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Expression of the Human Atypical Kinase ADCK3 Rescues Coenzyme Q Biosynthesis and Phosphorylation of Coq Polypeptides in Yeast *coq8* Mutants

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Abstract

Coenzyme Q (ubiquinone or Q) is a lipid electron and proton carrier in the electron transport chain. In yeast *Saccharomyces cerevisiae* eleven genes, designated *COQ1* through *COQ9*, *YAH1* and *ARH1*, have been identified as being required for Q biosynthesis. One of these genes, *COQ8* (*ABC1*) encodes an atypical protein kinase, containing six (I, II, III, VIB, VII, and VIII) of the twelve motifs characteristically present in canonical protein kinases. Here we characterize seven distinct Q-less *coq8* yeast mutants, and show that unlike the *coq8* null mutant, each maintained normal steady state levels of the Coq8 polypeptide. The phosphorylation states of Coq polypeptides were determined with two-dimensional gel analyses. Coq3p, Coq5p, and Coq7p were phosphorylated in a Coq8p dependent manner. Expression of a human homolog of Coq8p, ADCK3(CABC1) bearing an amino-terminal yeast mitochondrial leader sequence, rescued growth of yeast *coq8* mutants on medium containing a nonfermentable carbon source and partially restored biosynthesis of Q₆. The phosphorylation state of several of the yeast Coq polypeptides was also rescued, indicating a profound conservation of yeast Coq8p and human ADCK3 protein kinase function in Q biosynthesis.

Keywords

coenzyme Q; ubiquinone; *Saccharomyces cerevisiae*; mitochondria; lipid metabolism; protein kinase

1. Introduction

Coenzyme Q (ubiquinone or Q)³ is an essential lipid component and plays a well-known role in respiratory electron and proton transport in energy metabolism. Q, the oxidized quinone accepts electrons from NADH via complex I or succinate via complex II, and the

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reduced hydroquinone (QH₂) is oxidized by complex III where the electrons are transferred to cytochrome *c*. Q also functions as the electron acceptor in a number of metabolic oxidation reactions including amino acid catabolism and fatty acid beta oxidation via acyl-CoA dehydrogenases [1], glycerol-3-phosphate, dihydroorotate [2], and the oxidation of sulfide [3]. QH₂ is an important lipid-soluble chain-terminating antioxidant [4], and may also function as a co-antioxidant to maintain levels of vitamin E in membranes of cells and lipoproteins [5]. Q and Q analogs have been shown to be capable of inhibiting, activating, or occupying sites of the mitochondrial permeability transition pore [6-8]. The mitochondrial permeability transition pore is involved in apoptosis through the opening of a non-selective channel that disrupts membrane potential and causes hypotonic swelling of the inner membrane, a process that appears to be conserved from yeast to plants and animals [9,10].

Cells utilize isoprenoid biosynthetic pathways (mevalonate or methylerythritol) to produce the polyisoprenyl tail of Q_n, where *n* designates the number of isoprene units; Q₁₀, Q₉, Q₈ and Q₆ in human, *Caenorhabditis elegans*, *Escherichia coli*, and *Saccharomyces cerevisiae*, respectively [11]. Coq1p in *S. cerevisiae*, IspB in *E. coli*, or a complex of PDSS1 and PDSS2 in human cells synthesize the polyisoprene diphosphate tail precursor of Q_n. The aromatic ring precursors supplying the quinone ring of Q include 4-hydroxybenzoic acid (4-HB) derived from either chorismic acid or tyrosine [12]. Recently, para-aminobenzoic acid (pABA) was discovered to function as a ring precursor of Q in *S. cerevisiae* [13,14]. Coq2p is required for the prenylation of either 4-HB or pABA to form 3-hexaprenyl-4-hydroxy benzoic acid or 3-hexaprenyl-4-amino-benzoic acid [13]. *S. cerevisiae* requires at least nine additional polypeptides (Coq3-Coq9, Yah1, and Arh1) for biosynthesis of Q₆ [11,14,15].

Although much progress has been made in determining the enzymatic functions of the Coq proteins, the functions of Coq4p and Coq9p in Q biosynthesis are not known, and new information on the putative kinase function of Coq8p is described in this work. In *S. cerevisiae*, Coq4p and Coq9p are members of a high molecular mass multi-subunit mitochondrial complex together with the *O*-methyltransferase Coq3p, the *C*-methyltransferase Coq5p, and the hydroxylases Coq6p and Coq7p, required for ring modifications forming Q [16-18]. A zinc-binding motif identified in Coq4p was postulated to be important for Coq4p function as a scaffolding protein [17]. Recently, Rea *et al.*, [19] derived a model of yeast Coq4p by taking advantage of the structure determined by the Northeast Structural Genomics consortium of a Coq4p homolog from *Nostoc sp. PCC7120* (Alr8543) that crystallized with a bound geranylgeranyl monophosphate and a magnesium ion. The predicted Coq4p structure is consistent with the idea that Coq4p may bind the polyisoprene tail of a Q-intermediate, possibly serving as an important anchor for the Q-multisubunit biosynthetic complex.

The yeast *COQ8* gene was originally identified as *ABC1* (activator of the *bc*₁ complex) in a multicopy suppression screen of a specific *chs2* mutant allele (*chs2*-223) and proposed to function as a cytochrome *b* chaperone [20], necessary for *bc*₁ complex function [21]. However, the decrease in *bc*₁ complex in *coq8* mutants can be explained fully by the requirement of *COQ8* for Q biosynthesis [22]. Yeast *coq8* mutants lack Q₆ and the growth defect in media containing a nonfermentable carbon source can be rescued by the addition of exogenous Q₆ to the growth medium. The suppression of the *chs2* mutation was shown to be

³The abbreviations used are

2D-IEF/SDS-PAGE two dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Q coenzyme Q

ORF open reading frame

due to a neighboring tRNA and not to *COQ8* [23]. *E. coli* UbiB and *Proventencia stuartii* AarF are prokaryotic Coq8 homologs required for Q biosynthesis; mutants accumulate octaprenyl-phenol, the Q-intermediate expected from a block at the first hydroxylase step [24,25]. Patients with mutations in *ADCK3/CABC1* (a homolog of yeast *COQ8*) exhibit progressive neurological disorders with childhood onset cerebellar ataxia and atrophy and have decreased Q₁₀ content in muscle, fibroblast, and lymphoblast cells [26] [27]. Thus, in prokaryotes, yeast, and human cells, the principal and conserved function of the Coq8 polypeptide is its requirement for Q biosynthesis.

Yeast Coq8p, *E. coli* UbiB, and human ADCK3 are members of an atypical kinase family, first identified by Leonard *et al.* [28], as potential protein kinases harboring motifs I, II, VIB and VII. Subsequent analyses identified ADCK1-5 as novel human kinases [29], containing six (I, II, III, VIB, VII, and VIII) of the twelve motifs characteristically present in canonical protein kinases [27]. Patients with Q₁₀ deficiencies harboring mutations in *ADCK3* indicated it is required for Q₁₀ biosynthesis in humans. In these studies, expression of human ADCK3 did not rescue yeast *coq8* mutants. However, introduction of the human mutations into the corresponding yeast *COQ8* gene impaired growth of yeast on nonfermentable carbon sources and resulted in decreased Q₆ content [26,27]. These findings suggested that yeast Coq8p and human ADCK3 may function as kinase required for Q biosynthesis. There is also evidence that Coq8p may function to regulate Q biosynthesis, as overexpression of yeast Coq8p has been shown to rescue a yeast *coq9* nonsense mutant [18,30], a yeast *coq10* null mutant [31,32], and to restore synthesis of DMQ₆ in a *coq7* null mutant [33]. Tauche *et al.* [34] utilized two dimensional IEF-SDS PAGE and tagged forms of yeast Coq polypeptides and determined that phosphorylation of yeast Coq3p is dependent on Coq8. They also demonstrated that the phosphorylation state of Coq3 affects its association with the Coq high molecular mass polypeptide complex. Thus, Coq8p appears to be essential for the formation of a multi-subunit Q-biosynthetic complex. It is intriguing that the first O-methylation step catalyzed by yeast Coq3 was identified as a potential regulated site of Q biosynthesis in response to high glucose, and it was speculated that a cAMP-dependent protein kinase might be involved in mediating this regulation [35].

In this study we have analyzed mutations in Coq8 that result in Q biosynthetic defects. Initially, we employed site directed mutagenesis to target the catalytic lysine in kinase motif II of Coq8p. Although yeast *coq8* null mutants expressing the Coq8-K216A polypeptide lacked Q₆, the Coq8-K216A polypeptide was not stable, and the phenotype of this strain mirrored the *coq8* null mutant. Several Coq polypeptides are unstable in the *coq8* null mutant (including Coq4p, Coq6p, Coq7p and Coq9p) [18]. To assess the role of Coq8p as a potential kinase, our goal was to analyze *coq8* mutants that retained normal steady state levels of Coq8p. Therefore we examined the collection of yeast *coq8* mutants in order to identify those that retained normal steady state levels of Coq8p. Seven distinct yeast *coq8* amino acid substitution mutants have been characterized and a subset of these mutants used to investigate the phosphorylation state of Coq3 and other Coq polypeptides. We show that expression of human ADCK3 bearing an amino-terminal mitochondrial leader sequence in yeast *coq8* mutants rescues both synthesis of Q₆ and the phosphorylation state of several of the yeast Coq polypeptides, indicating a profound conservation of protein kinase function in Q biosynthesis.

2. Materials and Methods

2.1 Strains and growth media

The yeast strains used in this study are listed in Table 1. Growth media were prepared as described [36]. Media included YPD (1% yeast extract, 2% peptone, 2% dextrose), YPG (1% yeast extract, 2% peptone, 3% glycerol) and YPGal + 0.1% Dextrose (1% yeast extract,

2% peptone, 2% galactose, 0.1% dextrose). SDC consisted of 0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4 , 0.5% $(\text{NH}_4)_2\text{SO}_4$ and amino acids were added at final concentrations as described in [37]. SD-ade, SD-his, SD-leu, SD-met, SD-trp and SD-ura consisted of SDC media minus adenine, histidine, leucine, methionine, tryptophan and uracil respectively. Solid media contained 2% agar. All materials were obtained from Difco, Fisher or Sigma.

2.2 Sporulation and Tetrad Analysis

Diploid cells were grown to a cell concentration of $\text{OD}_{600} = 2.5$ in YPD. Yeast cells from 1 ml of culture were pelleted by centrifugation $12,000 \times g$, 30 seconds. The cell pellet was washed with sterile water and pelleted by centrifugation again. Cells were then resuspended in 1 ml sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% dextrose) and incubated at 30°C for 2 to 3 days. Cells were pelleted by centrifugation, and then resuspended in zymolyase buffer (1 M sorbitol, 50 mM Tris-Cl pH 7.5, 6 mg/ml zymolyase-20T (MP Biomedicals)). Cells were incubated for 15 or 30 min at 30°C . The zymolyase digestion was stopped by diluting the sample with four volumes of water and placing it on ice. The procedure for analysis and dissection of haploid spores was as described [36]. Yeast cells were transformed with plasmid DNA as described [38].

2.3 Subcloning of COQ8 and ADCK3

The plasmids used in this study are listed in Table 2. To construct *COQ8* with a biotin accepting sequence at its C-terminus, a DNA segment was amplified with the primers pBC8-1 (5' CGCGGAATTCACCAGCATTTTGGATAATTC 3' -600 to -589 of *COQ8*) and pBC8-2 (5' CGCGGAATTCAACTTTATAGCAAAAATCT 3' +1503 to +1484 of *COQ8*). The DNA fragment was digested with EcoRI (sites underlined) and then ligated into the EcoRI site of the yeast/*E. coli* shuttle vector containing the biotinylated *Propionibacterium shermanii* transcarboxylase sequence, Yep352-Bio6 [39] [37] [40]. The resulting plasmid, pBT8-1, contains *COQ8* in frame with a carboxyl-terminal biotinylation site.

The plasmid MHS1011 containing human *ADCK3* cDNA was obtained from Open Biosystems. To generate a low copy plasmid containing the open reading frame of *ADCK3* subcloned behind the *S. cerevisiae* *CYC1* promoter, MHS1011 was digested with XhoI and EcoRI. The DNA fragment containing *ADCK3* was purified with a Qiaquick Gel Extraction Kit (Qiagen) and the purified DNA treated with Klenow. A low copy plasmid containing the *CYC1* promoter, pAH01 [41], was digested with HindIII, treated with Klenow and then treated with bacterial alkaline phosphatase. The purified DNA fragment containing *ADCK3* was then ligated into the treated pAH01 plasmid. The plasmid with *ADCK3* in the correct orientation was designated psCABC1. To generate a high copy plasmid containing *ADCK3* subcloned behind the *CYC1* promoter, psCABC1 was digested with KpnI and SpeI to excise a DNA fragment containing the *CYC1* promoter and *ADCK3*. This DNA fragment was then ligated into the high copy plasmid pCH1 [41], also digested with KpnI and SpeI. This plasmid was designated pmCABC1.

Two other yeast expression plasmids were constructed so that the ORF of human *ADCK3* would contain an amino-terminal mitochondrial-targeting leader sequence (from yeast *COQ3*), and expressed from the yeast *CYC1* promoter (see Table 2). A forward primer, ADCK3For (5'-clamp-ClaI-(+1 to +31)-3' of *ADCK3*) and a reverse complement primer, ADCK3Rev (5'-clamp-KpnI-(+1944 to +1927)-3' of *ADCK3*) were used with MHS1011 as template DNA to generate a PCR-amplified segment of DNA containing the ORF of *ADCK3*. The resulting DNA was digested with ClaI and KpnI and inserted into the

corresponding restriction sites of pQM [41] or pRCM (Table 2), to generate plcADCK3 (low copy) and pmcADCK3 (multi copy), respectively.

2.4 Site Directed Mutagenesis

Primer mediated site directed mutagenesis was performed as described [42]. Primer pairs used to generate *coq8-K216A* were KAbc1AF4 (5' TCTTAAGAACTATACAGAACGGGAGAATTTC 3' +55 to +89 bp) with KAbc1AR3 (5' CAGGATATTGAATTGCGACAACCACTCTTT 3' +661 to +632 bp) and KAbc1AF3 (5' AAAGAGTGGTTGTCGCAATTCAATATCCTG 3' +632 to 661 bp) with KAbc1AR4 (5' CATTCGTACGCCCTTTTCTATCTCTCAATGTGG 3' +1194 to 1160 bp). Nucleotides that are underlined are the mutated nucleotides. The PCR fragments were digested with AflIII and BsiWI. The plasmids p3HN4 [22], p4HN4 [23] and pBT8-1 were treated with AflIII and BsiWI to remove a fragment of *COQ8*. The AflIII/BsiWI digested PCR fragment containing the K216A mutation was then ligated into the digested plasmids to generate pK216A, p4K216A and pBT8KA, respectively. pK216A and p4K216A are single copy and multicopy plasmids that contain the *coq8* gene with the K216A mutation. The pBT8KA plasmid contains the K216A mutation of *coq8* and a carboxyl-terminal biotinylation site.

2.5 Genomic DNA Purification and Sequencing

Yeast strains were grown overnight in 5 ml YPD at 30°C. Yeast cells from 0.5 ml of culture were pelleted by centrifugation at 12,000×g for 1 min. Genomic DNA was purified using the Wizard Genomic DNA Kit (Promega). For sequencing, the *COQ8* locus was amplified in two overlapping segments. The first segment was amplified with the primers Abc1Seq-F (5' ACTCGAGAAAAGCAATCTGGTAGATTATGGG 3' -300 to -276 (underlined) relative to the *COQ8* ATG) and Abc1Seq-R (5' CCTCGAGTTAACTTTATAGGCAAAAATCTCTT 3' +1506 to +1481 (underlined) relative to the *COQ8* ATG). The second segment was amplified with the primers KAbc1AF1 (5' CGTCTCTTAAGAACTATACAGAACG 3' +55 to +74 relative to the *COQ8* ATG) and Abc1Seq-R2 (5' TCTGTTTTATCTTTTTTTTTTGTTCCTTCGAGATT 3' +1640 to +1607 from the *COQ8* ATG). The UCLA Sequencing and Genotyping Core determined the nucleotide sequence of each of the PCR products.

2.6 Purification of Mitochondria

Yeast cells were grown in 1 liter of YPGal + 0.1% Dextrose and harvested at an OD₆₀₀ of approximately 1. Crude mitochondria were isolated and purified on a Histodenz (Sigma) gradient as described in [43]. Protein concentration was measured by bicinchoninic acid protein assay (Pierce).

2.7 Submitochondrial localization

Mitochondria were hypotonically shocked for 20 min on ice, with 5 volumes of 20 mM HEPES-KOH pH 7.4, to generate mitoplasts. Mitoplasts were pelleted by centrifugation (14,000×g, 10 min) and the supernatant saved as the intermembrane space fraction (IMS). Mitoplasts were sonicated and the matrix (supernatant) and membrane (pellet) components separated by centrifugation (100,000×g, 30 min). Mitoplasts were also alkaline extracted with 0.1 M Na₂CO₃ pH 11.5 for 20 min at 4°C, and subjected to centrifugation to separate matrix (supernatant), peripheral membrane (supernatant) and integral membrane (pellet) proteins. Proteinase K protection assays were as described [44]. Mitoplasts were pelleted and resuspended in isotonic buffer (0.6 M sorbitol, 20 mM HEPES-KOH pH 7.4) with or without the addition of 100 µg/ml proteinase K (Fisher Scientific) and 1% Triton X-100, for 1 h at 4°C. Proteins were precipitated with 10% trichloroacetic acid and resuspended in

SDS-PAGE sample buffer. SDS-polyacrylamide denaturing gels were prepared containing 10% acrylamide as described [45].

2.8 2D-Isoelectric focusing (IEF)/SDS-PAGE

IEF-PAGE was performed as described [34]. Approximately 200 µg of purified mitochondria from each strain were sedimented by centrifugation (14000×g, 10 min) at 4°C, and resuspended in standard Rehydration Solution (7 M urea, 2 M thiourea, 4 % CHAPS, 0.5 % Ampholytes 3-10, 0.0002% bromophenol blue) to a final concentration of 1.0 µg/µl. The sample was sedimented again (14000×g, 10 min) and de-streak reagent (GE Healthcare; final concentration of 1.25%) was added to the sample as a reducing agent. For in-gel hydration, 200 µl of diluted samples were applied to Immobiline DryStrips (GE Healthcare, 13cm, pH 3-11 NL) and incubated overnight at room temperature. IEF was performed in the Bio-Rad Protean Isoelectric Focusing Cell (Bio-Rad) with four steps of voltage, (4 h at 100 V, 2 h at 250 V, 5 h gradient up to 4000 V, and a further 80,000 Volt-h at constant 4000 V) at 20°C with a maximum current of 50 µA. The strips were equilibrated first for 20 min in Equilibration Buffer (75 mM Tris-HCl, pH 8.8, 6 M urea, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue, 20 mg/ml dithiothreitol), and then another 20 min in Equilibration Buffer with 25 mg/ml iodoacetamide. For the second dimension, the strips were applied to Criterion 8-16% PAGE gels in a Bio-Rad Criterion Dodeca Electrophoresis Cell. For phosphatase treatment, isolated mitochondria (200 µg protein) were resuspended in 19 µL λ-phosphatase buffer (New England Bio Labs; 50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM dithiothreitol, 0.01% Brij 35) containing complete EDTA-free Protease Inhibitor Cocktail (Roche). Samples were incubated for 45 min at 30°C after addition of 1 uL λ-phosphatase (100 U/ uL) (New England Bio Labs).

2.9 Immunoblot analyses

Proteins were transferred from SDS-polyacrylamide gels to polyvinylidene difluoride (PVDF) membrane, and then incubated in 2% non-fat milk and probed with primary antibodies (generated in rabbits) to yeast mitochondrial proteins at the following dilutions: Coq1p, 1:10,000; Coq2p, 1:1000; Coq3p, 1:1000; Coq4p, 1:1000; Coq5p, 1:10,000; Coq6p, 1:250; Coq7p, 1:1000; Coq8p, 1:100; Coq9p, 1:1000; Secondary antibody goat anti-rabbit IgG, heavy and light chain specific peroxidase conjugate (Calbiochem) were used at 1:10,000. A mouse monoclonal antibody for Rip1p was used at 1:10,000 and the secondary antibody was goat anti-mouse peroxidase conjugate. Supersignal West Pico Chemiluminescent kit (Pierce) was used for detection of polypeptides on Western blots.

2.10 Lipid Extraction and Analysis

Yeast cells were grown in YPGal + 0.1% dextrose. Q was extracted from yeast whole cells as described [13,46]. Lipids were separated and analyzed essentially as described [13]. In brief, reverse-phase HPLC with tandem mass spectrometry was used to detect and quantify Q₆ and Q₄ was used as an internal standard. The mass spectrometer was an Applied Biosystems-MDS Sciex 4000 Q Trap (Hybrid triple-quad linear ion trap analyzer with Autosampler, and a Turbo-V source equipped with ESI and APCI sources).

3. Results

3.1 Coq8p is a peripheral membrane protein located on the matrix side of the inner membrane

Coq8p is localized to the mitochondria [22] and a c-myc-tagged form of Coq8p is located within the mitochondrial matrix [34]. Recent analyses of yeast Coq8p have identified a membrane spanning region [47]. Although the Coq8-c-myc tagged polypeptide was shown

to rescue growth on glycerol [34], Q content was not reported. Other tagged forms of Coq polypeptides have been observed to rescue growth on glycerol, but the Q content is not restored to wild-type [14], and in some cases the tagged form of the polypeptide was found to be mislocalized in the rescued yeast strains [48]. Thus it is important to localize the native form of the Coq8 polypeptide. We used antibodies recognizing Coq8p to determine the submitochondrial location of the untagged polypeptide. W303-1A yeast mitochondria were isolated and a hypotonic shock was performed to rupture the outer membrane, leaving the inner membranes intact, generating mitoplasts. Mitoplasts were sonicated to disrupt the remaining membranes, allowing separation of soluble matrix proteins and insoluble membrane associated proteins. Mitoplasts were also treated with alkaline buffer to extract proteins peripherally associated with the inner membrane [49]. Western blot analysis of these samples showed that Coq8p remained with the membrane components after sonication and was released into the supernatant after alkaline treatment, but not after sonication, indicating that Coq8p is a peripherally associated membrane protein (Fig. 1A). A proteinase K protection assay on purified mitochondria, or mitochondria subjected to hypotonic buffer to release intermembrane space components, identified Coq8p as located on the matrix side of the inner membrane (Fig. 1B). The results show that Coq8p is a peripherally associated membrane protein located on the matrix side of the inner membrane.

3.2 Mutation of the catalytic lysine-216 to alanine in Coq8p disrupts Q biosynthesis

Amino acid sequence analyses of yeast Coq8p, human ADCK3, and homologues have identified protein kinase motifs [27-29]. The lysine present in motif II is believed to be involved in the transfer of protons during phosphorylation [50]. Previous studies have shown that mutation of this lysine to alanine in a variety of other protein kinases results in the loss of kinase function, but preserves structure [51]. To determine the role of this conserved lysine in Coq8p function, site directed mutagenesis was used to convert lysine-216 to alanine. The *coq8Δ* yeast strain, W303ΔABC1, was transformed with plasmids containing either wild-type or *coq8-K216A* and tested for growth on plate media containing glycerol as sole non-fermentable carbon source. No growth was detected after three to ten days of incubation of transformants expressing Coq8-K216A (Fig. 2). Identical results were obtained with another *coq8Δ* null mutant FYμABC1, in a distinct genetic background, (data not shown).

3.3 Steady State Polypeptide Levels in *coq8-K216A*

Previous work has shown that deletions of any of the *COQ1-COQ9* genes results in a decrease in the steady state levels of several of the other Coq polypeptides [18]. For example, *coq8* null mutants have decreased levels of Coq4, Coq6, Coq7, and Coq9 polypeptides [18], and the formation or integrity of the Q-biosynthetic complex is affected by the absence of Coq8p [34]. To determine the steady state level of the Coq8-K216A polypeptide, mitochondria from yeast expressing Coq8p-K216A were purified and steady state polypeptide levels were determined by Western blot. Polypeptide levels of Coq8p-K216A when expressed from a low copy plasmid are diminished relative to wild-type or the null mutant expressing wild-type Coq8 (W303ΔABC1:p3HN4) (Fig. 3), and steady state levels of Coq4p are also decreased. Thus, it is not possible to discern whether low Coq4p levels are due to the absence of kinase activity, or to the low level of the Coq8p-K216A.

Previous studies have suggested that Q or a Q biosynthetic intermediate may be necessary for Coq polypeptide complex stability [52,53]. For example, addition of Q₆ to growth media has been shown to increase steady state levels of Coq3 and Coq4p polypeptides in *coq1* and *coq7* null mutants [52,53], and addition of Q₆ to yeast *coq7* null mutant cultures restores assembly of a biosynthetic complex enabling synthesis of DMQ₆, the penultimate intermediate in Q biosynthesis [33]. Addition of Q₆ to cultures of *coq8* null mutants has

been shown to restore growth on media containing glycerol or ethanol as the sole nonfermentable carbon source [22]. However, addition of Q₆ to cultures of W303ΔABC1:pK216A yeast did not rescue steady state Coq8 polypeptide levels. Similarly, addition of Q₆ did not restore the Q-biosynthetic complex in *coq8* null mutants [34]. Antibodies against the β subunit of the F₁-ATPase and the Complex III Rieske iron-sulfur complex, Rip1, were used to verify equivalent sample loading, and indicate that disruption of the Coq-polypeptide complex has no effect on steady state levels of representative components in mitochondrial respiratory complexes. In summary, the very low steady state levels of the Coq8-K216A polypeptide produces the same limitations as the *coq8* null mutant; thus, we sought to identify *coq8* mutants that retained normal steady state levels of Coq8p.

3.4 Characterization of yeast *coq8* point mutants with normal steady state level of Coq8p

Eight different mutants in the G75/*coq8* complementation group [54] whose *coq8* gene defects had not previously been characterized were studied, with the goal of identifying mutants that retained steady state levels of Coq8p. These mutants were obtained via mutagenesis of the parental wild-type strain D273-10B/A1 [55,56]. Each of the original *coq8* mutants within this collection of strains (Table 1) was shown to be defective for growth on ethanol and/or glycerol as a carbon source, indicating a defect in respiration. The respiratory deficiency stems from recessive mutations in a nuclear gene, as they were complemented by a ρ^0 strain with a normal complement of nuclear genes but lacking mitochondrial DNA (Table 1). Each strain was cultured and subjected to analysis by Western blot. Seven of the mutants expressed Coq8p polypeptide in amounts comparable to wild type, W303-1A (Fig. 4). The *coq8* loci in each of these seven mutants were amplified by PCR and sequence analysis revealed that each mutant harbored a unique nucleotide substitution mutation resulting in amino acid substitution (Table 3). Four of the mutations affect residues within known kinase motifs (*coq8-3*, A197V in motif I; *coq8-6*, D229N in motif III; and *coq8-8*, D346N and *coq8-4*, N348Y, both in motif VIB; Fig. 5). The remaining three mutations result in glycine to aspartate substitution and are present in regions of unknown function (*coq8-2*, G89D; *coq8-5*, G130D; and *coq8-7*, G475D; Fig. 5).

Derivatives of each of these seven *coq8* point mutant yeast strains were prepared by mating to W303-1A, sporulation, and dissection of tetrads to yield respiratory defective mutants with designated auxotrophies (Table 1). Each of the *coq8* mutant strains were rescued for growth on glycerol-medium following transformation with p3HN4 (low copy yeast *COQ8*), while transformation with pRS426 (multicopy yeast empty vector) failed to rescue growth. Two of the *coq8* point mutants were chosen for further characterization: the W222 point mutant (*coq8-5*; G130D) was chosen because it mirrors a human ADCK3 mutation (G272D) present in a conserved region of unknown function and that is associated with disease and a lower level of Q₁₀ content in skeletal muscle [26], and the W182 *coq8* point mutant (*coq8-3*; A197V) was selected because it is located within conserved kinase motif I, and we anticipated that it would disrupt kinase activity of Coq8p. The rescue of W183 and W222, harboring the respective *coq8-3* or *coq8-5* allele, is shown (Fig. 6). To determine the effect of the mutations on Q₆ content, lipids were extracted from wild-type and mutant yeast, and Q₆ levels were measured with a QT4000 mass spectrometer coupled in-line with reversed phase HPLC. Q₆ was not detected in W303ΔCOQ8, W183, or W222 harboring empty vector, pRS426. In contrast, Q₆ levels were restored in W303ΔCOQ8, W183, and W222 mutant yeast harboring the p3HN4 plasmid (low copy yeast *ABC1/COQ8*) (Fig. 7). The rescue is less efficient in the point mutants (W183 and W222) than in the *coq8* null mutant, perhaps due to interference by the mutant Coq8 polypeptide.

3.5 Expression of the human ADCK3 polypeptide rescues yeast *coq8* mutants

A human homolog *ADCK3* shows 38% amino acid sequence identity with *S. cerevisiae* Coq8p. However, the human *ADCK3* gene has not been shown to rescue the yeast *coq8* mutant. To determine if *ADCK3* might be an ortholog of yeast *COQ8*, its corresponding cDNA was subcloned into yeast expression vectors. Prior studies showed that rescue of yeast *coq* mutants by heterologous *COQ* genes required expression from multicopy vectors [46,57]. In other cases, rescue by an *E. coli* homolog of Coq5p was observed for certain *coq5* point mutants but not *coq5* null mutants [58]. Neither low copy nor high copy plasmids expressing *ADCK3* from the yeast *CYC1* promoter (Table 2) were found to rescue yeast *coq8* mutants [59]. It seemed possible that the human ADCK3 polypeptide may not be efficiently transported to the yeast mitochondrial matrix. To remedy this potential problem, we then tested plasmids expressing *ADCK3* ORF with an amino-terminal yeast mitochondrial leader sequence (plcADCK3 and pmcADCK3). The resulting plasmids were transformed into a *coq8* null mutant (W303ΔABC1) as well as yeast strains containing the *coq8-3* (W183) and *coq8-5* (W222) mutant alleles. To test rescue, yeast *coq8* mutants harboring ADCK3 were plated on medium containing the non-fermentable carbon source, glycerol. Each of the *coq8* mutants tested were rescued by pmcADCK3, and rescue was also observed after 10 days with plcADCK3 (Fig. 6). The pmcADCK3 plasmid partially restored Q₆ content in yeast lipid extracts from transformed yeast (Fig. 7). Yeast *abc1/coq8* mutants transformed with pmcADCK3 contained a small amount of Q₆, while the same mutants transformed with the empty vector (pRS426) contained no detectable Q₆.

The long incubation time needed to observe the rescue of the W222 *coq8* mutant by low copy ADCK3, and the *coq8* null mutant by multicopy ADCK3 probably reflects the time necessary to accumulate a sufficient amount of Q₆. Yeast growth is observed in the spot containing only 20,000 cells (2 μl of yeast cells at A_{600nm} = 1.0 or 1×10⁷ cells/ml). Hence, it seems unlikely that spontaneous secondary mutations could account for the rescue on YPG, since they would not be expected to arise at such a high frequency. While there does not seem to be a strong correlation between the rescued growth in Fig. 6 and the Q₆ content shown in Fig. 7, it seems likely that the content of Q₆ as measured in YPGal liquid medium (Fig. 7) may not be indicative of the Q₆ content in cells tested for growth on YPGlycerol. In fact, yeast *coq7* mutants rescued with a heterologous gene and cultured on YPGal have been shown to have lower Q₆ content as compared to the same cells cultured on YPG [53]. In summary, human ADCK3 is an ortholog of yeast Coq8p, since its expression rescues both growth on nonfermentable carbon source and Q₆ biosynthesis in yeast *coq8* mutants.

3.6 Coq8p is required for steady state levels of other Coq polypeptides

Previous studies have investigated the effects of deletions in the *COQ* genes on the steady state protein levels of Coq1p-Coq10p [18]. To examine whether *coq8* point mutants destabilize other Coq polypeptides, mitochondria isolated from W183, W222, W303-1A (WT), and W303ΔABC1 were analyzed on immunoblots with antisera against the Coq1 – Coq9 proteins (Fig. 8). Mitochondria from W183 and W222 contained steady state levels of Coq8p, which was absent in W303ΔABC1. Steady state levels of Coq1p, Coq2p, Coq3p, Coq5p, and Coq6p were observed in each of the *coq8* mutant strains. As reported by Tauche et al., [34], we observed that the presence of phosphatase inhibitors during mitochondria isolation preserved the steady state levels of Coq3p. Interestingly, W183 contained steady state levels of Coq4p and Coq7p, which were either dramatically decreased or absent in W222 and W303ΔCOQ8. All three *coq8* mutant strains had dramatically decreased levels of Coq9p compared to wild type. Expression of human *ADCK3* was able to restore steady state level of Coq4p in W222, and steady state level of Coq7p in both W222 and W303ΔCOQ8 strains. However, Coq9p level in the *ADCK3* transformed yeast remained significantly decreased compared to wild-type yeast.

3.7 Coq3p, Coq5p, and Coq7p are putative substrates of Coq8p

Phosphorylation of Coq3p has been shown to depend on the presence of Coq8p [34]. 2D-IEF/SDS-PAGE was performed to address whether other Coq polypeptides might also serve as substrates for Coq8p. Purified mitochondria from wild-type W303-1A and from *coq8* mutants (W183, W222, and W303ΔABC8) were subjected to 2D-IEF/SDS-PAGE as described in Materials and Methods. Whereas previous studies made use of a tagged version of Coq3p [34], we utilized antibodies to Coq3p to detect the polypeptide in its natural state (Fig. 9). The detection of Coq3p revealed two spots in wild type in the pH range of 5–6, which is consistent with the predicted isoelectric point of 5.44 for Coq3p (Table 1S). The leftward, more acidic spot was no longer present in the phosphatase-treated wild-type mitochondria, identifying it as a phosphorylated isoform of Coq3p. These studies confirm the observation that W303ΔABC1 contained only the unphosphorylated isoform of Coq3p [34], and allowed us to use Coq3p as an internal marker in these analyses. Interestingly, W183 does contain a small fraction of phosphorylated Coq3p, suggesting this point mutation did not completely eliminate the kinase activity of Coq8p. On the other hand, W222 contained only the unphosphorylated isoform of Coq3p, with migration patterns similar to the *coq8* null mutant. Expression of human ADCK3 was able to restore the phosphorylation state of Coq3p in both *coq8* null mutant and W222 point mutant, although to different degrees.

Detection of Coq5p and Coq7p with antibodies to these polypeptides revealed multiple spots for each (Fig. 10). For Coq5p, wild-type mitochondria contained at least four spots in the pH range of 5 to 6, consistent with the predicted isoelectric point of Coq5p (Table 1S). The two most acidic spots disappeared in wild-type mitochondria upon phosphatase treatment, indicating those two spots represent two different phosphorylation states of Coq5p. Interestingly, W183, W222, and W303ΔABC1 contained three spots, lacking the most acidic spot observed in wild type, indicating that Coq8p is responsible for the most acidic phosphorylated isoforms of Coq5p. This result suggests there may be another kinase working together with Coq8p on Coq5p, which is responsible for some Coq5p phosphorylation. The phosphorylation pattern of Coq5p is restored to wild type when ADCK3 was expressed in the point mutants W222 and W183. For Coq7p, three spots were observed in wild-type mitochondria in the pH range of 7 to 8, consistent with the predicted isoelectric point of Coq7p (Table 1S). The left two spots disappeared upon phosphatase treatment, indicating those two spots are phosphorylated isoforms of Coq7p. Coq7p is not detected in the *coq8* null mutant (Fig. 10) since it is destabilized in the W303ΔABC1 strain (Fig. 8). In contrast, the W183 point mutant contained two spots, lacking the most acidic spot as compared to the wild type. However, it is difficult to discern whether a distinct kinase acts on Coq7p, or whether the A197V point mutation in Coq8p of W183 fails to completely eliminate the kinase activity. As shown in Fig. 8, W222 mitochondria do not contain stable levels of Coq7p. Expression of human ADCK3 is able to restore the phosphorylation pattern of Coq7p in both *coq8* point mutants, but only the steady-state level of Coq7p is restored in *coq8* null mutant (Figs. 8 and 10).

4. Discussion

S. cerevisiae Coq8 polypeptide, and its human homologue ADCK3, have been shown to be required for biosynthesis of Q [22,26,27]. Yeast Coq8p is known to be required for the phosphorylation of the yeast Coq3 polypeptide, and this phosphorylation is important for the stability or formation of a multisubunit Coq polypeptide Q-biosynthetic complex [34]. In this work we have identified and utilized *coq8* mutants that retain normal steady state levels of the Coq8 polypeptide and determined the effects of Coq8 point mutations on Q₆ content, Coq polypeptide steady state levels, and the phosphorylated states of Coq3p, Coq5p, and Coq7p. The results suggest a profound functional conservation of kinase activity of human

ADCK3, which when expressed in yeast *coq8* mutant strains, rescues both Q₆ synthesis and phosphorylation of yeast Coq3, Coq5 and Coq7 polypeptide substrates.

Initially, we introduced a K216A substitution within kinase motif II of Coq8p, because the corresponding mutation of this residue in other kinases resulted in correctly folded but kinase-inactive enzymes [51]. However, levels of the K216A-Coq8 polypeptide were profoundly decreased as compared to wild-type Coq8p. Consequently, steady state levels of the other Coq polypeptides were also decreased (for example Coq4p), as observed previously in *coq8* null mutants [18]. For this reason we screened a collection of yeast *coq8* mutants [54], with the goal of identifying *coq8* mutant strains that retained normal steady state levels of the Coq8 polypeptide.

Seven distinct *coq8* missense mutations were characterized in yeast mutants that produced a wild-type steady state level of Coq8p. Four of the seven yeast *coq8* mutations affect residues that reside within known kinase subdomains (Fig. 5). A197V is located in subdomain I, which surrounds the bound ATP [51]. This subdomain in the majority of eukaryotic protein kinases contains the amino acid sequence G-x-G-x-x-G, present in many nucleotide-binding proteins. This motif helps form a β -strand-turn- β -strand structure that holds the α and β phosphate groups of ATP [51]. In Coq8p, this motif contains two alanine residues in place of the first two glycine residues (A-A-A-S-I-G). While the A197V (underlined) might cause steric hindrance and interfere with binding of phosphate moieties, our results indicate that the yeast W183 (*coq8-3*) mutant harboring A197V-Coq8p retained some kinase activity. Another mutation affected the aspartate in subdomain III (D229N), believed to help stabilize interactions between the invariant lysine of subdomain II and the phosphate groups of ATP [60,61]. Disruption of the negative charge at that position would directly affect its ability to form a salt-bridge with the lysine. Two other mutations, D346N and N348Y, affected residues with kinase subdomain VIB, identified by the D-x-x-x-x-N motif that forms a catalytic loop. The invariant D residue of this loop is believed to be the catalytic base that accepts the proton from the amino acid substrate hydroxyl [51]. Thus D346N-Coq8p would be expected to slow or prevent phosphorylation. Distinct classes of eukaryotic protein kinases uniformly retain the invariant terminal D and N residues of motif VIB, but show variations of the “x” residues that mediate peptide substrate recognition [62-64]. For example, many serine and threonine kinases typically contain the sequence D-L-K-P-E-N, while tyrosine kinases contain D-L-R-A-A-N or D-L-A-A-R-N [65]. The sequence D-P-N-W-A-N constitutes another distinct motif that is highly conserved among the Coq8/ADCK3 homologues (Fig. 5). The yeast *coq8* mutation resulting in N348Y (underlined) would likely disrupt the structure of this catalytic loop.

Three *coq8* missense mutations (G89D, G130D, and G475D) are present in regions of unknown function. Both G89D and G130D are located near or in conserved regions. Patients harboring ADCK3 mutations resulting in R213W, G272V or G272D (corresponding to R77 and G130 in yeast Coq8, respectively) had deficiencies in muscle Q₁₀ content [26]. Introduction of the human mutations into the corresponding positions of yeast Coq8 (R77WCoq8, G130VCoq8, or G130DCoq8) and expression in yeast *coq8* null mutants, was shown to result in greatly decreased or absent Q₆ content, respectively [26]. We confirm that the corresponding yeast *coq8* mutant harboring G130D-Coq8p lacked Q₆ and further determined that it failed to phosphorylate Coq3p and Coq7p, and was deficient in phosphorylation of Coq5p.

The mutation in *coq8-7* (G475D) is near the sequence P-P-P-E-E-T-Y-S-L-H-R-K-x-x-G, identifying a remarkably conserved region among the Coq8 and human ADCK3 and ADCK4 homologues (Figure 5). A BLAST search with this sequence identifies a similar segment in the *Bacillus subtilis* protein, RsbU. This sequence is well conserved near the

amino-terminus of RsbU homologues (Fig. 12). In crystal structures of RsbU, this region forms an α -helix and several conserved residues (including P44 and E45 in *B. subtilis*) are required for interaction with a neighboring α -helix [66]. This interaction stabilizes the RsbU polypeptide (a phosphatase), allowing it to form a homodimer necessary for binding of RsbU to RsbT (a kinase). The RsbU/RsbT interaction (termed partner switching) is controlled by serine phosphorylation and mediates stress response to environmental and nutritional signals in *B. subtilis*, and contributes to pathogenicity and enhanced intracellular growth of *Staphylococcus aureus* and *Listeria monocytogenes* [67,68]. In Coq8p, this motif is located near the carboxyl-terminus. It is conceivable that in yeast Coq8 and human ADCK3, this region mediates a similar protein-protein interaction required for Q biosynthesis. Tauche *et al.* [34] determined that yeast Coq8p forms homomeric complexes. In fact, the rescue mediated by expression of wild-type yeast *COQ8* was much less robust in yeast *coq8* point mutants as compared to the *coq8* null mutant (Fig. 7), suggesting that dysfunctional Coq8p present in the point mutants may interfere with functional interaction(s) of wild-type Coq8p, either with itself or with partner polypeptides.

Tauche *et al.* [34] discovered that Coq3p is a putative phosphorylation substrate of Coq8p using a tagged version of Coq3p. Here, we utilized antibodies to the Coq3 polypeptide to confirm this result, and extended the studies by examining Coq polypeptide steady state levels and phosphorylation status in wild-type, *coq8* null and two of the *coq8* point mutants that retained high steady state levels of the Coq8 polypeptide. The *coq8* null mutant and W222 (*coq8*-5; G130D) were found to possess only the unphosphorylated isoform of Coq3p. However, W183 (*coq8*-3; A197V) retained phosphorylated Coq3p, suggesting that the A197V mutation did not eliminate the kinase activity of Coq8p, and allowed phosphorylation of Coq3p. W183 retained high steady state levels of Coq1 through Coq8 polypeptides, although levels of Coq9p were decreased relative to wild type. In contrast, W222 had dramatically lower levels of Coq4p and lacked Coq7p and Coq9p, a phenotype similar to the *coq8* null mutant. One interpretation is that phosphorylated forms of Coq3p are required to preserve stability of Coq4, Coq7 and Coq9 polypeptides. In contrast, the W222 mutant lacked phosphorylated forms of Coq3p, contributing to profound destabilization of Coq4, Coq7 and Coq9 polypeptides. Alternatively, the mutation in W183 that allows for detectable steady state levels of Coq9p may in turn stabilize the other Coq polypeptides.

Coq7p and Coq5p were also found to be substrates of Coq8p. Wild-type yeast and the W183 *coq8*-3 mutant each contained normal steady state levels of Coq7p, and W183 retained one of the two phosphorylated Coq7 isoforms. In contrast, the *coq8* null and W222 mutant both lacked steady state levels of Coq7p. We propose that the steady state level of Coq7p depends on Coq8 kinase activity, and that phosphorylation of Coq7p prevented Coq7p from destabilization. Coq7 has been previously identified as a regulated step in Q biosynthesis [33]. We further demonstrated that Coq5p also contained multiple phosphorylated isoforms. However, not all of the phosphorylation states of Coq5p were Coq8p dependent. Both the *coq8* null mutant and the W222 *coq8* point mutant lacked only the most acidic Coq5 spot in the 2D-IEF analyses. Three of the four Coq5 spots observed in wild-type mitochondria disappeared upon phosphatase treatment, suggesting that there is another kinase (or kinases) acting on Coq5p. Whether these phosphorylated isoforms of Coq5 are essential for Q synthesis still remains a question.

In these analyses we were not able to resolve phosphorylation states of several of the Coq polypeptides. Coq4p did not appear to be phosphorylated, since it migrated as one spot, and showed similar mobility with or without phosphatase treatment (data not shown). Coq2, Coq6, Coq8, Coq9 and Coq10 polypeptides were not detectable on 2D-IEF gels, even though they can be readily detected in isolated mitochondria by Western blot of SDS-PAGE

gels. Although multiple spots of Coq1p were detected on 2D-IEF gels, no signal was detected following phosphatase treatment, thus the phosphorylation state of Coq1p is ambiguous (data not shown).

Steady state levels of Coq9p mirrored those of Coq7p, and thus it is tempting to speculate that Coq8 may also phosphorylate Coq9p. In fact, Coq9p has been detected in a yeast phosphoproteome analysis and is phosphorylated at T34 [69]. Currently, this is the only reported phosphorylation site in any yeast Coq polypeptide [70]. The amino terminal sequence of Coq9 residue is not conserved in metazoan Coq9 sequences, and it is interesting that T34 may be located within the predicted mitochondrial leader sequence of yeast Coq9p (Table 1S). Overexpression of *COQ8* is known to suppress a nonsense (Q₁₅₁STOP) *coq9* mutant [18], and was attributed to a detectable presence of Coq9p due to read-through. Overexpression of *COQ8* restores the steady state level of Coq4p in several *coq* null mutants, but not in the *coq9* null mutant [71]. While experiments have not revealed a complex between Coq8p and Coq9p [30], it is certainly possible that phosphorylation of Coq9p by Coq8p might be necessary to form a Q biosynthetic complex and so preserve the otherwise unstable Coq polypeptides known to interact with Coq9, including Coq4p, Coq6p, and Coq7p. It is likely that such Coq8-mediated phosphorylation of Coq polypeptides might function to regulate Q₆ synthesis, and thus also account for the *COQ8* multicopy suppression of the *coq10* mutant [31,71].

Expression of the Coq8 human homolog, ADCK3, rescued the growth of yeast *coq8* mutants on non-fermentable carbon source and enabled synthesis of Q₆. Although ADCK3 (CABC1) has been localized to mitochondria [72], the rescue observed here required the addition of an amino-terminal yeast mitochondrial leader sequence to ADCK3 (pmcADCK3). In the yeast W222 and *coq8* null mutants, pmcADCK3 rescued phosphorylation of Coq3p and Coq7p. The rescue afforded by pmcADCK3 was most dramatic in the W222 mutant because restoration of phosphorylated Coq3p and Coq7p coincided with significantly increased steady state levels of Coq7p, Coq4p, and Coq9p. ADCK3 also restored the phosphorylation state of Coq5p in W222. The effect of pmcADCK3 on the steady state level of Coq polypeptides was less significant in the *coq8* null mutant. We speculate that the presence of the Coq8-G130D polypeptide may facilitate the phosphorylation or stabilization of yeast Coq polypeptides by human ADCK3. It is important to note that detection of phosphorylation by 2D-IEF and Western blot can detect whether or not a protein is phosphorylated, but does not indicate whether the polypeptide is correctly phosphorylated. While the rescue observed is dramatic, growth is much slower and the content of Q₆ is much lower in the ADCK3 rescued mutants as compared to mutants rescued with yeast *COQ8*. ADCK3 shares 38% sequence identity with yeast Coq8p (Fig. 5), thus the activity of ADCK3 might not be ideal for the yeast substrates.

Patients harboring missense, deletion, or nonsense mutations in both copies of *ADCK3* seem to have a distinct and milder presentation of symptoms as compared to patients with defects in other genes necessary for Q biosynthesis [26,27,73]. For example patients with severe mutations resulting in nonsense mediated decay of *ADCK3* mRNA, develop progressive cerebellar ataxia, but indicate that ADCK3 itself is not essential for life [73]. In contrast, patients with *PDSS1*, *PDSS2*, *COQ2*, or *COQ9* mutations generally exhibit a severe multisystemic infantile form of Q deficiency, and develop renal disease [74]. In the clinical setting it is important to identify Q-deficiencies in patients as early as possible, since treatment with oral Q₁₀ can sometimes elicit dramatic improvement [74]. Partial rescue of kidney disease with oral Q₁₀ supplements has been observed in the *Pdss2* mouse model of kidney disease [75]. Yeast *coq8* null mutants are indistinguishable from the other yeast *coq* mutants (*coq1-coq7* and *coq9*); they lack Q, have severe respiratory deficiency, and exhibit similar sensitivity to oxidative stress [11,15]. Therefore, it seems likely that one or more of

the other human homologues of *ADCK3* must also function in Q biosynthesis, perhaps supplying a partial overlap of function. Consistent with this idea is that fibroblasts cultured from patients with defects in *ADCK3* have normal Q₁₀ content [26]. The best candidate appears to be *ADCK4*, since it shares highest sequence identity. However, the function of *ADCK4* in Q biosynthesis is not yet clear.

It is intriguing that plants contain numerous homologues of Coq8/*ADCK3*. *Arabidopsis thaliana ABC1A* is an ortholog of yeast Coq8p, and when expressed in yeast *coq8(abc1)* mutants, rescues the Q-deficient respiratory defect [76]. However, four distinct *A. thaliana* Coq8 homologues are present within plastoglobules, which are located within chloroplasts and appear to be important in lipid metabolism, including the synthesis and storage of other prenylated molecules such as plastoquinone, phylloquinone, and tocopherols [77]. It will be important to determine the function of plant Coq8(*Abc1*) homologues in the synthesis and regulation of these prenylated lipid molecules. Indeed, the stress sensitivity observed in *A. thaliana* strains harboring insertion mutations in the *AtOSA1* gene (a Coq8 homologue) is consistent with a defect in synthesis of one of the prenylated quinone antioxidant lipids [47].

In summary, there is a striking conservation of Q biosynthesis from yeast to humans [11,15]. This can now be extended to include the conserved function of yeast Coq8p and human *ADCK3*. Both yeast Coq8p and human *ADCK3* appear to function as kinases recognizing conserved Coq polypeptide substrates, including Coq3p, Coq7p, and Coq5p. This phosphorylation is key to the formation or maintenance of a Q multisubunit complex [34] and may modulate biosynthesis of Q.

Research Highlights of Xie LX et al

1. Yeast *coq8* mutants were identified that maintain normal steady state levels of Coq8p
2. *S. cerevisiae* Coq8 polypeptide is required for phosphorylation of Coq3, Coq5 and Coq7
3. The human Coq8 homolog *ADCK3* expressed in yeast *coq8* mutants rescues Q biosynthesis
4. Human *ADCK3* rescues phosphorylation of yeast Coq polypeptides in yeast *coq8* mutants
5. The results indicate a profound conservation of Coq8/*ADCK3* function in Q synthesis

Supplementary Material

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Fig. 1.

Coq8p is a peripheral membrane protein, associated with the mitochondrial inner membrane on the matrix side. **A.** Coq8p is peripherally associated with mitochondria membranes. Nycodenz purified mitochondria (M) were hypotonically swelled to generate the intermembrane space (IMS) fraction and mitoplasts. Mitoplasts were then treated with 0.1 M Na₂CO₃ pH 11.5, or sonicated. The treated mitoplasts were centrifuged and the supernatant (Sup) and pellet (Pel) fractions analyzed. **B.** Coq8p is located on the matrix side of the inner membrane. Mitochondria and mitoplasts were treated with Proteinase K with and without 1% Triton X-100. The β subunit of F₁-ATPase (F₁β) is a peripheral membrane protein [83], cytochrome *b*₂ (Cyt *b*₂) is located in the intermembrane space [84], cytochrome *c*₁ (Cyt *c*₁) is an integral membrane protein [85], malate dehydrogenase (Mdh1) [86], and Hsp60p [87], are localized to the matrix.

Fig. 2.

The Lysine216 in kinase subdomain II of Coq8 is required for rescue of glycerol growth in a yeast *coq8* null mutant. The yeast *coq8* null mutant (W303 Δ ABC1), was transformed with plasmids containing either wild-type *COQ8* or *coq8-K216A*. p3HN4 (*COQ8*), and pK216A (*coq8-K216A*) are low-copy vectors; p4HN4 (*COQ8*), p4K216A (*coq8-K216A*), pBT8-1 (*COQ8*) and pBT8KA (*coq8-K216A*) are multi-copy vectors. Yeast transformants were incubated 3 days on YPG plate media at 30 °C.

Fig. 3.

The yeast Coq8-K216A polypeptide is not present at normal steady state levels. Yeast were grown in 1 liter of YPGal medium with 0.1% dextrose and harvested at an OD_{600nm} of approximately 4. W303ΔABC1:pK216A + Q₆ was grown in YPGal + 0.1% Dextrose media supplemented with 1.2 μM Q₆. Purified mitochondria from W303-1A, W303ΔABC1 and W303ΔABC1 harboring the designated plasmids were subjected to Western blot analysis.

Fig. 4.

Seven yeast *coq8* mutant strains have normal steady state levels of the Coq8 polypeptide. The designated yeast strains were grown in YPD, lysed with 2% SDS and 425-600 μ m glass beads (Sigma). Aliquots of whole cell lysates (corresponding to 0.1 OD₆₀₀ of yeast cells) were separated by SDS-PAGE and then transferred to a PVDF membrane for western blot analyses with antibodies to Coq8p.

Fig. 5.

Amino acid alignment of Abc1/Coq8p homologues. An alignment of Abc1/Coq8p and its homologues was created using DNASTAR Megalign. Clustal method was used with PAM250 residue weight table. Homologues are *E. coli* (GenBank™ accession number P27854), *S. pombe* (GenBank™ accession number CAA62818), *A. thaliana* (GenBank™ accession number AAC72875), *C. elegans* (GenBank™ accession number AAA62560), *D. melanogaster* (GenBank™ accession number NP_572836) and humans (*ADCK1*, *ADCK2*, *ADCK3/CABC1*, *ADCK4* and *ADCK5*. GenBank™ accession numbers NP_065154, NP_443085, BAB91363, NP_079152 and NP_777582, respectively). *S. cerevisiae coq8* mutant alleles and their mutations identified in this study (Table 3) are indicated on the alignment by *arrows*. Mutations detected in CABC1/ADCK3 in human patients [26,27,73] are indicated with *triangles*. Kinase subdomains I, II, III, VIB, VII and VIII, the G-x-G-x-x-G glycine rich region and the D-x-x-x-x-N catalytic loop are indicated. The invariant lysine of subdomain II is designated with an asterisk. A highly conserved motif in Coq8p with remarkable sequence identity to a region in the *Bacillus subtilis* protein RsbU is indicated by the hatched bar (▨). Numbers on the left indicate amino acid position relative to the start codon. Identical residues are shaded.

Fig. 6.

Expression of the human ADCK3 polypeptide rescues the growth of yeast *coq8* mutants on non-fermentable carbon source media. Yeast *coq8* point mutants W183 and W222, and *coq8* null mutant (W303 Δ ABC1) were transformed with the designated plasmids: p3HN4, yeast low-copy *COQ8*; plcADCK3, low-copy ADCK3; pmcADCK3, multi-copy ADCK3; or pRS426, empty vector. Each strain was cultured overnight in SD-Ura selective media, the optical density (A_{600nm}) was adjusted to 1.0, and 2 μ l of 1:5 serial dilutions were spotted onto plate media, corresponding to a final A_{600nm} of 1.0, 0.2, 0.04, 0.008, 0.0016, or 0.00032. A, C, & E, Growth is depicted on SD-Ura plates after three days of incubation at 30°C; B, D, & F, YPG plates after 10 days of incubation at 30°C.

Fig. 7.

Expression of the human ADCK3 polypeptide partially restores Q₆ content in yeast *coq8* mutants. Yeast cells were cultured in YPGal + 0.1% dextrose and collected at an optical density ($A_{600\text{nm}}$) of about 1.0. The content of Q₆ in yeast cell lipid extracts was determined by HPLC/MS-MS as described in Experimental Procedures. Yeast *coq8* mutants were transformed with plasmids expressing yeast *ABC1/COQ8* (low-copy, p3HN4), human CABC1/ADCK3 (multi-copy, pmcADCK3), or empty vector control (multi-copy, pRS426). Each bar represents a total of four measurements from two independent samples each with two injections. Standard deviations are represented with error bars. Q₄ was used as internal standard.

Fig. 8.

Expression of the human ADCK3 polypeptide in yeast *coq8* mutants partially restores steady state levels of some of the yeast Coq polypeptides. Mitochondria were isolated from wild-type yeast (W303-1A) or from the indicated *coq8* mutant yeast strains with or without pmcADCK3 (a multi-copy plasmid with ADCK3). Isolated mitochondria (20 μg protein) from the designated yeast strains were separated by 10% SDS-PAGE and then transferred to a PVDF membrane for immunoblotting with antibodies to one of the Coq polypeptides (Coq1-Coq9), or to malate dehydrogenase (Mdh1), as a loading control.

Fig. 9.

Expression of the human ADCK3 polypeptide in yeast *coq8* mutants partially restores the phosphorylation state of the yeast Coq3 polypeptide. Isolated mitochondria (200 μ g protein) from wild-type yeast (W303-1A), or from the designated *coq8* mutants, W183, W222, or W303 Δ COQ8, either without or with pmcADCK3 (as indicated), were separated by two-dimensional electrophoresis, with isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension, followed by transfer to PVDF membranes for Western analysis. Where indicated, wild-type mitochondria were treated with phosphatase prior to rehydration and IEF. Coq3p was detected with anti-Coq3p antibody (1:1000 dilution). The two dashed lines indicate the positions of Coq3p isoforms. The pH range for IEF is indicated.

Fig. 10.

Expression of the human ADCK3 polypeptide in yeast *coq8* mutants partially restores the phosphorylation state of the yeast Coq5 and Coq7 polypeptides. Isolated mitochondria (200 μ g protein) from wild-type yeast (W303-1A), or from the designated *coq8* mutants, W183, W222, or W303 Δ COQ8, either without or with pmcADCK3 (as indicated), were analyzed as described in Fig 7. Where indicated, wild-type mitochondria were treated with phosphatase prior to rehydration and IEF. Coq5p and Coq7p were detected with the designated antibodies (1:1000 dilution). The dashed lines designate the alignment and relative positions of the Coq5p and Coq7p isoforms. The pH range for IEF is indicated.

Coq8 Sc	460	RLCPPPEETYS	SLHRKFSGI
Coq8 Ce	681	RLTSPPEEIYS	SLHRKLSFC
Adck3 Hs	600	RLVPPPEETYS	SLHRKMGGs
RsbU Bs	39	EHQIPPEEII	SIHRKVLKE
RsbU BI	39	EHQVPPEEII	SIHRKVLSE
RsbU Bh	40	EQKVSPEELV	SLHRTVLSD

Fig. 11.

The carboxyl terminal segment of Coq8 homologues and the amino terminal segment of RsbU homologues share a region of high identity. In RsbU the sequence designated by the black bar forms an alpha-helix, and side chains interact with a neighboring alpha-helix in a RsbU homodimer. Numbers indicate the amino acid position of the segment. The RsbU homologues shown are from *Bacillus subtilis* (GenBank accession number NP_388351), *Bacillus licheniformis* (GenBank accession number YP_077796), and *Bacillus halodurans* (GenBank accession number NP_241392). Shading indicates identical residues present in the majority of the sequences.

Table 1

Genotypes and Sources of *S. cerevisiae* Strains

Strain	Genotype	Source
W303-1A	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i>	R. Rothstein ^a
W303ΔABC1	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq8::HIS3</i>	[22]
FY251	MAT a <i>ura3-52 his3Δ200 leu2Δ1 trp1Δ63</i>	F. Winston ^b
FYΔABC1	MAT a <i>ura3-52 hisΔA200 leu2Δ1 trp1Δ63 coq8::TRP1</i>	[22]
CH130-A1	MAT a <i>his3-1,15 leu2-3,112 trp1-1 ura3-1 coq8-1</i>	[22]
CF130-2B	MAT a <i>his3Δ200 ura3-52 trp1Δ63 coq8-1</i>	C130 ^c × FY251
C177	MAT a <i>met6 coq8-2</i>	A. Tzagoloff ^c
W177-3B	MAT a <i>his3-1,15 leu2-3,112 met6 ura3-1 coq8-2</i>	C177 × W303-1A
C183	MAT a <i>met6 coq8-3</i>	A. Tzagoloff ^c
W183-2A	MAT a <i>his3-1,15 trp1-1 ura3-1 coq8-3</i>	C183 × W303-1A
C194	MAT a <i>met6 coq8-4</i>	A. Tzagoloff ^c
W194-3A	MAT a <i>ade2-1 his3-1,15 met6 ura3-1 coq8-4</i>	C194 × W303-1A
C222	MAT a <i>met6 coq8-5</i>	A. Tzagoloff ^c
W222-7D	MAT a <i>ade2-1 his3-1,15 leu2-3,112 ura3-1 coq8-5</i>	C222 × W303-1A
C240	MAT a <i>met6 coq8-6</i>	A. Tzagoloff ^c
W240-1A	MAT a <i>leu2-3,112 ura3-1 coq8-6</i>	C240 × W303-1A
C275	MAT a <i>met6 coq8-9</i>	A. Tzagoloff ^c
C308	MAT a <i>met6 coq8-7</i>	A. Tzagoloff ^c
W308-4C	MAT a <i>ade2-1 his3-1,15 met6 trp1-1 ura3-1 coq8-7</i>	C308 × W303-1A
C315	MAT a <i>met6 coq8-8</i>	A. Tzagoloff ^c
W315-4A	MAT a <i>trp1-1 ura3-1 coq8-8</i>	C315 × W303-1A
JM6	MAT a, <i>his4 ρ⁰</i>	[78]
JM8	MAT a <i>ade1 ρ⁰</i>	[78]

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Table 2

Plasmid constructs used in this study

Plasmid	Genes	Copy number	Source
pRS316	Yeast shuttle vector only	Low copy	[79]
pRS426	Yeast shuttle vector only	Multi copy	[80]
pAH01	Yeast vector with <i>CYC1</i> promoter	Low copy	[81]
pCH1	Yeast vector with <i>CYC1</i> promoter	Multi copy	[81]
pQM	pAH01 with <i>COQ3</i> mito leader	Low copy	[41]
pRCM	pCH1 with <i>COQ3</i> mito leader	Multi copy	Saiki, R. and Morvaridi, S.
p3HN4	Yeast <i>ABC1/COQ8</i>	Low copy	[82]
pK216A	Yeast <i>abc1/coq8-K216A</i>	Low copy	This work
p4HN4	Yeast <i>ABC1/COQ8</i>	Multi copy	[23]
p4K216A	Yeast <i>abc1/coq8-K216A</i>	Multi copy	This work
pBT8-1	Yeast <i>ABC1/COQ8</i> -biotin	Multi copy	This work
pBT8KA	Yeast <i>abc1/coq8-K216A</i> -biotin	Multi copy	This work
psCABC1	pAH01/Human <i>CABC1/ADCK3</i> with <i>CYC1</i> promoter	Low copy	This work
pmCABC1	pCH1/Human <i>CABC1/ADCK3</i> with <i>CYC1</i> promoter	Multi copy	This work
plcADCK3	pQM/Human <i>CABC1/ADCK3</i> with <i>CYC1</i> promoter and <i>COQ3</i> mito leader	Low copy	This work
pmcADCK3	pRCM/ Human <i>CABC1/ADCK3</i> with <i>CYC1</i> promoter and <i>COQ3</i> mito leader	Multi copy	This work

Table 3Amino Acid and Nucleotide Substitutions of *coq8* Alleles

Strain (<i>abc1/coq8</i> allele)	Amino acid substitution (nucleic acid mutation) ^a
C130 (<i>coq8-1</i>)	Not determined
C177 (<i>coq8-2</i>)	G89D (G266A)
C183 (<i>coq8-3</i>)	A197V (C590T)
C194 (<i>coq8-4</i>)	N348Y (A1042T)
C222 (<i>coq8-5</i>)	G130D (G389A)
C240 (<i>coq8-6</i>)	D229N (G685A)
C275 (<i>coq8-9</i>)	Not determined
C308 (<i>coq8-7</i>)	G475D (G1424A)
C315 (<i>coq8-8</i>)	D346N (G1036A)

^a Positions of mutations are relative to the ATG start codon of *ABC1/COQ8*.