loading control.

(B) Western blot analysis of OXPHOS subunits in fibroblasts of the subject and two controls (C2 and C3) transduced with or without WT *CHCHD4* cDNA. β -actin was used as a loading control.

(C) BN-PAGE on fibroblasts from S and 2 controls transduced with or without WT *CHCHD4* cDNA.

fibroblasts of the subject compared to controls (Figures 3A and S2), suggesting that the *CHCHD4* variants affect the stability of the protein. *CHCHD4* mediates the import of several mitochondrial proteins, including complex I and complex IV subunits and/or assembly factors. Consistent with this, we could observe a severe decrease of various OXPHOS subunits, Grim19, and UQCRC2 in fibroblasts with a relative sparing of ATP5A (Figures 3B and S3). Moreover, the mitochondrially encoded COXII subunit was also decreased, suggesting a destabilization of at least OXPHOS complex IV. Finally, to assess the assembly status and function of the OXPHOS system, we carried out BN-PAGE analyses, which revealed an obvious complex I and IV assembly defect (Figures 3C and S4).

To demonstrate that variations in the *CHCHD4* gene were responsible for the observed molecular phenotype

of subject-derived fibroblasts and therefore are likely disease causing in nature, we undertook functional complementation analyses in control and subject-derived fibroblasts. Fibroblasts were transduced with lentiviral particles expressing WT *CHCHD4* using pD2109 (Atum) for 12 h and then incubated for an additional 40 days until the cells were harvested. Stable transduction with lentiviral particles expressing WT *CHCHD4* cDNA (transcript variant 1) restored the steady-state levels of GRIM19, UQCRC2, and COXII proteins as well as a complex I and IV assembly defect (Figures 3B and 3C).

To assess the effect of the identified *CHCHD4* variants on various cellular functions, we performed proteomics analysis in subject and control fibroblasts transduced with or without WT *CHCHD4* cDNA. The proteomics analysis allowed the quantification of 8211 proteins, 603 of which were significantly downregulated in the fibroblasts of the subject compared to the control group (t test, false discovery rate [FDR] = 0.05). Overall, mitochondrial proteins were found to be mostly downregulated (17% of the downregulated proteins were mitochondrial proteins against 3% of the upregulated proteins) (Figure 4A; Table S2). *CHCHD4* was significantly decreased in subject fibroblasts (Figure 4A) and transduction with WT *CHCHD4* cDNA significantly increased its level in subject-derived fibroblasts (Figure S1C). As expected, the TMEM43 protein level was reduced in subject-derived fibroblasts compared to the control and not modified by overexpression of WT *CHCHD4* cDNA (Figure S1C).

Reactome pathway analysis identified respiratory electron transport and complex I biogenesis as the main dysregulated pathways within our dataset (FDR = 1.4×10^{-14} ; Figures S1A and S1B; Table S2). We then systematically looked at OXPHOS structure proteins and assembly factors and detected decreased levels of a large part of nucleus-encoded complex I subunits (Figures 4B and 4C; Table S2). Among them, only NDUFA8, NDUFB8, NDUFB10, and NDUFS5 are known *CHCHD4* targets,² suggesting a secondary defect of other subunits related to complex I assembly defect. Most complex IV subunits were also significantly decreased (Figures 4B and 4D; Table S2). Two complex IV assembly factors, COA7 and COA6, were also reduced (FC -3.6 and -2.76, respectively) as well as SCO2, COX19, COX11, and COX17 to a lesser extent (FC -2.08 to -1.07; Table S2). Accordingly, COX6B1 complex IV subunit and the COA6, COA7, SCO2, and COX17 assembly factors are *CHCHD4* targets.² No significant modification of nucleus-encoded subunits or assembly factors of complexes II, III, and V was observed (data not shown). Interestingly, several complex I and IV subunits encoded by mtDNA were also significantly decreased, but they do not require mitochondrial import, as they are synthesized within mitochondria (Figure 4E; Table S2). This decrease should be related to the decreased stability of complexes I and IV rather to abnormal mitochondrial protein synthesis, as factors

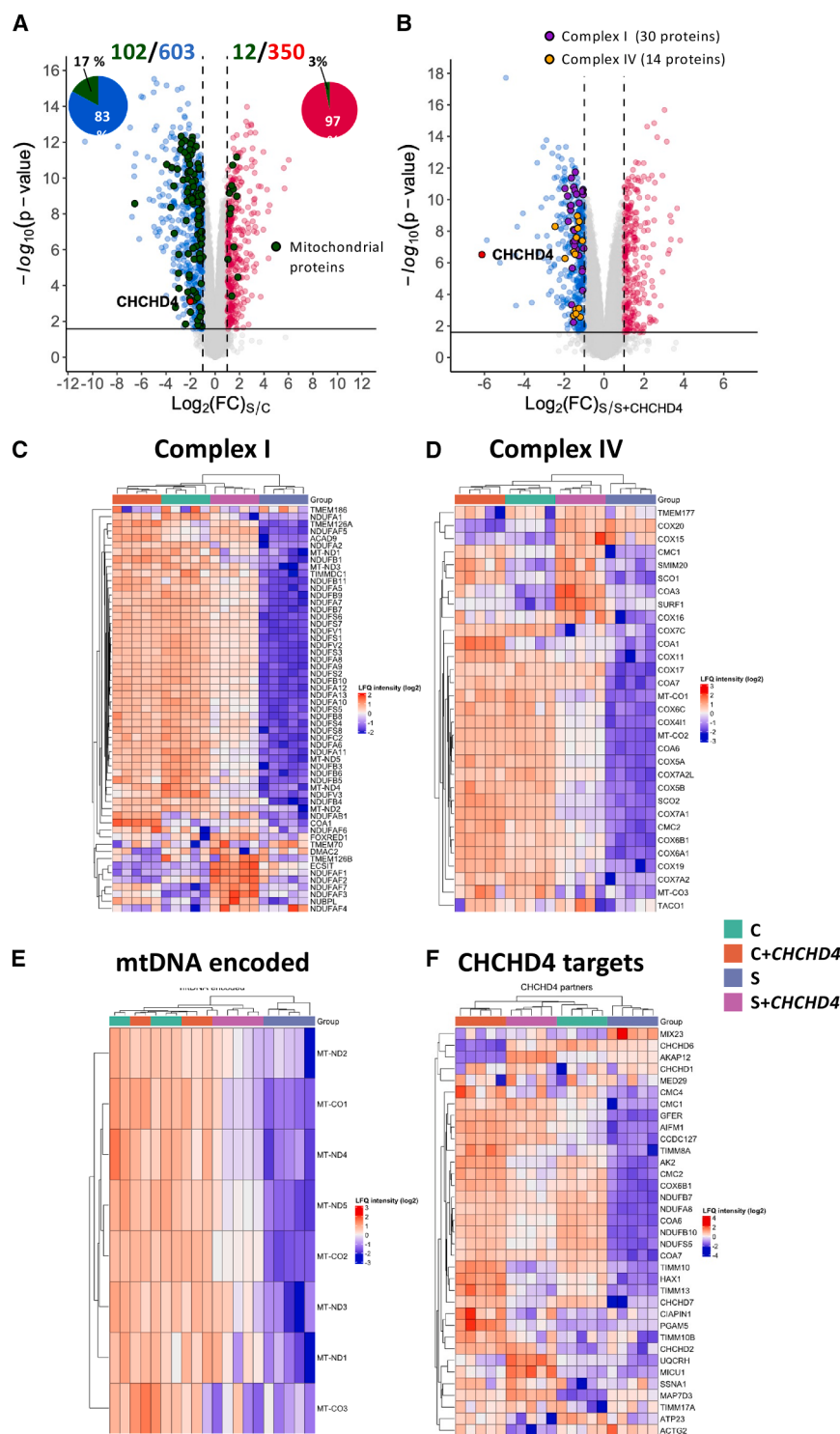


Figure 4. Proteomics analysis of subject and control fibroblasts transduced or not by WT *CHCHD4* cDNA

(A) Volcano plot representing the statistical comparison of the protein label-free quantification (LFQ) intensities of subject fibroblasts vs. control fibroblasts (FC = 2, FDR = 0.05). Proportions of up- and downregulated mitochondrial proteins are reported. Mitochondrial proteins (green) were retrieved from Mitocarta and literature data. CHCHD4 is shown in red.

(B) Volcano plot representing the statistical comparison of the protein LFQ intensities of subject fibroblasts vs. subject fibroblasts transduced with WT *CHCHD4* cDNA (FC = 2, FDR = 0.05). Complex I and IV proteins are reported.

(C–F) Heatmap representation depicting the dysregulated complex I (C) and complex IV (D) subunits and/or assembly factors, mtDNA-encoded proteins (E), and CHCHD4 targets (F) in different samples.

defect and that improvement of protein import significantly rescues OXPHOS function.

CHCHD4 forms a stable complex with AIFM1 through a direct interaction with its N-terminal part¹⁹ for mitochondrial protein import, but only a minor decrease of AIFM1 was observed in the subject's fibroblasts (Figure S1C; Table S2). This is consistent with previous reports showing that knockdown of CHCHD4 does not affect the AIF amount.¹⁹ CHCHD4 also interacts with GFER protein, essential for protein folding and import of a subset of mitochondrial proteins as part of the DRS. Notably, CHCHD4 downregulation has been shown to decrease the mitochondrial import of GFER.²⁰ Accordingly, the subject's fibroblasts displayed a medium decrease of GFER, and overexpression of WT *CHCHD4* cDNA increased it in the subject's fibroblasts (Figure S1C; Table S2).

involved in mitochondrial translation were mostly not changed in the subject's fibroblasts (Table S2), whereas downregulation of CHCHD4 has been previously shown to decrease several mitoribosome proteins.¹⁸ Interestingly, overexpression of WT *CHCHD4* cDNA in subject's fibroblasts increased the level of almost all of these proteins (Figures 4 and S1C; Table S2), demonstrating that *CHCHD4* variants are the cause of the OXPHOS

CHCHD4 has a central role in mitochondrial protein import and has several targets and interacting factors. CHCHD-containing proteins are imported with the help of the CHCHD4 import machinery,² but no major modification of these proteins was observed in subject-derived cells (Figure 4F; Table S2). Similarly, very few components of translocase of outer and inner mitochondrial membrane machinery were modified despite several proteins

of this machinery being substrates for CHCHD4²¹ (Figure 4F; Table S2). Finally, the additional CHCHD4 partner proteins^{22,23} were not significantly modified in the subject's fibroblasts except those involved in complexes I and IV (Figure 4F; Table S2).

CHCHD4 is not only involved in mitochondrial protein import but also in the export of ISCs synthesized inside the mitochondria⁶ that are then incorporated in cytosolic or nuclear proteins involved in essential cellular pathways such as iron homeostasis (IRP2 and FBXL5), nuclear DNA polymerases, helicases or exonucleases, or interferon response (RSAD2).^{24–26} Accordingly, CHCHD4 downregulation in HEK293 cells lowered the activity and stability of several cytosolic ISC-containing proteins.⁶ Among all known non-mitochondrial ISC-containing proteins, only one, the AOX1 aldehyde oxidase, a cytosolic drug-metabolizing enzyme,²⁷ was significantly decreased in the subject's fibroblasts (FC –3.26) and increased by overexpression of WT *CHCHD4* cDNA (Figure S1C).

Finally, none of the proteins and enzymes involved in mtDNA maintenance, mtRNA metabolism, nucleotide metabolism, the tricarboxylic acid cycle, mitochondrial lipid metabolism, fatty acid oxidation, CoQ₁₀ synthesis, mitochondrial dynamics and surveillance, amino acid metabolism, or mitochondrial transport were downregulated in CHCHD4-deficient fibroblasts (data not shown), suggesting that these proteins do not depend on CHCHD4 for protein import. In keeping with this, neither the regulator of the mitochondrial Ca²⁺ uniporter MICU1, a well-known target of CHCHD4,²³ nor other proteins involved in mitochondrial calcium metabolism were changed in the subject's fibroblasts (data not shown). Furthermore, proteomics analysis did not detect other non-mitochondrial deregulated pathways, suggesting that *CHCHD4* variants primarily affect OXPHOS proteins.

Discussion

In conclusion, this work shows that a CHCHD4 (MIA40) defect in humans is associated with a severe mitochondrial disease, resulting mainly from an OXPHOS function and assembly defect. Nevertheless, this defect does not alter other well-known functions depending on CHCHD4, such as Ca²⁺ signaling, or cytosolic ISC-containing proteins, suggesting that the residual amount of CHCHD4 is sufficient to maintain these functions. This confirms a central role of CHCHD4 in OXPHOS protein import, but why other functions are not affected is intriguing. Moreover, these results add to rare causes of protein import defects associated with pathogenic variants in *AIFM1*, *GFER*, *TIMM50* (MIM: 607381; translocase of inner mitochondrial membrane 50), *TIMM8B* (MIM: 606659), *TIMM22* (MIM: 607251), *TOMM7* (MIM: 607980; translocase of outer mitochondrial mem-

brane 7), *MAGMAS/PAM16* (MIM: 614336; presequence translocase-associated motor 16), and *DNAJC19* (MIM: 608977, DNAJ/HSP40 homolog, subfamily C, member 19)²⁸ and emphasizes the wide clinical heterogeneity associated with this cause of genetic diseases.

Limitations of the study

The coexistence of a complete *CHCHD4* deletion and the p.Ser2Phe variation leads to a significant decrease in CHCHD4 protein, and it is not possible to determine whether the p.Ser2Phe variation leads to a total loss of protein function or whether some residual activity remains.

We present here a single case that does not allow us to determine the natural history of CHCHD4, and identification of additional individuals with *CHCHD4* variants will allow us to better define the clinical and biochemical consequences of these very rare variations.

Data and code availability

The genome sequencing data supporting the current study have not been deposited in a public repository because consent was not obtained for this.

The published article includes all other data generated or analyzed during this study, or it is readily available from the authors.

Proteomic data submission

The mass spectrometry proteomics data have been deposited into the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD069027.

ClinVar submission

The accession number for the c.5C>T (p.Ser2Phe) variant is VCV004795219.1. The accession number for the CHCHD4 deletion is SCV007338468.

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Declaration of interests

The authors declare no competing interests.

Supplemental information

Supplemental information can be found online at <https://doi.org/10.1016/j.xhgg.2026.100615>.

Web resources

AlphaFold Protein Structure Database, <https://alphafold.ebi.ac.uk/>

MassDynamics 2.0 software, <https://massdynamics.com>

OMIM, <http://www.omim.org/>

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