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RESEARCH ARTICLE

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Mutations in COQ8B (ADCK4) found in patients with steroid-resistant nephrotic syndrome alter COQ8B function

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Abstract

Mutations in *COQ8B* cause steroid-resistant nephrotic syndrome with variable neurological involvement. In yeast, *COQ8* encodes a protein required for coenzyme Q (CoQ) biosynthesis, whose precise role is not clear. Humans harbor two paralog genes: *COQ8A* and *COQ8B* (previously termed *ADCK3* and *ADCK4*). We have found that COQ8B is a mitochondrial matrix protein peripherally associated with the inner membrane. *COQ8B* can complement a Δ*COQ8* yeast strain when its mitochondrial targeting sequence (MTS) is replaced by a yeast MTS. This model was employed to validate *COQ8B* mutations, and to establish genotype–phenotype correlations. All mutations affected respiratory growth, but there was no correlation between mutation type and the severity of the phenotype. In fact, contrary to the case of *COQ2*, where residual CoQ biosynthesis correlates with clinical severity, patients harboring hypomorphic *COQ8B* alleles did not display a different phenotype compared with those with null mutations. These data also suggest that the system is redundant, and that other proteins (probably COQ8A) may partially compensate for the absence of COQ8B. Finally, a *COQ8B* polymorphism, present in 50% of the European population (NM_024876.3:c.521A > G, p.His174Arg), affects stability of the protein and could represent a risk factor for secondary CoQ deficiencies or for other complex traits.

KEYWORDS
coenzyme Q deficiency, steroid-resistant nephrotic syndrome, yeast, COQ8B, mitochondrial nephropathy

1 | INTRODUCTION

Coenzyme Q (CoQ) is a small lipophilic molecule involved in a series of crucial cellular processes. It is composed of a quinone group and of a polyisoprenoid of variable length in different species: six units in yeast (CoQ_6), nine in mice (CoQ_9), and 10 in humans (CoQ_{10}). CoQ is an electron shuttle in the mitochondrial respiratory chain, a cofactor of several other mitochondrial dehydrogenases, and of uncoupling proteins. Besides these roles, it acts as a modulator of the permeability transition pore, and is one of the most important cellular antioxidants. CoQ is synthesized ubiquitously; in mammals, the pathway requires the products of at least 16 different genes (*PDSS1*, *PDSS2*, *COQ2*, *COQ3*, *COQ4*, *COQ5*, *COQ6*, *COQ7*, *COQ8A*, *COQ8B*, *COQ9*, *COQ10A*, *COQ10B*, *FDX1L*, *FDXR*, and *ALDH3A1*) (Desbats, Lunardi, Doimo, Trevisson, & Salviati, 2015a; Doimo et al., 2014a; Payet et al., 2016). Mutations in nine of these genes (*PDSS1*, *PDSS2*, *COQ2*, *COQ4*, *COQ6*, *COQ7*, *COQ8A*, *COQ8B*, and *COQ9*) cause primary CoQ deficiency a severe, but potentially treatable, mitochondrial cytopathy (Acosta et al., 2016).

CoQ deficiency is associated with a variety of clinical manifestations. A subgroup of defects is characterized by renal involvement, manifesting as steroid-resistant nephrotic syndrome (SRNS) (Emma, Montini, Parikh, & Salviati, 2016).

CoQ deficiency may also be secondary to defects in genes unrelated to CoQ biosynthesis (Yubero et al., 2016). For example, for still unknown reasons, up to 30% of patients with mitochondrial myopathy may have reduced levels of CoQ in skeletal muscle (Sacconi et al., 2010).

Despite its importance, the knowledge on the biosynthesis pathway and on its regulation is still scarce. The synthesis of the isoprenoid tail of CoQ shares the initial steps with cholesterol in the cytoplasm, whereas the terminal steps take place within mitochondria, and are catalyzed by a multienzyme complex associated with the mitochondrial inner membrane (MIM) facing the matrix side (Tran & Clarke, 2007).

In yeast, one of the component**s** of this complex is the product of the *COQ8* gene. *COQ8* encodes for a protein with the features of an atypical kinase, which is thought to have a regulatory function in CoQ biosynthesis. To date, however, only indirect evidence of its activity has been provided (Xie et al., 2011), and recent findings question its role as a protein kinase (Stefely et al., 2016).

Yeast lacking *COQ8* do not synthesize CoQ, cannot produce ATP through oxidative phosphorylation, and are unable to grow on non-fermentable carbon sources such as glycerol (Do, Hsu, Jonassen, Lee, & Clarke, 2001).

Yeast *COQ8* has two human homologues, *COQ8A* and *COQ8B* (the legacy nomenclature identified them as *ADCK3* and *ADCK4* (Doimo et al., 2014a). The precise relationship of these two genes is still under scrutiny. Interestingly, both have been associated with primary CoQ deficiency albeit with completely different phenotypes. *COQ8A* mutations cause cerebellar ataxia and encephalopathy (Lagier-Tourenne et al., 2008; Mollet, et al., 2008), whereas *COQ8B* defects cause SRNS and central nervous system involvement is observed only in a minority of patients (who anyway do not develop ataxia) (Ashraf et al., 2013; Korkmaz et al., 2016).

In this work, we have developed a yeast model to study COQ8B mutations involved in human disease.

2 | MATERIALS AND METHODS

2.1 | Patients

Patients and their mutations were described previously (Ashraf et al., 2013; Korkmaz et al., 2016). We did not have direct access to patient data and all information was obtained from the literature. This is why no formal approval from the ethical committee was necessary. Table 1 summarizes genotypes and phenotypes. The age of onset varies from 1 to 27 years of age.

2.2 | Yeast strains, media, and plasmid transformation

The *W303ΔCOQ8* strain (MATa; ade2-1; his3-1_15; leu2-3_112; trp1-1; ura3-1 *YGL119W*::*kanMX4*) was generated by disrupting the endogenous gene by homologous recombination with a kanamycin-resistance cassette. The inactivation procedure has been detailed elsewhere (Vetro et al., 2017). Growth minimum medium SM Glu (2% glucose, 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, histidine 10 mg/l, leucine 60 mg/l, tryptophan 20 mg/l) was supplemented with amino acids in order to cover the yeast auxotrophies except for uracil. For mitochondrial purification (for Western blot and biochemical analyses), 0.1% of glucose was added to YPGly medium (1% yeast extract, 2% peptone, and 3% glycerol) in order to allow growth of null mutants(Gigante et al., 2017). When necessary, 500 μg/ml of doxycyclin was added to the YPGly medium. Yeast DNA transformations were performed with the PEG-lithium acetate method as previously described (Vetro et al., 2017).

2.3 | Vectors construction and mutagenesis

Sequences of all oligonucleotides used in this study can be found in Supplementary Table S1. *yCOQ8* was amplified from yeast genomic DNA, cloned into pCR8/GW/TOPO vector (Thermo Fisher, Waltham, MA, USA) and then transferred to the centromeric (CEN) pCM189 yeast expression vector adapted to the Gateway cloning system (Thermo Fisher, Waltham, MA, USA). The coding sequence of human *COQ8B* was amplified from human cDNA and cloned into pCR8/GW/TOPO vector (Thermo Fisher, Waltham, MA, USA). We used a reverse primer that included the sequence encoding either the V5 or the HA tag. $COQ8B^{V5}$ was then subcloned into the pCM189 yeast expression vector, whereas $COQ8^{HA}$ was cloned into the pCDNA5 using the Gateway cloning system.

The *yCOQ8-COQ8B* and *yCOQ3-COQ8B* hybrid genes were constructed by sequential PCR as described (Nguyen et al., 2014) and cloned in pCM189. Both constructs encode the V5 epitope on the 3′terminus.

All mutagenesis reactions were performed on fragments cloned in pCR8/GW/TOPO vector using the QuikChange Lightning site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA). The correctness of all constructs was confirmed by direct sequencing.

2.4 | PK accessibility assay

Crude mitochondria from HEK293 cells were isolated as described (Frezza, Cipolat, & Scorrano, 2007). For PK accessibility assay, 200 μg of mitochondria or mitoplasts obtained by hypotonic swelling (2 mM Hepes pH 7,4) was incubated with proteinase K (100 μg/μl) in the presence or absence of 0.1% Triton X-100. Samples were incubated on ice during 30 min and reaction was stopped by addition of Protease Inhibitor Cocktail (Sigma, Saint Louis, MO, USA) and 2 mM PMSF (Sigma, Saint Louis, MO, USA). Proteins were precipitated with TCA and centrifuged for 30 min at 16,000*g*, followed by SDS-PAGE and immunoblot.

2.5 | Carbonate extraction

To assess COQ8B association to membranes, 120 μg of intact mitochondria were sonicated (4 pulses 2 sec, 40% duty cycle, microtip) or treated with 200 mM Na_2CO_3 pH 11.5 or with 2% Triton X-100 for

TABLE 1 Genotype and phenotypes of patients considered in this study

Mutation 1	Type	Residual activity	Mutation 2	Type	Residual activity	No. of patients	Onset (years)	Age at end stage renal disease
p.Arg178Trp	Mis	Yes	p.Arg178Trp	Mis	Yes	2	7 13	7 NA
p.Phe215Lfs*14	Trunc	(No)	p.Arg477Gln	Mis	NT	2	12 13	12 13
p.Asp286Gly	Mis	Yes	p.Glu483*	Trunc	No	3	3 9 14	NA NA 15
p.Arg320Trp	Mis	Yes	p.Arg320Trp	Mis	Yes	2	12 20	17 23
p.Arg343Trp	Miss	NT	p.Arg343Trp	Mis	NT	2	18 20	19 20
p.His400Asnfs*11	Trunc	(No)	p.His400Asnfs*11	Mis	(No)	1	<1	1
p.Glu447Glyfs*10	Trunc	(No)	p.Glu447Glyfs*10	Trunc	(No)	8	7 7 13 14 16 17 25 27	12 NA 16 17 16 18 35 31

Mis, missense; Trunc, truncating; NT, not tested; NA, data non available/

45 min on ice. Soluble and insoluble fractions were obtained by centrifugation of samples at 12,000*g* for 10 min. Proteins were precipitated with TCA following centrifugation for 30 min at 16,000*g* and subjected to SDS PAGE and immunoblot.

2.6 | Immunoblot analysis

The following primary antibodies were used in this work: yeast porin (MSA08; Mitoscience, Cambridge, UK), V5 (R960-25; Invitrogen, Carlsbad, CA, USA), HA (11 867 423 001; Roche, Basel, Switzerland), TOM20 (sc-11415; Santa Cruz, Dallas, TX, USA), OPA-1 (612606; BD Biosciences, San Jose, CA, USA), GRP75 (sc-13967; Santa Cruz, Dallas, TX, USA), ADCK4 (LS-C119206; LsBIO, Seattle, WA, USA), SCO2 (sc-49110; Santa Cruz, Dallas, TX, USA), SDHA (459200; Molecular Probes, Eugene, OR, USA), human porin (MSA03; Mitoscience, Cambridge, UK), and CytC (556433; BD Pharmigen, San Diego, CA, USA).

2.7 | Mitochondrial purification and respiratory chain measurement

Yeast cells were grown in 500 ml of 0.1 % glucose YPGly medium (for some experiments also in presence of 150 μg/ml of doxycyclin) for 24 hr with shaking (250 rpm) at 30°C and at an initial OD_{600} of approximately 1. Crude mitochondria were obtained after a DTT, Zymolyase, and yeast homogenization with a glass/glass potter and then were used for the measurement of the respiratory complex II + III activity as previously described (Desbats et al., 2015b; Spinazzi et al., 2011) using citrate synthase activity for normalization.

2.8 | Molecular modeling

Molecular modeling was performed as described (Trevisson et al., 2009) using as template the structure of human COQ8A (PDB code 4PED).

3 | RESULTS

3.1 | COQ8B is a mitochondrial matrix protein peripherally associated with the inner membrane

Both COQ8A and COQ8B proteins share significant sequence conservation with yeast COQ8; however, yeast COQ8 and human COQ8B display a classical N-terminal mitochondrial targeting sequence (MTS) (composed of amino acids 1–29 in COQ8 and 1–34 in COQ8B), whereas COQ8A has a much longer N-terminal sequence, without the typical characteristics of a MTS (Cullen et al., 2016). A blast search using the first 205 amino acids of COQ8B as a bait found homologous sequences only in vertebrates, but not in other chordates or in lower eukaryotes.

To verify the mitochondrial localization of COQ8B, we performed a proteinase K protection assay. Crude mitochondrial preparations from HEK293 cells overexpressing an-HA-Tagged version of the protein ($COQ8B^{HA}$) were incubated with Proteinase K in isolation buffer, in hypotonic buffer, which causes mitochondrial swelling and disrupts the mitochondrial outer membrane (MOM), or Triton X100, which disrupts both, the MOM and the MIM. After SDS-PAGE and blotting, membranes were probed with antibodies against TOM20 (a MOM protein), the HA tag, cytochrome *c* (Cyt *c*) (a MIM protein), or GRP75 (a mitochondrial matrix protein).

As seen in Figure 1A, TOM20 is degraded even in hypotonic buffer, whereas the $COQ8B^{HA}$ signal disappears only after Triton X100 treatment, the same as GRP75, indicating that the C-Terminus of the protein is localized to the mitochondrial matrix.

Because COQ8B is predicted to have a transmembrane domain (Stefely et al., 2015), in a second set of experiments, we evaluated the relationship of COQ8B with the MIM. Crude mitochondrial preparations of HEK293 cells expressing COQ8B-HA were treated with

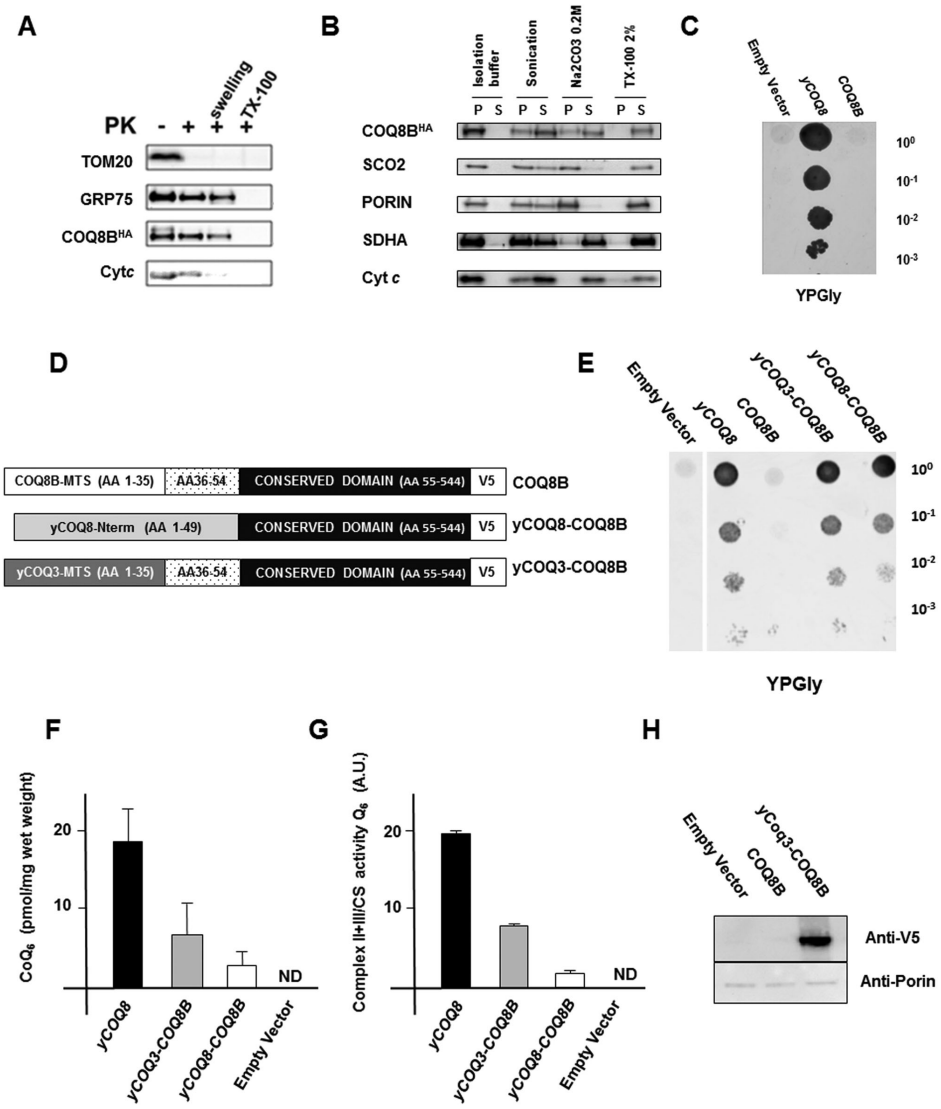

FIGURE 1 **A**: Mitochondrial localization of COQ8B by PK assay. Immunoblots of COQ8B-HA compared with MOM marker TOM20 and mitochondrial matrix marker GPR75 from enriched mitochondrial fractions of HEK293 cells. **B**: Carbonate extraction experiments to determinate COQ8B association to membranes. Immunoblots of COQ8B were compared with SCO2 and porin (integral membranes proteins), SDHA (a peripheral MIM protein) and cytochrome *c* (a soluble protein). P, pellets; S, soluble fraction. **C**: Human *COQ8B* cannot complement Δ*COQ8* yeast strains. **D**: Structure of the different *COQ8B* constructs used in this work. COQ8B amino acids 36–54 were not conserved during evolution. **E**: Both hybrid constructions *yCOQ8-COQ8B* and *yCOQ3-COQ8B* can complement Δ*COQ8* yeast even when expressed from a low-copy centromeric vector. **F**: Coenzyme Q_6 levels and (**G**) complex II + III activity normalized to citrate synthase (CS) in complemented yeast strain. ND, not detectable. **H**: Anti-V5 immunoblot of Δ*COQ8* yeast expressing *COQ8B*, *yCOQ3-COQ8B* or the empty vector. For the last three experiments, yeast strains were grown in YPGly medium supplemented with 0.1% glucose. Porin was used as loading control

sonication, Na_2CO_3, or Triton X100 and precipitated with trichloroacetic acid. After centrifugation, supernatants and resuspended pellets were separated by SDS-PAGE and blotted. Membranes were probed with antibodies anti Cyt *c*, a soluble protein, SDHA (a peripheral MIM protein), SCO2 and porin (integral membrane proteins), and COQ8B.

We detected only a weak association with the MIM (Figure 1B), similar to that of other peripheral proteins like SDHA (which in turn behaves like COQ proteins such as COQ5 (Nguyen et al., 2014)), whereas SCO2, a single-pass transmembrane protein (Lode, et al., 2002), was released from the pellet only after treatment with Triton. These finding are consisted with what has been reported for yeast COQ8 (Xie et al., 2011)

3.2 | Human COQ8B can complement yeast ΔCOQ8 mutants

To verify that functional equivalence of human COQ8B and yeast COQ8, we asked whether the human gene could complement the yeast Δ*COQ8* strain. The human gene was initially cloned into the pCM189 yeast expression vector, a low-copy CEN vector in which expression is driven by the relatively strong *CYC1* promoter. This construct was

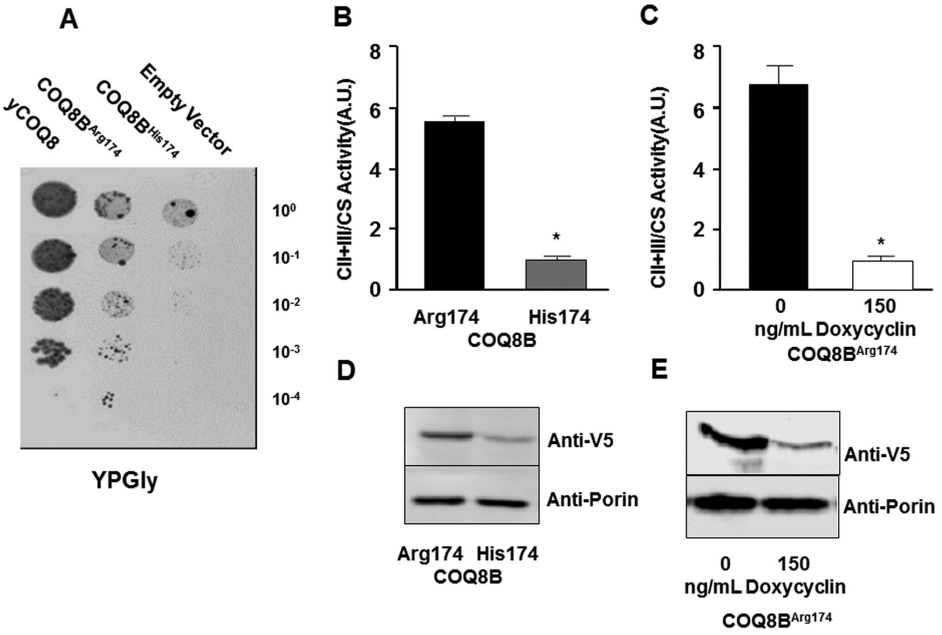

FIGURE 2 **A**: Impaired respiratory growth of the strain carrying *COQ8B*^{His174} compared with the strain carrying *COQ8B*^{Arg174}. (**B**) Complex II + III activity and (**C**) immunoblot against the V5 tag in the same strains. **D**: Complex II + III activity, and (**E**) immunoblot against the V5 tag, in the *COQ8B*^{Arg174} strain grown in standard medium or in the presence of 150 μg/ml of doxycyclin

not able to complement Δ*COQ8* yeast and no growth was observed in Glycerol medium (Figure 1C). Because human MTS often do not perform well in yeast (Desbats et al., 2016), we constructed a hybrid gene in which we substituted the N-terminus of human COQ8B (including the MTS) with that of yCOQ8 (Figure 1D). Because the MTS of yeast Coq3 also performs very well for hybrid yeast-human constructs (Xie et al., 2011), we synthesized a second hybrid gene in which this MTS replaced that of the human protein (yCOQ3-COQ8B). The cleavage site of the MTS in COQ8B was mapped using MitoProt II (Claros, 1995) between Gly35 and Pro36, whereas in the case of the Coq3 MTS, we used the previously described cleavage site between Asp35 and Ala36) (Hsu, Poon, Shepherd, Myles, & Clarke, 1996).

As seen in Figure 1E, F, and G, both constructs restored respiratory growth, coenzyme Q_6 (CoQ_6) production, and complex II + III activity in Δ*COQ8* yeast. Isolated activities of complexes II and III were normal (not shown). These data suggest that the negative results with the wild-type human COQ8B should be attributed to faulty mitochondrial importation by the human MTS. Because the construct with the Coq3 MTS performed better than that with yCOQ8 MTS, we used it for the subsequent experiments.

The activity of respiratory chain complex II + III (if isolated complex II and III activities are normal) is strictly dependent on CoQ content in the MIM (in both yeast and human cells), and (as seen also in Figure 1F and G) there is a good correlation between these two parameters (Desbats et al., 2015b; Gigante et al., 2017; Salviati et al., 2012). In our hands, determination of complex II + III activity provided more precise and reproducible results than direct CoQ quantification. Therefore, even though it is an indirect assay, we favored this approach for subsequent experiments.

When we analyzed these strains by western blot analysis (Figure 2D), we failed to detect any signal in cells expressing native human COQ8B, while the hybrid protein was present. Even though we cannot in principle rule out that the gene is not expressed, we favor the explanation that the unimported human protein is rapidly degraded.

3.3 | A common COQ8B polymorphism affects COQ8B stability

During the construction of the *COQ8B* expression plasmids, we realized that compared to the reference sequence NM_024876.3 almost half of the clones contained a single A > G variation at position 521 (c.521A > G). This change causes the substitution of a histidine with an arginine at position 174 (p.His174Arg). This variation is reported in dbSNP (accession rs3865452) and in the ExAC databases with an allelic frequency of approximately 50% in Europeans (exac.broadinstitute.org). A search of the GenBank database (www.ncbi.nlm.nih.gov) showed that most mammalian species (including other primates) carry the Arg174 allele (which represents the ancestral human allele), while His174 is found in camelides, in *Manis javanica* (Sunda pangolin), in *Eptesicus fuscus* (big brown bat), in *Bubalus bubalis* (water buffalo), and in *Neotoma lepida* (desert woodrat) indicating that the His174 allele originated independently in these species.

We then asked whether this variant impacts COQ8B function. We therefore evaluated the performance of *COQ8B*^{His174} versus *COQ8B*^{Arg174} in our yeast system. Interestingly, yeast expressing *COQ8B*^{Arg174} grew better in non-fermentable medium (Figure 2E), and had higher complex II + III activity compared with those expressing *COQ8B*^{His174} (Figure 2F). Western blot analysis showed a reduction of the steady state levels of COQ8B^{His174} compared with COQ8B^{Arg174} (Figure 2G). A similar reduction in both complex II + III activity (Figure 2H) and protein steady state levels was observed by expressing the *COQ8B*^{Arg174} in the presence of 150 ng/ml doxycycline (Figure 2I),

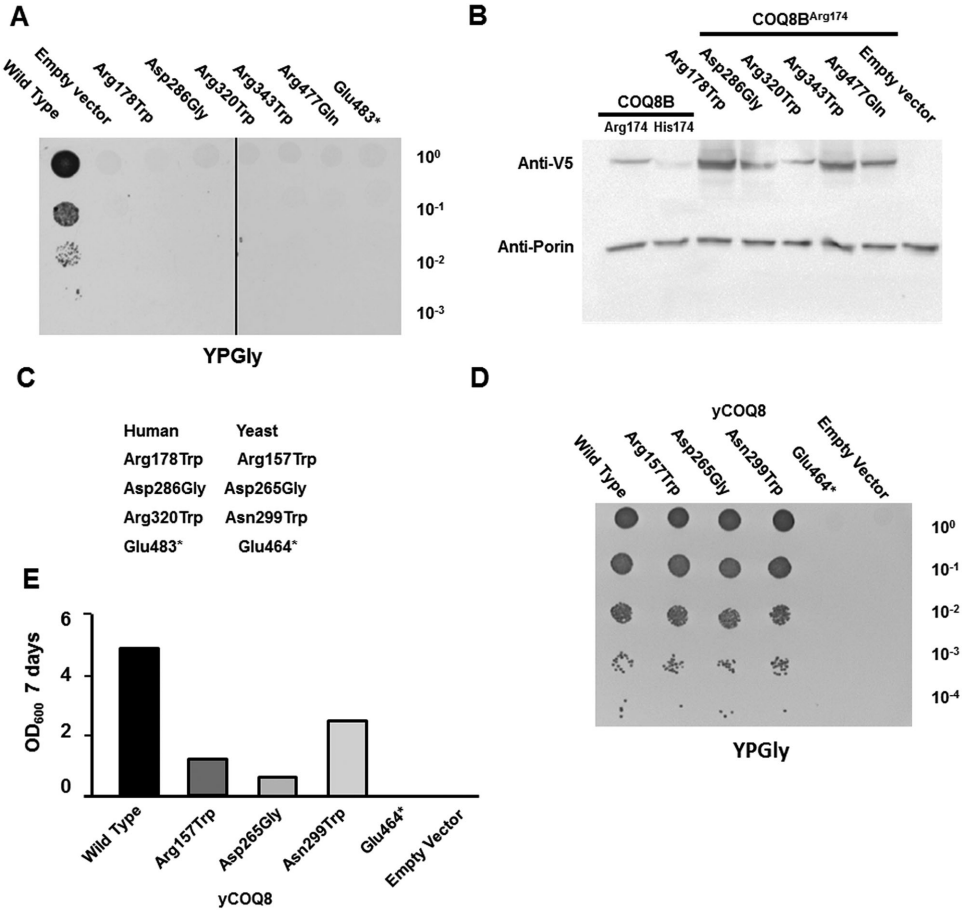

FIGURE 3 **A**: Missense *COQ8B* mutations found in patients with SRNS were not able to complement Δ*COQ8* yeast and no growth was observed in glycerol medium. **B**: Immunoblot against the V5 tag showing the steady-state levels of the mutated versions of COQ8B. **C**: Human *COQ8B* mutations and their corresponding mutations in *yCOQ8*. **D**: Effect of the *yCOQ8* mutations when the strains were growth in glycerol medium. **E**: Growth of the same strains in liquid YPGly medium in the presence of 500 μg/ml doxycycline after 7 days

which represses expression of the pCM189 plasmid. These data suggest that His174 affects stability of COQ8B, without a significant alteration of its "specific activity" (whatever it might be).

3.4 | COQ8B mutations found in patients with SRNS impair COQ8B function

To validate the pathogenicity of the missense mutations identified in the initial cohort of *COQ8B* patients, we mutagenized our *yCOQ3-COQ8B*^{Arg174} construct and expressed the different mutants in the *W303-*Δ*COQ8* strain. All mutants completely abolished yeast growth on non-fermentable medium confirming that they have a negative impact on COQ8B function (Figure 3A). The system can discriminate the mutations from the polymorphisms of residue 174 since the latter still allow residual growth in selective medium. Western blot analysis showed that steady state levels of the mutants were not particularly decreased, while we confirm the reduction associated with the His174 allele (Figure 3B). Curiously, in the case of Arg178Trp and Arg343Trp, protein levels appear to be increased, as if the mutant protein was indeed more stable than the wild-type. However, these results should be taken with care, and must be confirmed in a mammalian model. Yet, none of these mutants seem to drastically affect stability of the protein.

Because heterologous expression of human genes in yeast may result in suboptimal complementation, it may be difficult to distinguish between hypomorphic and truly null alleles (Doimo et al., 2014b). We therefore examined the effect of three missense mutations (Arg178Trp, Asp286Gly, and Arg320Trp) as well as the truncating Glu483* mutation on the yeast gene. We mutagenized the corresponding residues of yCOQ8 (Arg157, Asp265, and Glu464) (Figure 3C). Because human Arg320 corresponds to Asn299 in yCOQ8, residue Asn299 of yCOQ8 was mutagenized to both Arg and Trp.

When we expressed the different constructs in the *W303-*Δ*COQ8* strain, those encoding the missense variants still allowed virtually normal growth in glycerol plates, while the truncating mutation completely abolished yeast growth, indicating that ablation of the C-terminal portion of the protein completely abolishes its activity (Figure 3D). We then employed a growth assay in liquid medium containing 500 μg/ml doxycyclin (to reduce *yCOQ8* expression form the *CYC1* promoter to near-physiological levels); p.Arg157Trp, p.Asp265Gly, and p.Asn299Trp partially reduced growth compared to the wild-type, indicating that they are indeed hypomorphic alleles, whereas no

growth was observed with Glu464* (Figure 3E). No effect was noted with the Asn299Arg change neither in plates nor in liquid medium (not shown), suggesting that this change is virtually harmless.

4 | DISCUSSION

Although COQ8 is essential for COQ biosynthesis in yeast, its precise function is not clear, but it probably has a regulatory role in the biosynthetic process (Acosta et al., 2016). There are two human paralogs of *yCOQ8*, *COQ8A*, and *COQ8B*. They share a common C-terminal domain, but while COQ8B has a MTS, overlapping that of yCOQ8, COQ8A has a longer N-terminus, lacking a canonical MTS, which nevertheless targets the protein to mitochondrial *cristae* (Cullen et al., 2016). The precise role of this peculiar N-terminus of COQ8A is unknown.

Localization data confirm that COQ8B is a mitochondrial protein, loosely associated with the inner membrane, like other COQ proteins such as COQ5 (Nguyen et al., 2014) and it does not appear to be an integral membrane protein like it had been suggested by in-silico predictions. These data are in accordance with those obtained experimentally with yeast COQ8 and COQ8A (Cullen et al., 2016; Xie et al., 2011).

Human *COQ8B* may complement Δ*COQ8* yeast even when expressed from a low-copy plasmid (albeit with a relatively strong promoter) provided that its MTS is substituted with a yeast MTS.

By definition, orthologs are genes in different species that evolved from a common ancestral gene and have retained the same function in the course of evolution. The main problem with heterologous expression in yeast of human genes encoding for mitochondrial proteins, is that the human MTS perform erratically in in *Saccharomyces cerevisiae* (Desbats et al., 2016). For example, human *COQ2* per se does not complement yeast, and complementation was observed only when non-physiological targeting sequences were used (and the phenomenon was strain-specific) (Desbats et al., 2016; Forsgren et al., 2004; Lopez-Martin et al., 2007). Human COQ5 performs much better in yeast when its presequence is substituted by the yeast one (Nguyen et al., 2014). In the case of *OPA1*, complementation was also achieved using hybrid constructs, which included the MTS and the transmembrane domain (Nasca et al., 2017; Nolli et al., 2015). In all these cases, these genes are considered orthologs of the yeast gene. In our experiments, we were not able to observe complementation with wild-type *COQ8B*, but we did detect it when we substituted its MTS with one that is known to perform well in yeast. COQ3 is a methyltransferase and (apart from being involved in the same biochemical pathway) is totally unrelated to COQ8. Substituting the MTS of human COQ8B with that of COQ3 does not affect the actual function of the protein, it simply improves its delivery to mitochondria.

COQ8A and *COQ8B* have been already defined as co-orthologs of yeast COQ8 (Stefely et al., 2016). However, because COQ8B has a structure that resembles more closely that of yeast COQ8, we argue that it should be considered the actual ortholog of yeast COQ8, whereas COQ8A should be considered a paralogous gene, which evolved relatively recently in the course of evolution, and which could have a (partially) different role in the CqQ biosynthetic process.

Interestingly, all reported patients with mutations in *COQ2* or *COQ6* (the two COQ genes for which mutations were most extensively characterized) harbored at least one hypomorphic allele. In fact, C-terminal frameshift mutations of both *COQ2* and *COQ6*, that were initially thought to be null alleles, were shown to still allow some residual function (Desbats et al., 2016; Doimo et al., 2014a). This is in agreement with the notion that a complete block in CoQ biosynthesis is lethal. In the case of *COQ8B* the situation is different. The most 3′ truncating mutation Glu483* completely abolishes COQ8B function; this suggests that the C-terminal portion of the protein is indispensable for COQ8B function and that upstream nonsense/frameshift mutations should be considered equally deleterious. Therefore, there are *COQ8B* patients who harbor two virtually null alleles, but residual CoQ biosynthesis is still present (as demonstrated by CoQ determinations in lymphoblastoid lines of these patients (Ashraf et al., 2013)), suggesting that the system is redundant, and that other genes may partially compensate for the absence of *COQ8B*. The most obvious candidate is *COQ8A*, however little is known about the exact relationships between these two genes. Interestingly, there are also *COQ8A* patients with two null alleles suggesting that the compensatory mechanism could be reciprocal (Lagier-Tourenne et al., 2008), however we cannot rule out the hypothesis that other *ADCK* genes, or other unrelated genes could be involved in the process. Further work on mammalian cellular and animal models is needed to address these issues.

Contrary to what we found for *COQ2*, where there is a strong relationship between the severity of symptoms and residual COQ2 function, there is no clear genotype–phenotype correlation for *COQ8B* mutants. In fact, the presence of two hypomorphic alleles (versus two null mutations) is not associated with milder phenotypes (in terms of age of onset or progression of disease). This last observation may imply that there could be sort of a threshold effect: mutations must impair COQ8B function below a certain threshold to cause the clinical phenotype, but it is not relevant whether the impairment is complete or if some residual activity is present. Probably it is the degree of activation of the compensatory mechanisms what in the end determines the actual phenotype of these patients.

Finally, none of the missense mutants seems to destabilize the COQ8B protein (whereas the His174 allele does reduce COQ8B stability). By modeling these residues on the human COQ8A protein, we noted that all affected residues are located at, or near, the surface of the protein (Figure 4), and none is in direct contact with the putative nucleotide binding site (Stefely et al., 2015), suggesting the possibility that these mutations affect interactions between COQ8B and other COQ proteins.

In our experiments, we did not perform quantification of *COQ8B* transcript levels in transformed yeast. However, because we are employing low copy CEN plasmids, this step is generally not considered essential. These vectors are episomal and are present in a fixed (and extremely stable) number of copies per yeast cell. Therefore, one can safely assume that expression levels are constant and mRNA analysis is usually not required (Alston et al., 2012; Baruffini et al., 2006). Conversely, fluctuations in the number of plasmid copies/cell are a potential problem with high copy (2 micron) plasmids (such as the pYES family), which may cause artifacts (Trevisson et al., 2009).

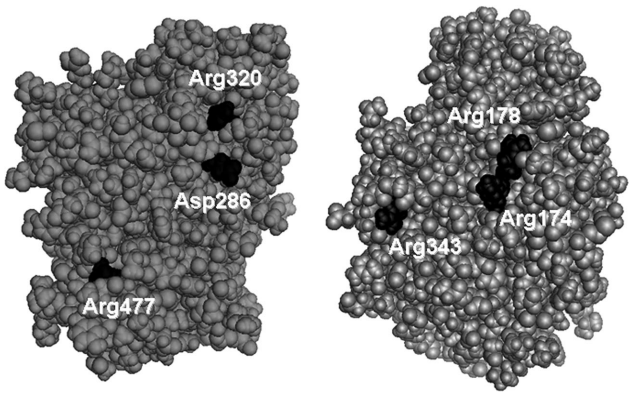

FIGURE 4 Mutated COQ8B residues modeled into the three-dimensional structure of COQ8A (PDB code 4PED)

The significance of the His174 allele is puzzling. It clearly affects COQ8B stability (although the yeast system could exaggerate this effect and could not reproduce exactly the situation in mammalian cells) but not the "specific activity" (whatever it might be) since complex II + III activity (an indirect measurement of CoQ levels) is similar in yeast expressing $COQ8B^{His174}$ or a comparable level of $COQ8B^{Arg174}$.

This variant is extremely frequent in the population (one quarter of Europeans is homozygous for His174 and an equal number for Arg174) so there is no question that they are both benign variants.

We did not have access to *COQ8B* patient data, and our cohort of COQ2 and COQ6 patients was too small to draw significant conclusions. Nevertheless, future studies are being planned to assess whether the His174 versus Arg174 genotype, may account for interindividual differences in CoQ levels. This polymorphism is also an interesting candidate for association studies. In particular, it will be interesting to assess whether or it could modulate the expressivity of defects of other *COQ* genes or of mitochondrial disease in general, or, more specifically, if it could determine the susceptibility to develop secondary CoQ deficiency. Moreover, similar studies could be performed for other forms of SRNS unrelated to COQ genes, and to evaluate its role as a risk factor for other types of complex disorders.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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