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Molecular characterization of the human COQ5 *C*-methyltransferase in coenzyme Q_{10} biosynthesis ☆

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ABSTRACT

Coq5 catalyzes the only *C*-methylation involved in the biosynthesis of coenzyme Q (Q or ubiquinone) in humans and yeast *Saccharomyces cerevisiae*. As one of eleven polypeptides required for Q production in yeast, Coq5 has also been shown to assemble with the multi-subunit complex termed the CoQ-synthome. In humans, mutations in several *COQ* genes cause primary Q deficiency, and a decrease in Q biosynthesis is associated with mitochondrial, cardiovascular, kidney and neurodegenerative diseases. In this study, we characterize the human COQ5 polypeptide and examine its complementation of yeast *coq5* point and null mutants. We show that human *COQ5* RNA is expressed in all tissues and that the COQ5 polypeptide is associated with the mitochondrial inner membrane on the matrix side. Previous work in yeast has shown that point mutations within or adjacent to conserved *COQ5* methyltransferase motifs result in a loss of Coq5 function but not Coq5 steady state levels. Here, we show that stabilization of the CoQ-synthome within *coq5* point mutants or by over-expression of *COQ8* in *coq5* null mutants permits the human *COQ5* homolog to partially restore *coq5* mutant growth on respiratory media and Q_6 content. Immunoblotting against the human COQ5 polypeptide in isolated yeast mitochondria shows that the human Coq5 polypeptide migrates in two-dimensional blue-native/SDS-PAGE at the same high molecular mass as other yeast Coq proteins. The results presented suggest that human and *Escherichia coli* Coq5 homologs expressed in yeast retain *C*-methyltransferase activity but are capable of rescuing the *coq5* yeast mutants only when the CoQ-synthome is assembled.

1. Introduction

Coenzyme Q (ubiquinone or Q) is an essential lipophilic electron carrier found within the mitochondrial inner membrane of eukaryotes and the plasma membrane of many prokaryotes. Q is composed of a polyisoprenoid "tail" that anchors it to the lipid membrane, and a benzoquinone "head" that confers the abilities to shuttle electrons from Complexes I and II to Complex III [1,2]. Q also acts as a cofactor of uncoupling proteins and several mitochondrial dehydrogenases, and in its reduced or hydroquinone form can quench lipid radical species as an antioxidant [3,4].

The biosynthesis of the isoprenoid tail derives from either the mevalonate or 1-deoxy-D-xylulose-5-phosphate pathways [5], with the number of isoprene units varying in different species: six in *Saccharomyces cerevisiae*, eight in *Escherichia coli*, and ten in humans. In *S. cerevisiae*, the hexaprenyl diphosphate tail is attached to either 4-hydroxybenzoic acid (4HB) or para-aminobenzoic acid (pABA), both of which have been shown to serve as aromatic ring precursors in *S. cerevisiae* Q_6 biosynthesis [6,7].

Abbreviations: 2D-BN-SDS/PAGE, two-dimensional blue-native-sodium dodecyl sulfate/polyacrylamide gel electrophoresis; DDMQ, demethyl-demethoxy-Q; $DDMQH_2$, demethyl-demethoxy-QH_2; DMQ, demethoxy-Q; DOD, drop-out growth medium with dextrose; 4HB, 4-hydroxybenzoic acid; HPLC, high performance liquid chromatography; IDMQ, 4-imino-demethoxy-Q; MRM, multiple reaction monitoring; MTase, methyltransferase; pABA, para-aminobenzoic acid; PK, proteinase K; Q, coenzyme Q or ubiquinone; QH_2, coenzyme QH_2, ubiquinol, or ubihydroquinone; SD, minimal synthetic media with dextrose; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; YPD, rich growth medium with dextrose; YPG, rich growth medium with glycerol; YPGal, rich growth medium with galactose

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Eleven *S. cerevisiae* genes (*COQ1-9*, *ARH1*, *YAH1*) are required for Q_6 biosynthesis, several of which encode proteins associated with the mitochondrial inner membrane in a multi-subunit complex termed the CoQ-synthome [8]. In the absence or deficiency of any one of these genes, Q_6 is not made and growth on medium containing a non-fermentable carbon source is not possible; the presence of each Coq polypeptide is essential for the assembly of the Coq polypeptide complex and for the proper function of each individual enzyme [9]. The over-expression of *COQ8*, which encodes a putative regulatory kinase, has been shown to restore steady state levels of several Coq polypeptides [10] and the assembly of the high molecular mass CoQ-synthome [8].

In humans, decreased levels of Q_{10} are associated with mitochondrial, cardiovascular, kidney and neurodegenerative diseases [3,11]. Mutations in *COQ* genes cause primary Q_{10} deficiency (OMIM #607426), one of the few treatable mitochondrial disorders; in fact some affected patients respond well to oral Q_{10} supplementation [12]. *COQ* genes are highly conserved throughout evolution, and several human *COQ* genes have complemented the corresponding yeast *coq* null mutant [11,13]. Previously, we have shown that expression of human ADCK3 (a yeast Coq8 ortholog) fused with an N-terminal yeast mitochondrial leader sequence rescued the growth of yeast *coq8* null mutants and restored de novo Q biosynthesis [14].

In this study, we report the cloning and functional characterization of the human ortholog of yeast *COQ5* and test its ability to complement yeast *coq5* point and null mutants. Coq5 catalyzes the only *C*-methylation involved in the synthesis of Q_6 in yeast [15]. Previous work in yeast has shown that certain point mutations within or adjacent to conserved *COQ5* methyltransferase motifs result in a loss of Coq5 methyltransferase function, but mutants harboring these alleles (*coq5-2*, *coq5-5*) still retain steady state levels of Coq5 protein [15,16]. Complementation of these yeast point mutants with *ubiE*, an *E. coli COQ5* homolog, restored respiration and *C*-methyltransferase activity [16]. Here, we examine the function of human COQ5, and show that expression of human COQ5 in yeast mutants identifies the functional conservation of the yeast and human Q biosynthetic pathways, with implications for the diagnosis and treatment of Q_{10} deficiencies in patients.

2. Material and methods

2.1. Yeast strains and growth media

S. cerevisiae strains used in this study are listed in Table 1. Media were prepared as described [17], and included: YPD (2% glucose, 1% yeast extract, 2% peptone), YPGal (2% galactose, 1% yeast extract, 2% peptone, 0.1% glucose), and YPG (3% glycerol, 1% yeast extract, 2% peptone). Synthetic dextrose/minimal medium (SD, SD–Ura, and SD–Ura–Leu) consisted of 0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4, and 0.5% $(NH_4)_2SO_4$, and amino acids (minus uracil or leucine for selective media) were added at final concentrations as described in [15]. Drop-out media with dextrose (DOD) lacking folate and para-amino benzoic acid and with proper amino acid selection were prepared as described in [18]. Plate media contained 2% bacto agar.

Table 1
Genotype and source of yeast strains.

Strain	Genotype	Source or reference
W303-1A	MAT **a** *ade2-1* his3-*1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303ΔCOQ5	MAT **a** *ade2-1* his3-*1,15 leu2-3,112 trp1-1 ura3-1 coq5::HIS3*	[15]
CH83-B3	MAT α *ade2-1 coq5-2 his3-1,15 ura3-1*	[16]
CH316-6B	MAT α *coq5-5 trp1-1 ura3-1*	[16]

[a] Dr. Rodney Rothstein, Department of Human Genetics, Columbia University.

2.2. Identification of human COQ5

The human *COQ5* gene (*hCOQ5*) cDNA sequence was identified by screening the Expressed Sequence Tag (EST) database with the tBLASTn algorithm (www.ncbi.nlm.nih.gov/blast) and the yeast protein sequence as bait [19].

2.3. Plasmid construction

Plasmids used in this study are listed in Table 2. Human *COQ5* was amplified from cDNA obtained from human skin fibroblasts [20] using primers COQ5F and COQ5-1077R. PCR products were cloned in pCRII TOPO (Invitrogen), and the high copy pYES2.1V5His yeast expression vectors (Invitrogen). The *COQ5* insert from pCRIITOPO-COQ5 was then cloned into the centromeric pCM189 vector (EUROSCARF) [21]. Yeast *COQ5* (yCOQ5) was amplified from genomic DNA obtained from a wild-type BY4741 strain using standard protocols and cloned into the same vectors. Amplification primers and conditions for all reactions are shown in Table S1. The hybrid yeast–human *COQ5* (yhCOQ5) gene was obtained by amplifying a 5′ segment of yCOQ5, corresponding to the mitochondrial targeting region (encoding aa 1–54; with primers yCOQ5F and hybridCOQ5R) and the 3′ of human *COQ5* (encoding aa 56–327; with primers hybridCOQ5F and COQ5-1037R). The two PCR products were joined with a sequential PCR protocol [22].

To generate the *COQ5-GFP* fusion gene, the human *COQ5* coding region (devoid of the termination codon) was amplified from pCRIITOPO-COQ5 with primers containing HindIII and PstI restriction sites, digested with HindIII and Pst1, and cloned into the pEGFP-N1 vector (Clontech) digested with the same enzymes. Finally a myc-tag was added to the C-terminus of hCOQ5 by PCR with primers COQ5F and COQ5mycR (Table S1) and cloned into the pCDNA3.1V5HisTOPO vector (Invitrogen) to yield pCOQ5-myc. All plasmid-constructs were sequenced to confirm the presence of the inserted DNA segments and to ascertain they were free of errors.

2.4. Northern blot and RACE

The probe for Northern blot analysis was obtained by EcoRI digestion of pCRII TOPO-COQ5 and was labeled and purified as described in [19] and hybridized to a commercial membrane (FirstChoice Human Blot 1 membrane–Ambion) containing 2 μg/lane of poly(A) + RNA from ten human tissues, previously used as described [23] and stripped. Radioactivity was detected with a Storm PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) after an overnight exposure. The 5′ and 3′ termini of *COQ5* transcripts were characterized by RACE (Rapid Amplification of cDNA Ends), a protocol that has been detailed elsewhere [19]. RNA was extracted from cultured skin fibroblasts and from HeLa cells. Primers are reported in Table S1.

2.5. Localization of human COQ5 polypeptide in HeLa and HEK cells

The COQ5-GFP construct was used to transfect HeLa cells stably expressing mitochondrial Red Florescent Protein (mtRFP) as previously reported [20]. Procedures for mitochondrial isolation, proteinase K protection assays, and carbonate extraction were performed as described [24]. Antibodies used are described in Table 3. Tagged versions of COQ5 were employed since our anti-COQ5 antibody detected only very faint signals in cultured cells.

2.6. Immunoprecipitation of hCOQ5 from cultured cells

HEK 293 cells were co-transfected with the pCOQ5-myc and pCOQ4-V5, and were harvested after 48 h and mitochondria were isolated as above. About 3–4 mg of mitochondria were resuspended in 1 ml of lysis buffer (150 mM NaCl; 10 mM Tris/HCl pH 7,4; 1 mM EDTA; 0.5% Triton X-100; protease and phosphatase inhibitors) and incubated

Table 2
Plasmids.

Plasmids	Relevant genes/markers	Source or reference
Yeast vectors		
pyCOQ5	pCM189 with *S. cerevisiae COQ5*, low copy	This work
phCOQ5	pCM189 with human *COQ5*, low copy	This work
pyhCOQ5	pCM189 with the first 55 amino acids of human COQ5 replaced with the first 54 amino acids of *S. cerevisiae* Coq5	This work
pYES-hCOQ5	pYES2.1V5His TOPOwith human *COQ5* high copy	This work
pUE3	pQM with *E. coli ubiE*; low copy	[29]
p4HN5 (hcCOQ8)	pRS426 with yeast *ABC1/COQ8*; high copy	C. He [a]
pRS316 (vector)	pRS316; low copy	Addgene
Human vectors		
pCOQ4-V5	Human COQ4-V5 fusion cloned in pCDNA3.1TOPO	[20]
pCOQ5-V5	Human COQ5-V5 fusion cloned in pCDNA3.1TOPO	This work
pCOQ5-myc	Human COQ5-myc fusion cloned in pCDNA3.1TOPO	This work
pCOQ5-GFP	Human COQ5-GFP fusion cloned in pEGFPN1 (Clontech)	This work

[a] Cuiwen H. He, Department of Chemistry and Biochemistry, UCLA.

with 400 μl of protein A-Sepharose (GE Lifesciences) 50% (v/v) for 1 h at 4 °C. An antibody-protein A-Sepharose 50% slurry was obtained by crosslinking anti-V5, anti-myc antibodies (or the preimmune serum), and incubated with 500 μl of the mitochondrial lysate overnight at 4 °C with rotation. The immune-complex was washed with lysis buffer 0.1% Triton X-100 and PBS (Life Technologies). The immune-precipitated proteins were eluted with SDS-Laemmli buffer without β-mercaptoethanol and heated for 10 min at 95 °C. The supernatant was collected, β-mercaptoethanol was added to a final concentration of 4%, heated again, and subjected to SDS-PAGE. Proteins were transferred to membranes, which were then probed with either anti-V5 or anti-myc antibodies.

2.7. Functional complementation of the yeast coq5 mutant

Yeast transformations with the plasmids described in Table 2 were performed with the PEG-lithium acetate method [25]. Transformed yeast strains were selected and maintained in SD–Ura or SD–Ura–Leu medium. For plate dilution assays, each strain was cultured overnight in synthetic dextrose medium with proper amino acid selection. Optical densities ($A_{600\ nm}$) of harvested cells were adjusted to 0.2, and 2 μl of 1:5 serial dilutions were spotted onto YPG, YPD, and SD plate media, corresponding to a final $A_{600\ nm}$ of 0.2, 0.04, 0.008, 0.0016, and 0.00032.

Table 3
Description and source of antibodies.

Antibody	Source
Atp2	Carla. M. Koehler[a]
Coq5 (yeast)	[16]
hCOQ5 (human)	Santa Cruz Biotechnology
Coq9	[9]
GRP75	Santa Cruz
OPA1	BD Biosciences
OAT	Abcam
Porin	Mitoscience
SDHA	Molecular Probes
TOM20	Santa Cruz
myc	Sigma
V5	Invitrogen

[a] Dr. Carla. M. Koehler, Department of Chemistry and Biochemistry, UCLA.

2.8. Lipid extraction and quantification of Q_6 by HPLC and tandem mass spectrometry

To analyze Q_6 content, yeast cells were seeded in SD with proper amino acid selection at 0.2 OD/ml and collected after 3.5 to 4 h during log phase (a total of 15–30 $A_{600\ nm}$ were collected). Lipid extraction and quantification of Q_6 in yeast cell lipid extracts were determined by HPLC/MS–MS as described [6,10]. Q_4 was used as internal standard (expected final concentration, 1 pmol/μl upon analysis). For de novo Q_6 labeling experiments, yeast were grown to log phase in SD medium with proper selection and then seeded at 0.07 OD/ml into DOD medium with proper amino acid selection supplemented with 8 μg/ml $^{13}C_6$-pABA or $^{13}C_6$-4HB (Cambridge Isotopes). Cultures were grown for 10 h to late log phase, and lipid extraction and quantification of $^{13}C_6$–Q_6 was determined by HPLC/MS–MS as described [6,10].

2.9. Preparation of mitochondria from yeast

The yeast strains were grown in selective media to log phase. An aliquot of the log phase culture was seeded into 600 ml YPGal + 0.1% dextrose to a final density of 0.05 $OD_{600\ nm}$ and incubated with shaking (250 rpm, 30 °C). The cells were harvested at $OD_{600\ nm}$ between 2 and 3. The purified mitochondria were isolated as described [18,26] in the presence of 1× EDTA-free protease inhibitor (Roche) and 1:100 phosphatase inhibitor cocktail sets I and II (Calbiochem). Pure mitochondria were flash frozen in liquid nitrogen and stored at −80 °C. The protein concentration was measured with a bicinchoninic acid assay with bovine serum albumin as standard (Thermo Scientific).

2.10. Two-dimensional blue-native SDS/PAGE

Aliquots of purified mitochondria (200 μg protein) were solubilized in 50 μl of 1.2% digitonin (Biosynth AG), 1× protease inhibitor (Roche), 1:100 phosphatase inhibitor cocktail sets I and II (Calbiochem) and 1× NativePAGE sample buffer (Invitrogen). Samples were incubated on ice for 1 h and mixed by pipetting up and down every 10 min. The soluble supernatant fraction was separated from the insoluble pellet by centrifugation in a Beckman Airfuge (100,000 ×g, 10 min, chilled rotor). NativePAGE 5% G-250 sample additive (Invitrogen) was added to the supernatant from 200 μg of digitonin-solubilized mitochondria (50 μl) to a final concentration of 2.5%. BN-PAGE was performed as described in NativePAGE user manual with NativePAGE 4–16% Bis–Tris gel 1.0 mm × 10 wells (Invitrogen). First dimension gel slices were soaked in heated 2× SDS sample buffer for 20 min before loading onto pre-cast 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P transfer membrane (Millipore), and blocked in 1% skim milk, phosphate-buffered saline, and 0.1% Tween-20 (phosphate buffered saline, pH 7.4, is composed of 0.141 M NaCl, 1.2 mM NaH_2PO_4, and 8.1 mM Na_2HPO_4). Membranes were treated with the following primary antibodies (Table 3): human Coq5, 1:2500, Coq5, 1:5000, Coq9, 1:1000; Atp2, 1:4000. Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Calbiochem) were used at a 1:10,000 dilution and visualized using the Alpha Innotech FluorChem FC2 Imaging System.

3. Results

3.1. Identification and genetic characterization of human COQ5

The gene encoding human *COQ5* spans to 26 kb on chromosome 12q24.31 and is comprised of seven exons. The open reading frame is 981 nucleotides long encoding a predicted 327 amino acid protein which contains a putative mitochondrial targeting sequence and shares 45% identity and 60% similarity with the yeast protein (excluding the

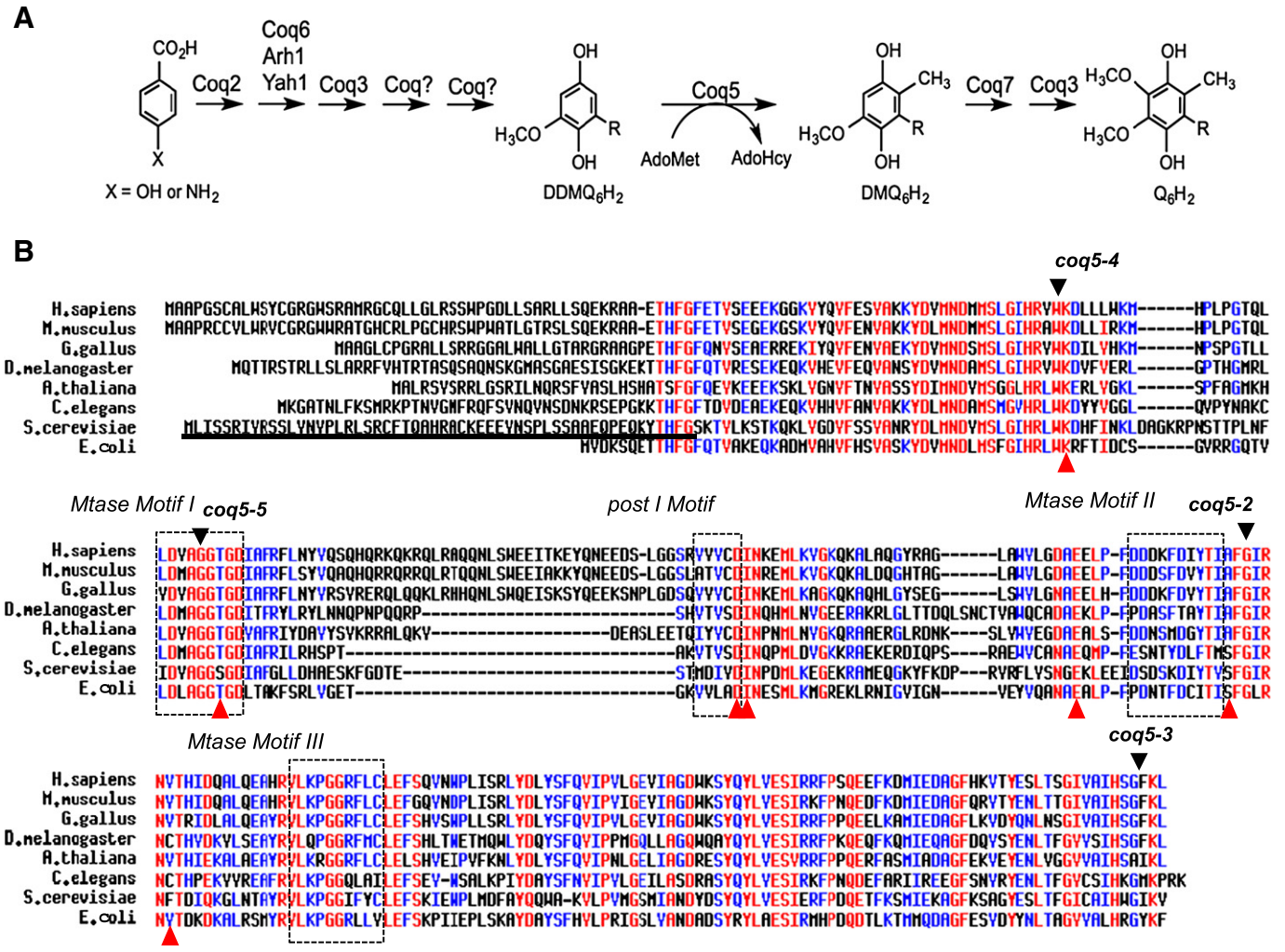

Fig. 1. Biosynthesis of Q in *S. cerevisiae* and amino acid alignment of COQ5 homologs. (A) The benzoquinone head group of Q derives from either the 4HB or pABA aromatic ring precursor, is prenylated by Coq2 and further modified by Coq polypeptides to make the Q_6-intermediate demethyl-demethoxy-hydroquinone ($DDMQ_6H_2$). Coq5 catalyzes the transfer of a methyl group from AdoMet to the C2 position of the hydroquinone ring to make demethoxy-Q_6 (DMQ_6H_2), which is ultimately converted into the fully substituted Q_6H_2. (B) Amino acid alignments of COQ5 homologs were generated by MultAlin. In red are highly conserved residues, in blue, moderately conserved residues. Methyltransferase motifs [38,39] are designated by the dashed-line boxes. The aligned sequences include: *Homo sapiens* COQ5 (84274), *Mus musculus* Coq5 (52064), *Gallus gallus* COQ5 (416975), *Drosophila melanogaster* CG2453 (32272), *Arabidopsis thaliana* AT5G57300 (835835), *Caenorhabditis elegans* COQ-5 (176099), *Saccharomyces cerevisiae* Coq5 (854930), and *Escherichia coli* UbiE (12932088). Single unique nucleotide substitutions in *coq5* mutant alleles producing amino acid substitutions are designated by arrowheads (▼) on the figure as G596A (G199D) for *coq5-2*, G911A (G304D) for *coq5-3*, G279A (W93STOP) for *coq5-4*, and G361A (G121R) for *coq5-5*. *S. cerevisiae* Coq5 residues making contacts with S-adenosyl-L-methionine [35] are identified (red triangles). The yeast DNA segment encoding the amino terminal 54 residues (underlined) replaced the corresponding segment of human COQ5 DNA to generate the hybrid yeast/human gene.

putative targeting region). Fig. 1B shows the amino acid alignment of COQ5 from different species. Northern blot analysis detected a single human *COQ5* transcript of about 1.5 kb expressed in all tissues tested (Fig. 2A). Human *COQ5* steady-state RNA levels were higher in liver, lung, placenta, and skeletal muscle, similar to what was detected for *COQ6*[23] (Fig.S1). RACE analysis on mRNA extracted from primary skin fibroblast detected a single transcription initiation site located 14 bp upstream of the ATG initiation codon, while the 3′ untranslated region (UTR) extends to 501 nucleotides downstream of the termination signal, and contains a non-canonical AGTAAA polyadenylation signal (Fig. 2B). The size of the transcript corresponds to the *hCOQ5* RNA observed by Northern blot. There was no bioinformatics evidence of *COQ5* pseudogenes, or other genes with significant similarity to *hCOQ5*. A search of the human EST database showed evidence of a second transcript that includes an additional 90 bp exon between exon 1 and exon 2 (Fig. 2C). This exon contains a termination codon so the predicted protein encoded by this transcript (which we have termed *COQ5* isoform 2) is only 70 aa. However, we did not detect this transcript in RNA extracted from cultured skin fibroblasts or skeletal muscle.

3.2. hCOQ5 encodes a mitochondrial matrix protein associated with hCOQ4

To determine the subcellular localization of human COQ5, we transfected HeLa cells stably expressing mitochondrial-targeted RFP [20] with a construct expressing a hCOQ5-GFP fusion protein. As seen in Fig. 3A, the green and red fluorescence signals coincide, indicating a mitochondrial localization of hCOQ5. To determine the precise sub-mitochondrial localization of hCOQ5, mitochondria prepared from cultured HEK293 cells transfected with pCOQ5-myc were subjected to treatment with proteinase K under different conditions (hypotonic buffer to disrupt outer membrane, or triton X-100 to disrupt both outer and inner membranes). Extracts were separated by SDS-PAGE and immunoblot analyses were performed with antibodies recognizing TOM20 (outer membrane protein), OPA1 (intermembrane space), GRP75 (matrix enzyme) and $COQ5^{Myc}$ (Fig. 3B, Table 3). The

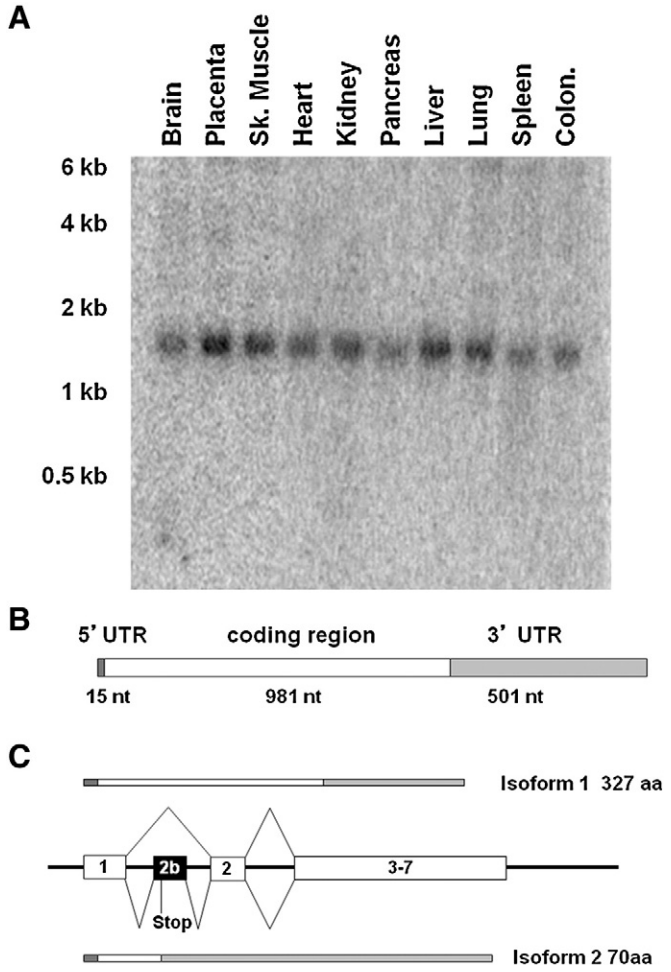

Fig. 2. Characterization of RNA steady-state levels, transcript isoforms, and gene structure of human *COQ5*. (A) Northern blot analysis of *COQ5* expression in different issues. Each lane contains 200 ng of polyA + mRNA. (B) Schematic structure of the principal transcript of human *COQ5* as determined by RACE experiments. (C) Genomic structure of the 5′ region of *COQ5* showing the alternatively spliced exon 2b and the resulting proteins.

hCOQ5 signal disappears only after treatment with triton X-100 indicating that hCOQ5 is localized to the mitochondrial matrix, similar to the GRP75 matrix marker.

To determine whether hCOQ5 is associated with the mitochondrial inner membrane, mitochondrial extracts were treated with sonication, sodium carbonate, or Triton X-100 (Fig. 3C). After treatment samples were precipitated with TCA and centrifuged. Aliquots of the supernatant and of the resuspended pellets were then separated with SDS-PAGE and polypeptides were detected with immune-blot analyses. Membranes were assayed with antibodies recognizing porin (an integral membrane protein), SDHA (a peripheral membrane protein), and $COQ5^{Myc}$. The association of hCOQ5 to the membrane is looser than SDHA, since sonication itself partially released the protein (Fig. 3C). Overall these results indicate that hCOQ5 is peripherally associated with the mitochondrial inner membrane on the matrix side, and confirm the findings of Chen et al. [27].

In yeast, Coq4 is a crucial organizer of the CoQ-synthome, a multisubunit complex required for Q biosynthesis [8]. To determine the association of COQ4 with COQ5 in human cells, we performed immune-precipitation assays. Tagged versions of COQ4-V5 and COQ5-myc were co-expressed in HEK293 cells. After cell harvesting, mitochondrial lysates were immune-precipitated with either anti-V5 or anti-myc antibodies. Immune-precipitates were separated by SDS-PAGE, and the proteins transferred to a membrane that was then probed with antibodies to the V5- or myc-epitopes. Fig. 3D shows that immune-precipitation of COQ4-V5 captures COQ5-myc and vice versa, indicating that these COQ proteins physically associate in a complex in human cells.

3.3. Expression of human or E. coli COQ5 homologs rescues yeast coq5Δ mutants over-expressing COQ8

We examined if expression of hCOQ5 could complement the Q_6 biosynthetic defect in *coq5*Δ mutant yeast. Human COQ5 expressed from either low- or high-copy expression plasmids failed to rescue growth of *coq5*Δ mutant yeast on medium containing glycerol as the sole carbon source (Fig. 4 and data not shown). Human proteins are sometimes not properly imported into yeast mitochondria [14,23]. Thus, a hybrid gene construct was prepared in which amino acids 1–55 of hCOQ5 were replaced with the first 54 amino acids of *S. cerevisiae* Coq5. This construct is referred to as yeast–human hybrid *COQ5* or yhCOQ5 (Table 2). As shown by a plate dilution assay, neither hCOQ5 nor yhCOQ5 rescues the growth of yeast *coq5*Δ on non-fermentable YPG medium (Fig. 4A). This result corroborates previous studies demonstrating that expression of the *E. coli* Coq5 homolog, UbiE, also failed to rescue growth of the yeast *coq5*Δ mutant on YPG medium [16].

We therefore asked whether the problem could be related to a failure of the human COQ5 protein to stabilize the Coq polypeptide complex in yeast *coq5*Δ cells. Recent work has shown that the over-expression of the putative Coq8 kinase in certain *coq* null mutants allows synthesis of late-stage Q-intermediates, restores steady-state levels of Coq polypeptides, and their association in a high molecular mass complex known as the CoQ-synthome [8,10,28]. To examine rescue of *coq5*Δ in the presence of the CoQ-synthome, yeast Coq8 was over-expressed on a high copy plasmid (hcCOQ8) and strains were grown on selective media (Fig. 4B). Co-expression of yeast Coq8 with either yhCOQ5 or *E. coli* UbiE, both of which contain an amino-terminal yeast mitochondrial leader sequence, rescues growth of *coq5*Δ on YPG medium (Fig. 4B). The rescue by hCOQ5 is less robust, suggesting that a yeast mitochondrial leader sequence is critical for efficient import of hCOQ5 into yeast mitochondria.

To examine Q_6 levels, lipids were extracted from yeast cells and subjected to HPLC and tandem mass spectrometry [10]. In the absence of yeast Coq8 over-expression, Q_6 was not detected in any of the *coq5*Δ mutant yeast strains (Fig. 4C). However, in the presence of yeast Coq8 over-expression, the Q_6 content is partially restored in strains expressing *E. coli* UbiE, yhCOQ5, or hCOQ5 (Fig. 4C). These results indicate that human and *E. coli* Coq5 homologs can rescue the defect in the yeast *coq5*Δ mutant only when other yeast Coq polypeptides are stabilized by Coq8 over-expression.

3.4. Expression of human and E. coli COQ5 homologs partially restores Q_6-content in coq5-2 and coq5-5 point mutants

To further examine whether expression of human COQ5 homologs is able to rescue *coq5* mutant strains retaining a stable CoQ-synthome, yeast *coq5* strains with point mutations within or adjacent to one of the methyltransferase motifs were employed (Fig. 1B). Two point mutants, *coq5-2* (CH83-B3) and *coq5-5* (CH316-6B) lack *C*-methyltransferase activity and Q_6 biosynthesis but retain steady state levels of Coq5 and other Coq polypeptides [15,16,29]. Expression of *E. coli* UbiE, hCOQ5 or yhCOQ5 in these yeast *coq5* point mutants rescued growth on rich glycerol (YPG) medium and partially restored Q_6 content (Fig. 5).

Expression of hCOQ5 results in detectable growth in the *coq5-5* mutant on respiratory media even though the content of Q_6 ranges from 0.4% to 3% of wild-type Q_6 content (Fig. 5C). Serial plate dilution assays show that growth on respiratory medium requires only a small amount of Q_6, as restoration of a low Q_6 content is sufficient to rescue plate growth of the *coq5-2* and *coq5-5* point mutants (Fig. 5).

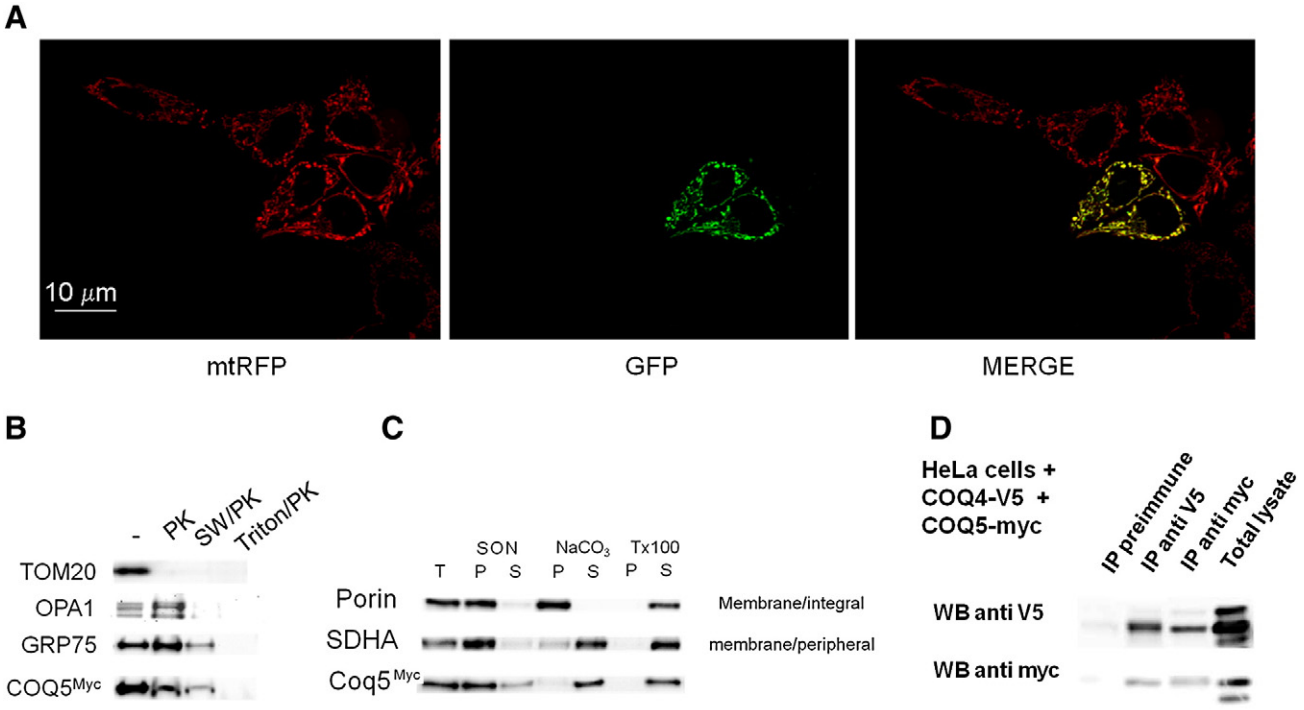

Fig. 3. Human COQ5 is located within the mitochondrial matrix and is peripherally associated with the inner membrane. (A) Co-localization of COQ5-GFP fusion proteins transiently expressed from pCOQ5-GFP in HeLa cells stably expressing mitochondria targeted RFP (mtRFP). (B) Proteinase K (PK) protection assays. Mitochondria were subjected to PK treatment directly after isolation and only outer membrane proteins are degraded (e.g. TOM20). Alternatively, mitochondria were treated with PK after treatment with hypotonic solution, which causes swelling (SW), and renders proteins of the inter-membrane space (e.g. OPA1) accessible to PK; or after treatment with a detergent (Triton X-100) which disrupts all mitochondrial membranes and allows PK degradation of all compartments including matrix proteins (e.g. GRP75). The susceptibility of COQ5 to PK follows the same pattern as GRP75, a mitochondrial matrix protein. (C) Membrane association assays. Mitochondrial membranes were prepared by sonication followed by centrifugation (12,000 *g* for 10 min) to generate pellet and supernatant fractions: isotonic buffer conditions (SON), $NaCO_3$ treatment (which solubilizes peripheral membrane proteins), or Triton X-100 (which solubilizes integral membrane proteins). Aliquots of the supernatants (S) and pellets (P) were separated by SDS-PAGE and proteins were detected with the designated antibodies. A sample of the total membrane preparation (T) was included as control. (D) Mitochondrial extracts of HEK293 cells co-expressing COQ5-myc and COQ4-V5 were immune-precipitated with anti-myc, anti-V5 or with pre-immune sera. Immune-precipitates were separated by SDS-PAGE, transferred to membranes and the tagged-polypeptides were detected with either anti-V5 or anti-myc antibodies.

3.5. Yeast coq5-5 mutants rescued with human COQ5 homologs show low de novo Q_6 biosynthesis and accumulation of Q_6-intermediates

The Q_6 levels measured in Figs. 4C and 5C account for total content of Q_6 present at the time of harvest; to assay the ability of mutants to synthesize new Q_6 de novo, *coq5-5* point mutants harboring hCOQ5 or yhCOQ5 were grown in medium containing either $^{13}C_6$-pABA or $^{13}C_6$-4HB. pABA and 4HB are two aromatic ring precursors of yeast Q_6 biosynthesis, and inclusion of $^{13}C_6$-pABA or $^{13}C_6$-4HB will result in the new synthesis of $^{13}C_6$–Q_6 detectable by its additional mass of +6 indicating the incorporation of a $^{13}C_6$-label. The yeast *coq5-5* point mutant rescued with either hCOQ5 or yhCOQ5 was able to synthesize similar

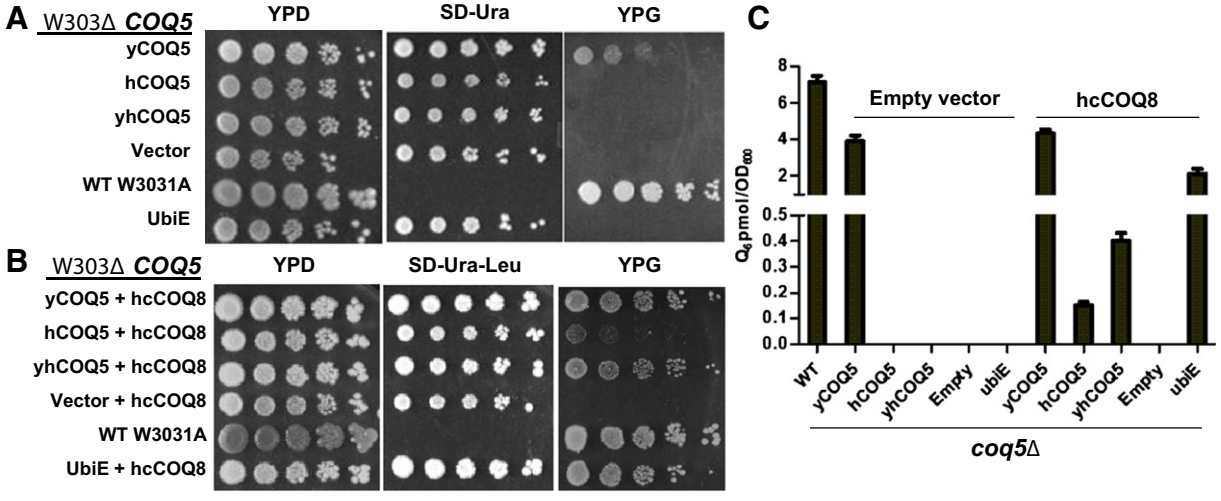

Fig. 4. *COQ5* homologs partially restore growth on respiratory medium and Q_6-content in yeast *coq5* null mutants over-expressing *COQ8*. Yeast *coq5* null mutants (W303Δ*COQ5*) were transformed with the designated plasmids without (A) or with (B) a high copy *COQ8* plasmid (hcCOQ8). Each mutant strain was cultured overnight in SD-selective media, adjusted to 0.2 optical density (OD), and 2 μl of 1:5 serial dilutions was spotted onto plate media. Plates are depicted after 3 days of incubation at 30 °C. (C) Yeast were seeded in SD–Ura at 0.2 OD and collected after 4.5 h. Q_6 content in cell lipid extracts was determined by HPLC/MS–MS as described [10] with a limit of detection of 10 fmol/μl. Each bar represents a total of four measurements from two independent samples each with two injections. Error bars represent standard deviations.

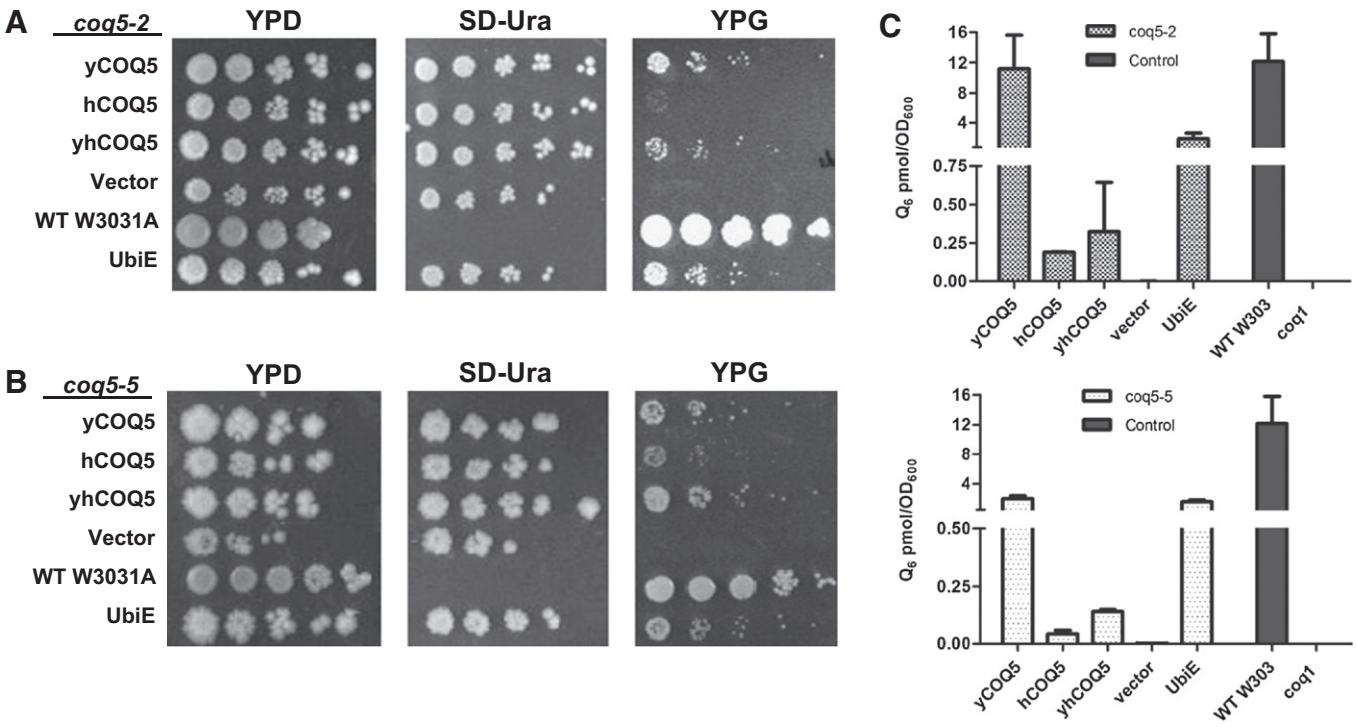

Fig. 5. *COQ5* homologs partially restore growth on respiratory medium and Q_6-content in yeast *coq5* point mutants. (A and B) Yeast were seeded in SD–Ura at 0.2 OD and collected after 3 h for Q_6 analysis and plate dilution assays. Plates are depicted after 3 days of incubation at 30 °C. (C) Q_6 content in cell lipid extracts was determined by HPLC/MS–MS as described [10]. Limit of detection was 50 amol/μl. Each bar represents a total of four measurements from two independent samples each with two injections. Error bars represent standard deviations.

amounts of $^{13}C_6$–Q_6 from either $^{13}C_6$-pABA (Fig. 6A) or $^{13}C_6$-4HB (Fig. 6B). However, the Q_6 synthesis was much less efficient as compared to either wild-type yeast or to the *coq5-5* mutant rescued by yeast *COQ5*.

The low content of newly synthesized $^{13}C_6$–Q_6 in the yeast *coq5-5* point mutants expressing hCOQ5 or yhCOQ5 suggested that Q_6-intermediates might accumulate in these strains. To determine this, lipid extracts were examined for the presence of $^{13}C_6$–Q_6 intermediates. Of particular interest is the substrate of Coq5, demethyl-demethoxy-Q_6 ($DDMQ_6$) (Fig. 1A). $DDMQ_6$ was first detected in *coq5*Δ strains over-expressing Coq8 [10]. $DDMQ_6$ is not detected in wild-type yeast, presumably because it is quickly methylated in the production of Q_6. We therefore determined whether $DDMQ_6$ could be detected in strains with less efficient Q_6 biosynthesis, for example in yeast *coq5-5* mutants expressing hCOQ5 or yhCOQ5.

The yeast *coq5-5* mutant harboring yhCOQ5 was cultured in the presence of $^{13}C_6$-pABA. Lipid extracts were prepared and subjected to reversed phase HPLC–MS/MS as described (Section 2.8). In extracts prepared from $^{13}C_6$-pABA-labeled yeast cells, a predicted precursor-to-product ion transition for $^{13}C_6$-labeled $DDMQ_6$ eluted at 4.69 min. The fragmentation spectrum (Fig. 7) revealed the $^{13}C_6$-$DDMQ_6$ $[M + H]^+$ precursor ion, the $^{13}C_6$-$DDMQ_6$ tropylium product ion $[M]^+$, and the $^{13}C_6$-$DDMQ_6$ chromenylium product ion $[M]^+$.

The levels of the $DDMQ_6$ and demethoxy-Q_6 (DMQ_6) were next examined in yeast *coq5* mutants expressing yhCOQ5 or *E. coli* UbiE (Fig. 8). DMQ_6 is detected in Q_6-producing yeast strains characterized so far and is a reflection of the Q_6-content [30]. In mutants rescued by yeast *COQ5* (blue trace in Fig. 8), DMQ_6 is detected, but not $DDMQ_6$, suggesting that yeast Coq5 efficiently methylates $DDMQ_6$ to make DMQ_6. In contrast, the yeast *coq5-5* mutant harboring the empty vector control accumulates $DDMQ_6$ (gray trace in Fig. 8A and C) but DMQ_6 is not detected (gray trace, Fig. 8B and D). Notably, *coq5-5* mutants harboring either yhCOQ5 or UbiE accumulate $DDMQ_6$ but also make small amounts of DMQ_6 (green and red traces, Fig. 8). We reason that the expression of yhCOQ5 or UbiE results in slower, less efficient methylation of $DDMQ_6$, leading to an accumulation of $DDMQ_6$ and lower levels DMQ_6. These data indicate that in this system yhCOQ5 is less efficient than yCOQ5 as a *C*-methyltransferase.

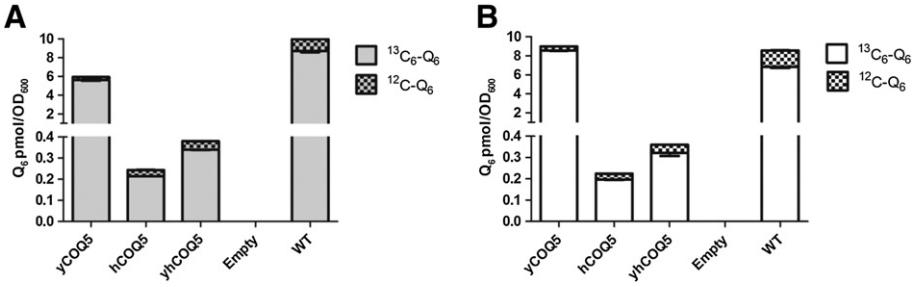

Fig. 6. *COQ5* homologs partially restore de novo Q_6 content of yeast *coq5-5* mutants. Yeast cells were seeded at 0.07 OD into DOD medium supplemented with (A) $^{13}C_6$-pABA or (B) $^{13}C_6$-4HB. After a 10-h incubation with label at 30 °C, $^{13}C_6$–Q_6 lipid extracts were determined by HPLC/MS–MS as in [10] with a limit of detection of 10 fmol/μl. Each bar represents two measurements from one sample. Error bars represent standard deviations.

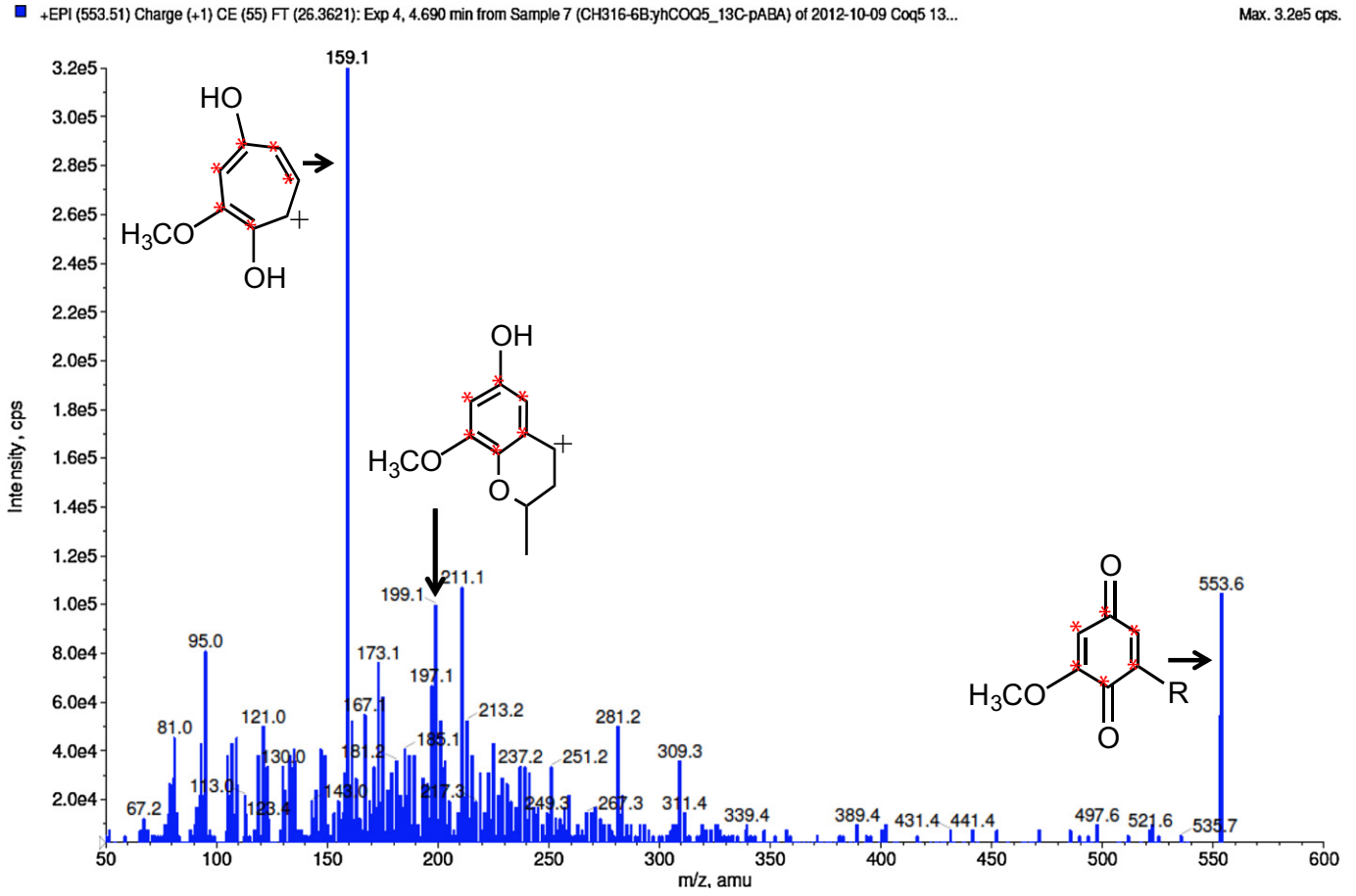

Fig. 7. Identification of de novo demethyl-demethoxy-Q_6 ($DDMQ_6$) in lipid extracts of *coq5-5*:yhCOQ5 yeast cells. $^{13}C_6$-$DDMQ_6$ was detected in the lipid extracts of *coq5-5*:yhCOQ5 cells grown in the presence of $^{13}C_6$-pABA as described in Materials and methods (Section 2.8). The $^{13}C_6$-$DDMQ_6$ $[M+H]^+$ precursor ion ($^{13}C_6^{12}C_{31}H_{55}O_3^+$; monoisotopic mass 553.4), the $^{13}C_6$-$DDMQ_6$ tropylium product ion $[M]^+$ ($^{13}C_6^{12}C_2H_9O_3^+$; monoisotopic mass 159.05), and the $^{13}C_6$-$DDMQ_6$ chromenylium product ion $[M]^+$ ($^{13}C_6^{12}C_5H_{13}O_3^+$; monoisotopic mass 199.1) are consistent with the presence of $^{13}C_6$-aromatic ring label as denoted by red asterisks.

3.6. Human Coq5 polypeptide co-migrates with Coq9 in a high molecular mass complex

The lower methyltransferase efficiency of the human and yeast–human COQ5 orthologs elicits an interesting question: what interaction, if any, do COQ5 orthologs have with the yeast CoQ-synthome? We probed this question in a preliminary way by determining whether the human COQ5 polypeptide co-migrated with yeast Coq polypeptides at high molecular mass. Previous studies have shown that the Coq9 polypeptide co-purifies with other yeast Coq polypeptides and migrates at high molecular mass in solubilized extracts of mitochondria [9]. Here, the antibody against yeast Coq9 was used as a benchmark for the presence of high molecular mass CoQ-synthome [8–10].

Mitochondria were purified from wild-type yeast and from *coq5*Δ mutants over-expressing yeast Coq8 in combination with yhCOQ5, yeast Coq5, or empty vector, and the digitonin extracts were separated by 2D-BN-SDS/PAGE. We used a polyclonal antibody against hCOQ5 that identified yhCOQ5 expressed in the yeast *coq5*Δ mutant (Fig. S2). The yhCOQ5 polypeptide was detected at high molecular mass (~650 kDa), although most of the signal was observed in a broad region around 66 kDa (Fig. 9A). Yeast Coq9 was also found to migrate with a pattern similar to the yhCOQ5 polypeptide on the same immune-blot membrane (Fig. 9A). In the *coq5*Δ strain co-expressing high copy yeast Coq8 and yeast Coq5, the yeast Coq5 polypeptide was detected at high molecular mass complex together with yeast Coq9 (~300 kDa) (Fig. 9B). Both yeast Coq5 and Coq9 were also present at lower mass (Fig. 9B). In the *coq5*Δ strain co-expressing high copy yeast Coq8 and vector control, no Coq5 is detected, while Coq9 is observed at both low and high molecular weight (Fig. 9C). In wild-type yeast Coq9 is present across a wide range of high molecular mass complexes, while Coq5 is present mainly in a broad region around 66 kDa, perhaps indicating the presence of Coq5 and Coq9 in sub-complexes.

4. Discussion

COQ5 encodes an essential protein involved in the biosynthetic pathway of Q_6 in *S. cerevisiae*. It is the only known *C*-methyltransferase enzyme involved in Q_6 synthesis, and catalyzes the methylation of demethyl-demethoxy-Q_6H_2 ($DDMQ_6H_2$) to form demethoxy-Q_6H_2 (DMQ_6H_2). In yeast, Coq5 and several *COQ* gene products are associated in the CoQ-synthome, a high molecular mass complex peripherally associated with the matrix side of the mitochondrial inner membrane [8]. In this study, we confirmed that the human protein is localized to the mitochondrial matrix and is peripherally associated with the inner membrane. We also showed that hCOQ5 physically associates with hCOQ4, indicating that a COQ multi-enzyme complex is also present in human cells.

Human *COQ5* RNA was expressed ubiquitously in human organ samples as assayed by Northern blotting (Fig. 3). Steady state RNA levels for *COQ2*, *COQ4*, and *COQ6* have been determined for a variety of human tissues [20,23,31]. In comparing the expression levels of these *COQ* genes, there does not seem to be a common pattern: *COQ5* had the highest expression levels in the placenta, liver, skeletal muscle, and lung, *COQ6* in skeletal muscle, kidney, and placenta, *COQ2* in skeletal

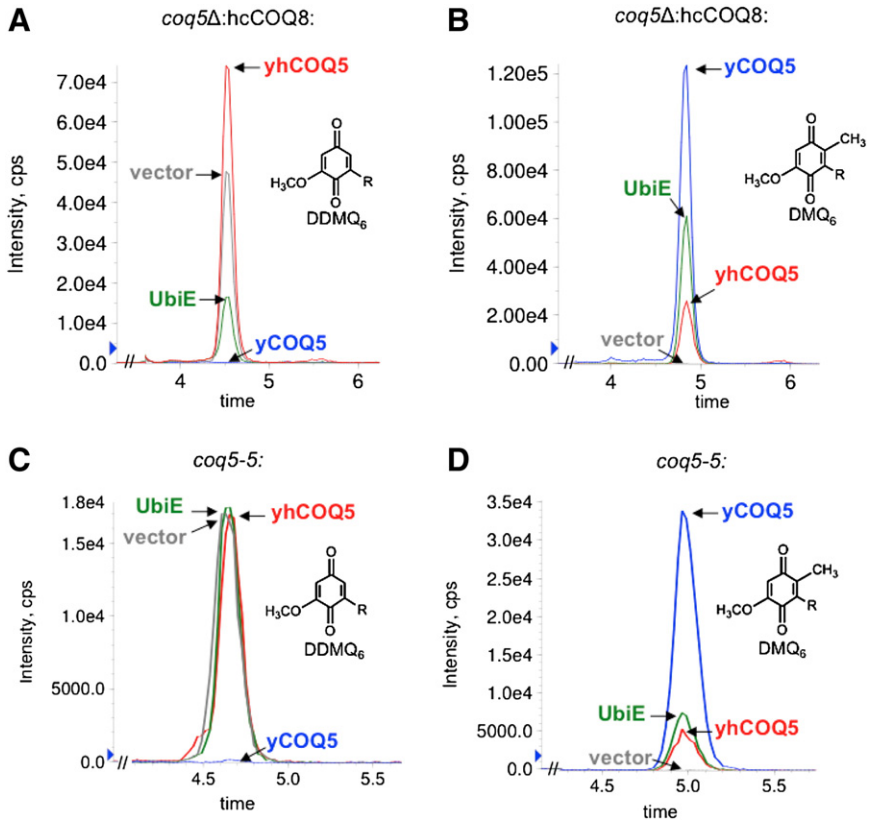

Fig. 8. Yeast *coq5* mutants rescued with *COQ5* homologs accumulate Q_6-intermediate $DDMQ_6$. The designated yeast *coq5* mutants were seeded in selective SD medium at 0.2 OD, collected after 3 h, and lipids were extracted for Q_6 analysis. MRM detected precursor-to-product ion transition 547.4/153.1 ($DDMQ_6$) in panels (A) and (C); and precursor-to-product ion transition 561.4/167.0 (DMQ_6) in panels (B) and (D). Red trace indicates the presence of the yeast-human hybrid COQ5, blue trace indicates yeast COQ5, green indicates *E. coli* UbiE, and gray indicates the empty vector control.

muscle and heart, while *COQ4* RNA abundance was highest in lung, colon and pancreas.

A search of the EST database revealed a possible second transcript encoding a truncated form of *COQ5*. The presence of transcripts encoding non-functional proteins is not unusual for *COQ* genes and has been reported also for *PDSS2*, *COQ4*, and *COQ6*[20,23,32]. The functional significance of the truncated proteins is still unclear, although they could play a regulatory role [33].

Despite a relatively high degree of homology to its yeast counterpart, the human COQ5 protein failed to complement yeast mutants with a deletion in *COQ5*, as determined by a lack of growth on non-fermentable carbon source and absence of Q_6. Rescue by human COQ5 was more efficient when the protein contained an appended *S. cerevisiae* Coq5 mitochondrial leader sequence. However, even under this condition, rescue was observed only in (1) yeast *coq5* point mutants harboring stable but catalytically inactive Coq5 polypeptide, or (2) yeast *coq5* null mutants over-expressing *COQ8*. We note that both conditions permit the stabilization of CoQ-synthome required for Q_6 biosynthesis in yeast. The results here suggest that *COQ5* orthologs can serve as functional *C*-methyltransferases and restore Q biosynthesis in the yeast *coq5* mutants only when the other yeast Coq polypeptides are preserved.

Interestingly, the expression of human COQ5 was recently shown to complement a *Schizosaccharomyces pombe coq5*Δ mutant for growth in medium containing a nonfermentable carbon source [34]. Unlike the *S. cerevisiae coq5*Δ mutant, the *S. pombe coq5*Δ mutant contained peaks tentatively identified as late-stage Q-intermediates, suggesting that the *S. pombe coq* deletion mutants do not have the same drastic "early intermediate" phenotype as observed in the *S. cerevisiae coq* deletion mutants. Indeed, human COQ homologs were shown to rescue each of the *S. pombe coq* deletion mutants, with the exception of Coq9 [34]. For both *S. pombe* and *S. cerevisiae*, expression of human COQ5 results in production of a very small amount of Q, as compared to wild-type cells. Several interspecific complementation studies suggest that such rescue of yeast growth on nonfermentable carbon sources can be accomplished with less than 0.2% of the normal levels of Q [14,23].

Yeast *coq5-5* mutants rescued by yeast Coq5 are able to efficiently methylate $DDMQ_6H_2$ to make DMQ_6H_2 and high amounts of Q_6H_2. Due to autoxidation, the $DDMQ_6$ and DMQ_6 quinones are the predominant forms recovered in lipid extracts. In contrast, the accumulation of the Coq5 substrate $DDMQ_6$ in yeast *coq5-5* mutants expressing either human COQ5 or *E. coli* UbiE indicates a slower or less efficient methylation of $DDMQ_6H_2$, and is also consistent with the lower levels of both DMQ_6 and Q_6. A recent crystal structure of the *S. cerevisiae* Coq5 polypeptide showed the structure to be a dimer, and identified amino acid residues at the dimer interface domain [35]. The residues at the yeast Coq5 dimer interface are not as highly conserved between Coq5 homologs as the active-site residues that contact S-adenosyl-L-methionine [35]. We speculate that the interaction of the yeast Coq5 dimer with the yeast CoQ-synthome enhances the catalytic efficiency of the *C*-methylation, and that the partial rescue observed by the heterologous COQ5 polypeptides results from a less efficient or absence of interaction with the yeast CoQ-synthome.

To explore this idea we examined whether the human COQ5 homolog might interact with the yeast CoQ-synthome as determined by separation of digitonin solubilized mitochondrial extracts with 2D-BN-SDS/PAGE. Small amounts of the hCOQ5 polypeptide were observed at high molecular mass in the *coq5* null mutant harboring yhCOQ5 with Coq8 over-expression. Similarly, small amounts of the yeast Coq5 polypeptide were observed at high molecular mass in the *coq5* null mutant harboring yeast *COQ5* with Coq8 over-expression. This may be due to the presence of over-expressed Coq8. However, we did not detect

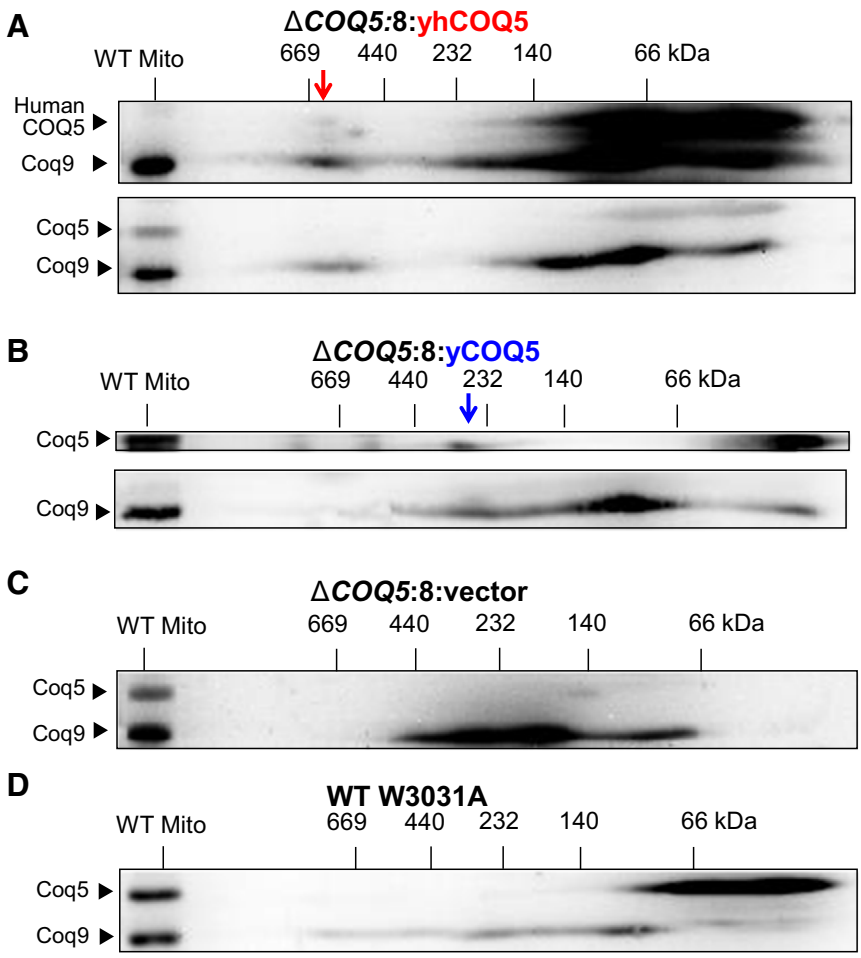

Fig. 9. Human COQ5 polypeptide co-migrates with yeast Coq9 at high molecular mass. Purified mitochondria (200 μg of protein) from the designated yeast strains were separated by 2D-BN-SDS/PAGE, and the membranes containing transferred proteins were probed with antibodies against human COQ5, yeast Coq9 or yeast Coq5. Mitochondria were isolated from *coq5* null with co-expression of Coq8 (hcCOQ8) and (A) yhCOQ5, (B) yCOQ5 and (C) empty vector. (D), Mitochondria were purified from wild-type yeast, W3031A. A sample of wild-type mitochondria separated just in the SDS-second dimension served as a positive control and is designated as WT Mito. The positions of molecular mass standards in the first dimension are indicated at the top of each panel.

Coq5 co-migrating with other Coq polypeptides at a high molecular mass in mitochondria prepared from wild-type yeast cells (Fig. 9D and unpublished results). It is very challenging to recover the intact CoQ-synthome. The complex must be first solubilized from the inner mitochondrial membrane with detergent, and depending on the separation conditions, may behave very differently. For example, although we readily detect the presence of the Coq5 polypeptide in complexes captured with tagged forms of Coq9 [9], only very small amounts of Coq5 are detected at high molecular mass by 2D-blue native-SDS PAGE [8]. Tauche et al. observed a Coq3–Coq5–Coq9 complex with myc-tagged Coq polypeptides [36]. We do not always observe the Coq5 polypeptide at high molecular mass when separated by size exclusion chromatography [37]. Based on these observations we think that it is very likely that Coq5 readily dissociates from the CoQ synthome. Obviously, the absence of co-migration does not rule out the possibility of a transient or weak physical association.

A physical interaction between the human COQ5 and the yeast Coq polypeptide complex may not be necessary for partial rescue of Q_6 content. The CoQ-synthome needs only to provide human COQ5 with its substrate $DDMQ_6H_2$, which may be accomplished with human COQ5 peripherally associated with the complex. Low Q_6 content and the presence of Q_6-intermediates suggest either inefficient or weak interaction with the Coq complex. The results presented here suggest that human and *E. coli* Coq5 homologs expressed in yeast retain C-methyltransferase function, but rescue the yeast *coq5* mutants only in the presence of the assembled yeast CoQ-synthome.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2014.08.007.

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