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Coenzyme Q biosynthesis in Saccharomyces cerevisiae: characterization of a Coq polypeptide biosynthetic complex and a coenzyme Q binding protein

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Coenzyme Q biosynthesis in *Saccharomyces cerevisiae*: characterization of a Coq polypeptide biosynthetic complex and a coenzyme Q binding protein

A dissertation submitted in partial satisfaction of the requirements for

the degree Doctor of Philosophy

in Biochemistry and Molecular Biology

by

Christopher Michael Allan

2014

ABSTRACT OF THE DISSERTATION

Coenzyme Q biosynthesis in *Saccharomyces cerevisiae*: characterization of a Coq polypeptide biosynthetic complex and a coenzyme Q binding protein

by

Christopher Michael Allan

Doctor of Philosophy in Biochemistry and Molecular Biology

University of California, Los Angeles, 2014

Professor Catherine F. Clarke, Chair

Coenzyme Q (Q or ubiquinone) is a redox-active lipid consisting of a fully substituted benzoquinone ring and a lipophilic polyisoprenoid moiety which serves to anchor Q in biological membranes. Q is essential to mitochondrial electron transport where it accepts electrons from NADH or succinate at complexes I and II respectively, and donates them to cytochrome *c* at complex III. In the yeast *Saccharomyces cerevisiae* Q is synthesized by the products of eleven nuclear-encoded genes: *COQ1-COQ9, YAH1,* and *ARH1*. The product of an additional gene, *COQ10*, is not required for the biosynthesis of Q but is necessary for its function in efficient electron transport and respiration.

Chapter 2 investigates the role of Coq10p in both mitochondrial respiration and *de novo* Q synthesis through analysis of *coq10* null mutants and the Coq10p prokaryotic homolog CC1736. Expression of CC1736 harboring a mitochondrial leader sequence in yeast *coq10* mutants restored respiration and growth on a non-fermentable carbon source, and binding assays

demonstrated that CC1736 is capable of binding different isoforms of Q and a late-stage Q biosynthetic intermediate. Lipid analysis of the *coq10* null mutant revealed significantly decreased *de novo* Q synthesis when compared to wild type, especially at early-log phase, indicating Coq10p is required for efficient *de novo* biosynthesis of Q.

Chapter 3 describes characterization of a mitochondrial multi-subunit Coq polypeptide complex required for Q biosynthesis. The complex was purified by tandem affinity purification of particular dual-tagged Coq proteins and Western blotting analysis of purified eluates showed that the biosynthetic complex includes Coq3p, Coq4p, Coq5p, Coq6p, Coq7p, and Coq9p. In addition, Coq8p was observed to co-purify with tagged Coq6p but not other tagged Coq proteins. The purified eluates were also subject to proteomic analysis to identify potentially novel binding partners, identifying two proteins, Ilv6p and YLR290C. Lipid analysis of the corresponding null mutants revealed that the *ylr290c* mutant has significantly reduced *de novo* Q synthesis, while the *ilv6* mutant synthesizes wild-type levels of Q. Tandem affinity purification of tagged YLR290C demonstrated that it is associated with Coq4p, Coq5p, and Coq7p.

The Coq polypeptide biosynthetic complex and the Q-binding protein Coq10p are conserved features of Q biosynthesis in mammals, and thus findings in the yeast model will be relevant to our understanding of Q biosynthesis and function in humans. Chapter 4 details future directions to this end to both further characterize the Q biosynthetic complex and its regulatory implications, as well as the potential role of Coq10p in both Q biosynthesis and respiratory electron transport.

The dissertation for Christopher Michael Allan is approved.

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Acknowledgments

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Chapter 2 is a reprint of an article that appears in *Biochimica et Biophysica Acta* (2013) **1831**, 776-791 and I would like to thank Elsevier for permission to use this copyrighted material. I also want to thank Elsevier and my colleague Dr. Letian Xie for permission to reprint Appendix I, an article that appears in *Biochimica et Biophysica Acta* (2011) **1811**, 348-360. I finally want

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During graduate school I had the opportunity to grow not only as a research scientist but as an effective communicator of science through my experience as a teaching assistant. My ability to comprehend and relate biochemistry to others was shaped by both the amazing faculty for whom I worked as well as the undergraduate students I taught. I would especially like to recognize and thank my research mentor Dr. Catherine F. Clarke, Dr. Richard L. Weiss, Dr. James W. Gober, and Dr. Steven G. Clarke. The passion and skill with which they taught biochemistry inspired me to always give my students my best and to never stop learning.

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Publications and Presentations

Allan CM, Awad AM, Johnson JS, Shirasaki DI, Wang C, Loo JA, Clarke CF. "Characterization of the mitochondrial coenzyme Q biosynthetic complex in *Saccharomyces cerevisiae*" **In preparation**.

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He CH, Xie LX, **Allan CM**, Tran UC, Clarke CF. (2014) "Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast *coq* null mutants" *Biochim Biophys Acta*. Jan 7; **1841**(4): 630-644.

Allan CM, Hill S, Morvaridi S, Saiki R, Johnson JS, Liau WS, Hirano K, Kawashima T, Ji Z, Loo JA, Shepherd JN, Clarke CF. (2012) "A conserved START domain coenzyme Q-binding polypeptide is required for efficient Q biosynthesis, respiratory electron transport, and antioxidant function in *Saccharomyces cerevisiae*" *Biochim Biophys Acta*. Dec 25; **1831**(4): 776-791.

Xie LX, Hsieh EJ, Watanabe S, **Allan CM**, Chen JY, Tran UC, Clarke CF. (2011) "Expression of the human atypical kinase *ADCK3* rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants" *Biochim Biophys Acta*. May 1; **1811**(5): 348-360.

Johnson JS, **Allan CM**, Clarke CF. (2012) "Associated polypeptides found within yeast mitochondria for the biosynthesis of coenzyme Q" Poster presented at SACNAS National Conference. October 11 – 14. Seattle, WA.

Allan CM, Hill S, Morvaridi S, Saiki R, Liau WS, Hirano K, Kawashima T, Clarke CF. (2011) "Characterization of CC1736, a conserved START domain protein and putative coenzyme Q-binding protein" Poster presented at LIPID MAPS Annual Meeting. May 2 – 3. La Jolla, CA.

Allan CM, Tran UC, Gulmezian M, Marbois B, Clarke CF. (2010) "Characterization of a coenzyme Q biosynthetic complex in *Saccharomyces cerevisiae*" Poster presented at the International Coenzyme Q Conference. May 27 – 30. Brussels, Belgium.

Allan CM, Tran UC, Gulmezian M, Marbois B, Clarke CF. (2010) "Characterization of a coenzyme Q biosynthetic complex in *Saccharomyces cerevisiae*" Poster presented at the American Society for Biochemistry and Molecular Biology Annual Meeting. April 24 – 28. Anaheim, CA.

Xie LX, Hsieh EJ, Watanabe S, **Allan CM**, Tran UC, Clarke CF. (2010) "Expression of human *ADCK3* restores coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *abc1*/*coq8* mutants" Poster presented at the American Society for Biochemistry and Molecular Biology Annual Meeting. April 24 – 28. Anaheim, CA.

Allan CM, Tran UC, Gulmezian M, Marbois B, Clarke CF. (2009) Biochemical characterization of Coq2 and Coq4 proteins, components of a coenzyme Q biosynthetic complex in *Saccharomyces cerevisiae* mitochondria" Poster presented at FASEB Summer Research Conference. July 5 – 10. Carefree, AZ.

Chapter 1

Coenzyme Q Function and Biosynthesis

Structure and function of Q

Coenzyme Q (Q or ubiquinone) is small lipophilic molecule consisting of a hydrophobic polyisoprenoid moiety and a fully-substituted benzoquinone ring, more specifically 2,3-dimethoxy-5-methyl-6-polyprenyl-1,4-benoquinone. Q is found in all eukaryotes and in α-, β-, and γ-proteobacteria (Nowicka and Kruk, 2010). The length of the polyisoprenoid group is species specific and contains 6, 8, 9, and 10 isoprenyl units in *Saccharomyces cerevisiae*, *Escherichia coli*, mice, and humans respectively (Crane, 1965). This functionality localizes Q to biological membranes where it is freely diffusible in the plane of the phosopholipid bilayer. The benzoquinone ring of Q is redox-active and is capable of carrying up to two electrons. The fully oxidized ubiquinone form may accept one electron to form the relatively stable ubisemiquinone radical ($QH^{\cdot}$), and then a second electron to form the fully reduced ubiquinol species (QH_2). This reaction is reversible (Figure 1).

The primary function of Q is to serve as a two electron carrier in respiratory electron transport, where it accepts electrons from NADH at complex I or succinate at complex II and donates them to cytochrome *c* at complex III (Brandt and Trumpower, 1994). Electron transfer within complex III occurs via the Q cycle in which there are two separate Q binding sites, Q_P and Q_N, allowing for efficient proton translocation across the membrane (Brandt and Trumpower, 1994). In eukaryotes this occurs in the inner mitochondrial membrane while in prokaryotes it occurs in the plasma membrane. *S. cerevisiae* lacks complex I and instead uses the internal or external NADH:Q oxidoreductases on the inner mitochondrial membrane which unlike complex I do not facilitate the concomitant translocation of protons across the membrane and are rotenone-insensitive (Grandier-Vazeille *et al.*, 2001).

Q also functions as an electron carrier in other aspects of metabolism including dihydroorotate dehydrogenase (pyrimidine synthesis), fatty acyl-CoA dehydrogenase (fatty acid β-oxidation), and other dehydrogenases that oxidize various substrates including choline, sarcosine, sulfide, and glycerol-3-phosphate (Lenaz and De Santis, 1985; Hildebrandt and Grieshaber, 2008; Bentinger *et al.*, 2010). Q has also been suggested to function as a cofactor for uncoupling proteins, however this role is controversial due to the different experimental systems used to assay this function as well as the controversy regarding the function of fatty acids and nucleotides on uncoupling protein activity (Divakaruni and Brand, 2011). In facultative anaerobic bacteria such as *E. coli* Q and QH_2 serve an important function in the Arc two-component system which controls transcriptional regulation in response to various respiratory growth conditions through the ArcB sensor kinase and the ArcA response regulator (Georgellis *et al.*, 2001; Malpica *et al.*, 2006). ArcB contains a pair of cysteines whose oxidation state controls the kinase activity of the protein and is dependent on the redox poise of Q and QH_2, which is dependent on the oxygen content of the environment (Sharma *et al.*, 2013).

QH_2 has also been demonstrated to function as a lipid-soluble antioxidant capable of mitigating lipid peroxidation and regenerating alpha-tocopherol (vitamin E) (Frei *et al.*, 1990; Turunen *et al.*, 2004). Yeast mutants incapable of synthesizing QH_2 are sensitive to oxidative stress caused by treatment with polyunsaturated fatty acids (Do *et al.*, 1996), an effect alleviated by substitution of the fatty acid bis-allylic hydrogen atoms with deuterium atoms (Hill *et al.*, 2011). Conversely, Q has also been implicated as a source of oxidative stress via superoxide formation by reverse electron transport from complex II to complex I in which electrons from succinate are transported to NAD^+ (Lambert and Brand, 2004). Superoxide may also be

generated at complex III by the reaction of molecular oxygen with $Q^{\cdot-}$ produced during the Q cycle (Jezek and Hlavata, 2005).

Biosynthesis of Q

The polyisoprenoid group of Q is synthesized from the five carbon compounds isopentenyl diphosphate and dimethylallyl diphosphate, which are synthesized from mevalonate in eukaryotes and 1-deoxy-D-xylulose-5-phosphate in prokaryotes (Olson and Rudney, 1983; Pennock and Threlfall, 1983; Rohmer *et al.*, 1996). Plants are capable of synthesizing their isoprenoid compounds from either mevalonate or 1-deoxy-D-xylulose-5-phosphate in their cytosol or chloroplasts respectively (Lichtenthaler, 1999). The polyisoprenoid group is synthesized and elongated via head-to-tail condensation with the loss of diphosphate in each coupling (Wang and Ohnuma, 2000).

The benzoquinone ring of Q is synthesized from 4-hydroxybenzoic acid (4HB) which is synthesized from tyrosine in animals, chorismate in *E. coli*, and both tyrosine and chorismate in *S. cerevisiae*, although the steps by which 4HB is produced in *S. cerevisiae* remain uncharacterized (Olson, 1966; Goewert, 1981; Olson and Rudney, 1983; Pennock and Threlfall, 1983; Siebert *et al.*, 1994; Dosselaere and Vanderleyden, 2001). This ring is modified by a series of hydroxylation, decarboxylation, and methyltransferase reactions to yield the fully-substituted ring found in Q (Tran and Clarke, 2007). Alternatively, *S. cerevisiae* may use para-aminobenzoic acid (pABA) as the precursor for the benzoquinone ring (Marbois *et al.*, 2010; Pierrel *et al.*, 2010).

In *S. cerevisiae* Q synthesis is dependent on eleven known mitochondrial proteins: Coq1p-Coq9p, Yah1p, and Arh1p. *COQ1-COQ9* were identified as complementation groups of Q-deficient yeast mutants (Tzagoloff and Dieckmann, 1990; Johnson *et al.*, 2005), while *YAH1* and *ARH1* were identified through lipid analysis of Yah1p- and Arh1p-depleted strains under

control of galactose-inducible promoters (Pierrel *et al.*, 2010). Deletion of any of these *COQ* genes results in a loss of Q synthesis and consequently a failure to respire and grow on a non-fermentable carbon source (Tran and Clarke, 2007; Pierrel *et al.*, 2010). Deletion of either *YAH1* or *ARH1* results in a loss in viability as the gene products are also required for biosynthesis of iron-sulfur clusters and heme A (Manzella *et al.*, 1998; Barros and Nobrega, 1999; Lange *et al.*, 2000; Li *et al.*, 2001; Barros *et al.*, 2002). There are currently steps in the yeast Q biosynthetic pathway (Figure 2), such as the decarboxylation reaction and subsequent ring hydroxylation, for which no enzyme has yet been characterized.

COQ1 encodes hexaprenyl diphosphate synthase which assembles the all *trans*-polyisoprenoid precursor (Figure 2, compound **3**) using isopentenyl diphosphate (Figure 2, compound **1**) and one molecule of dimethylallyl diphosphate which serves as a primer (Figure 2, compound **2**) (Ashby and Edwards, 1990), however geranyl diphosphate and farnesyl diphosphate have been shown to serve as primers for hexaprenyl diphosphate as well (Casey and Threlfall, 1978). Subcellular fractionation studies have shown that Coq1p is peripherally associated with the matrix-face of the inner mitochondrial membrane (Gin and Clarke, 2005). Coq1p and related eukaryotic polyprenyl diphosphate synthases contain seven highly conserved sequence motifs (Wang and Ohnuma, 2000). The *coq1* null mutant can be rescued by expression of polyprenyl diphosphate synthases from other organisms and produces Q isoforms with varying polyprenyl tail lengths, demonstrating that the length of the polyisoprenoid tail is specified by the polyprenyl diphosphate synthase (Okada *et al.*, 1998). Several Coq1p homologs from other organisms such as IspB in *E. coli*, Pdss1/Pdss2 in mice, and Dps1/Dlp1 in *Schizosaccharomyces pombe* form oligomeric complexes, a homodimer and heterotetramers respectively, that are essential for enzyme activity (Kainou *et al.*, 2001; Saiki *et al.*, 2008).

Unlike its homologs Coq1p appears to function as a monomer, however when expressed in the fission yeast *S. pombe* it forms a functional heteromeric complex with Dps1 (Zhang *et al.*, 2008).

COQ2 encodes 4-hydroxybenzoic acid:polyprenyltransferase which ligates hexaprenyl diphosphate to the aromatic ring precursors 4HB or pABA, forming 3-hexaprenyl-4-hydroxybenozic acid (HHB; Figure 2, compound **4**) or 3-hexaprenyl-4-aminobenzoic acid (HAB; Figure 2, compound **4**) respectively (Ashby *et al.*, 1992; Marbois *et al.*, 2010; Pierrel *et al.*, 2010). Numbering of the ring throughout the text for Q intermediates is designated according to the rules and recommendations of the International Union of Pure and Applied Chemistry (IUPAC). Coq2p is not specific for a particular polyprenyl diphosphate and will synthesize products of varying polyisoprenoid lengths (Melzer and Heide, 1994). Coq2p was predicted to have six transmembrane domains and was shown to be an integral membrane protein in the inner mitochondrial membrane by subcellular fractionation (Ashby *et al.*, 1992; He *et al.*, 2014). Mutations in the *E. coli ubiA* gene, which encodes a homolog to Coq2p with 31% identity, can be rescued by expression of *S. cerevisiae COQ2*, indicating a conservation of function (Suzuki *et al.*, 1994). The crystal structure of the *Aeropyrum pernix* Coq2p homolog UbiA, which shares 52% sequence similarity to *E. coli* UbiA, was determined and shown to contain nine transmembrane domains (Cheng and Li, 2014). The *A. pernix* UbiA structure demonstrates that the polyprenyl binding pocket has an unrestricted opening to the membrane, accounting for the promiscuity of substrate binding (Cheng and Li, 2014). The Phyre structure prediction server models the tertiary structure of Coq2p with nine transmembrane helices as it uses the *A. pernix* UbiA structure as a template, however Coq2p only has 21% identity with UbiA and most secondary structure prediction programs predict six transmembrane domains for Coq2p (Kelley and Sternberg, 2009; UniProt, 2014). In human cells another prenyltransferase

was characterized, UBIAD1, which is responsible for Q synthesis in the Golgi membrane where it is required for redox signaling and protection from lipid peroxidation (Mugoni *et al.*, 2013).

COQ3 encodes an *O*-methyltransferase that catalyzes two separate steps in the biosynthetic pathway, accounting for both methoxy groups in Q (Figure 2, compound **6** and $\mathbf{Q_6H_2}$) (Hsu *et al.*, 1996; Shepherd *et al.*, 1996; Poon *et al.*, 1999). Coq3p and its *E. coli* homolog UbiG share 40% sequence identity and *coq3* mutants can be rescued by expression UbiG harboring an amino-terminal mitochondrial leader sequence (Clarke *et al.*, 1991; Hsu *et al.*, 1996). Coq3p, UbiG, and other Coq3p homologs contain four sequence motifs characteristic of Class I *S*-adenosylmethionine-dependent methyltransferases which form a seven-strand twisted β sheet responsible for *S*-adenosylmethionine binding (Kagan and Clarke, 1994; Niewmierzycka and Clarke, 1999; Katz *et al.*, 2003; Petrossian and Clarke, 2009). Coq3p is phosphorylated in a Coq8p-dependent manner, and expression of the Coq8p human homolog ADCK3 in *coq8* mutants is able to restore Coq3p phosphorylation (Tauche *et al.*, 2008; Xie *et al.*, 2011). Subcellular fractionation has demonstrated that Coq3p is peripherally associated with the matrix-face of the inner mitochondrial membrane (Poon *et al.*, 1999).

COQ4 encodes a protein of unknown function and does not have sequence homology to any proteins of known function, however the *coq4* null mutant lacks Q and accumulates an early Q biosynthetic intermediate, indicating a role in Q biosynthesis (Belogrudov *et al.*, 2001). Coq4p appears to contain a conserved putative zinc-binding motif HDxxH-$(x)_{11}$-E, however the function if any of this motif is not known (Marbois *et al.*, 2009). The crystal structure of Alr8543, a Coq4p homolog from *Nostoc sp. PCC7120*, was solved and shown to be a dimer with each monomer containing a bound geranylgeranyl monophosphate as well as a bound

magnesium ion in the putative zinc-binding motif, suggesting a polyisoprenoid binding function for Coq4p in Q biosynthesis (Rea *et al.*, 2010). Coq4p has been shown to be peripherally associated with the matrix-face of the inner mitochondrial membrane (Belogrudov *et al.*, 2001).

COQ5 encodes a *C*-methyltransferase and attaches a methyl group directly to the 5-position of the demethyldemethoxy-Q_6H_2 (Figure 2, compound **8**) using *S*-adenosylmethionine as the methyl donor (Barkovich *et al.*, 1997; Baba *et al.*, 2004). The crystal structure of Coq5p with and without bound *S*-adenosylmethionine was determined and was found to exist as a dimer in both states, with slight conformational changes in the active site in the substrate-bound form (Dai *et al.*, 2014). Coq5p shares 44% sequence identity with its *E. coli* homolog UbiE and both proteins contain four conserved motifs consistent with class I *S*-adenosylmethionine-dependent methyltransferases (Barkovich *et al.*, 1997; Katz *et al.*, 2003; Petrossian and Clarke, 2009). *E. coli ubiE* mutants can be rescued by expression of yeast Coq5p, however expression of UbiE (harboring a mitochondrial targeting sequence) fails to rescue yeast *coq5* null mutants; however yeast *coq5* point mutants that retain stable levels of the Coq5p polypeptide are rescued by *E. coli* UbiE (Dibrov *et al.*, 1997; Baba *et al.*, 2004). Like Coq3p, Coq5p is also phosphorylated in a Coq8p-dependent manner and phosphorylation of Coq5p can be restored in *coq8* point mutants by expression of ADCK3 (Xie *et al.*, 2011). Two dimensional-isoelectric focusing (2D-IEF) data suggests that there are multiple phosphorylation states of Coq5p and that another kinase may be required in tandem with Coq8p (Xie *et al.*, 2011). Subcellular fractionation has shown that Coq5p is peripherally associated with the matrix-face of the inner mitochondrial membrane (Baba *et al.*, 2004).

COQ6 encodes a protein with homology to *E. coli* proteins UbiH and UbiF with sequence identities of 21% and 24% respectively (Gin *et al.*, 2003). These proteins have consensus sequences for ADP, FAD, and NAD(P)H binding suggesting that they function as flavin-dependent monoxygenases (Wierenga *et al.*, 1986; Eggink *et al.*, 1990; Eppink *et al.*, 1997; Clarke, 2000). While UbiH catalyzes hydroxylation at the C4 ring position in 2-methoxy-6-octaprenylphenol (Figure 3, compound **7**) and UbiF at the C6 position of 6-demethoxy-Q_8 (DMQ_8) (Figure 3, compound **9**), Coq6p is involved in hydroxylation at the C5 position of HHB and HAB (Figure 2, compound **4**) (Nakahigashi *et al.*, 1992; Gin *et al.*, 2003; Ozeir *et al.*, 2011). *E. coli* UbiI, a flavin-dependent hydroxylase with 25% sequence identity to Coq6p, was found to catalyze hydroxylation at the C6 position of 2-octaprenylphenol (Figure 3, compound **5**), the equivalent position to C5 of HHB and HAB, and expression of *ubiI* was found to rescue a yeast *coq6* null in the presence of multicopy *COQ8* (Chehade *et al.*, 2013). The Coq6p-dependent hydroxylation reaction is dependent on Yah1p and Arh1p, ferredoxin and ferredoxin reductase respectively, which are postulated to be the source electrons for the monoxygenase reaction (Pierrel *et al.*, 2010; Ozeir *et al.*, 2011). Yeast *coq6* null mutants expressing enzymatically inactive but structurally stable human COQ6 were rescued for Q synthesis and respiratory growth by addition of exogenous vanillic acid (4-hydroxy-3-methoxybenzoic acid), which presumably bypasses the Coq6p-dependent hydroxylation step (Doimo *et al.*, 2014). Coq6p has been shown to be peripherally associated with the matrix-face of the inner mitochondrial membrane (Gin *et al.*, 2003).

COQ7 was originally identified as *CAT5* and was thought to be involved in regulating activation of gluconeogenic genes, but this was later shown to be a secondary phenotype in response to a general loss of respiration (Proft *et al.*, 1995; Jonassen *et al.*, 1998). The function

of Coq7p in Q biosynthesis was first characterized through the observation that a *coq7* point mutant accumulated the intermediate 6-demethoxy-Q_6 (DMQ_6, Figure 2, compound **9**), suggesting it catalyzes a hydroxylation reaction (Marbois and Clarke, 1996). Coq7p was identified as a member of a di-iron carboxylate family of proteins that catalyze hydroxylation reactions and does not have significant homology to *E. coli* UbiF, which is flavin-dependent and also catalyzes hydroxylation of DMQ_8 (Figure 3) (Marbois and Clarke, 1996; Stenmark *et al.*, 2001). Expression of *E. coli* UbiF harboring a mitochondrial leader sequence complemented the *coq7* null mutant as well as a DMQ_6-producing *coq7* point mutant (Tran *et al.*, 2006). Coq7p was shown to have two phosphorylated isoforms however it is not clear whether Coq7p phosphorylation is dependent solely on Coq8p activity (Xie *et al.*, 2011). Expression of ADCK3 restores Coq7p phosphorylation in *coq8* point mutants (Xie *et al.*, 2011). Coq7p was shown to be phosphorylated *in vitro* at serine 20, serine 28, and threonine 32, and mutagenesis studies of these residues suggest that phosphorylation acts as a regulatory mechanism in Q biosynthesis and that dephosphorylation by Ptc7p activates Q biosynthesis (Martin-Montalvo *et al.*, 2011; Martin-Montalvo *et al.*, 2013). Interestingly these putative phosphorylation sites occur near the amino-terminal leader sequence of Coq7p, whose predicted mature amino-terminus is at isoleucine 23 (Xie *et al.*, 2011). Subcellular fractionation has shown that Coq7p is peripherally associated with the matrix-face of the inner mitochondrial membrane (He *et al.*, 2014).

COQ8 was originally identified as *ABC1* (activity of bc_1 complex) in a screen for suppressors of defects in cytochrome *b* translation as it was able to partially rescue a *cbs2* mutant, a translational activator required for cytochrome *b* translation (Rodel *et al.*, 1986; Bousquet *et al.*, 1991). This suppressor effect was later shown to be due to a $tRNA^{TRP}$ located downstream of *ABC1*/*COQ8* and the *coq8* null mutant was shown to be defective in Q

biosynthesis (Do *et al.*, 2001; Hsieh *et al.*, 2004). Analysis of the Coq8p sequence identified protein kinase motifs I, II, III, VIB, VII, and VIII suggesting it may be a member of the atypical protein kinase family (Leonard *et al.*, 1998; Lagier-Tourenne *et al.*, 2008). Coq8p has not been demonstrated to directly catalyze phosphorylation but three proteins have been demonstrated to be phosphorylated in a Coq8p-dependent manner: Coq3p, Coq5p, and Coq7p (Tauche *et al.*, 2008; Xie *et al.*, 2011). Expression of the human Coq8p homolog ADCK3 (harboring a yeast mitochondrial leader sequence) in *coq8* point mutants was able to partially restore Q biosynthesis and phosphorylation of Coq3p, Coq5p, and Coq7p (Xie *et al.*, 2011). Subcellular localization has shown Coq8p is peripherally associated with the matrix-face of the inner mitochondrial membrane (Xie *et al.*, 2011). The *E. coli* Coq8p homolog UbiB also contains conserved kinase motifs and indirectly facilitates the UbiI hydroxylation step in the *E. coli* Q biosynthetic pathway (Figure 3) (Poon *et al.*, 2000; Chehade *et al.*, 2013).

The product of *COQ9* was identified as required for Q biosynthesis and while it has no sequence homology to any protein domains of known function the *coq9* null mutant accumulates HHB, indicating Coq9p functions downstream of Coq1p and Coq2p (Johnson *et al.*, 2005). Overexpression of *COQ8* in a *coq9* nonsense mutant with decreased steady-state levels of Coq9p increases the stability of the Coq9p polypeptide, restoring growth on a nonfermentable carbon source (Hsieh *et al.*, 2007). Subcellular fractionation has shown Coq9p to be a peripheral membrane protein on the matrix side of the inner mitochondrial membrane (Hsieh *et al.*, 2007). Coq9p homologs have been found in eukaryotes but not prokaryotes, indicating that it is specific for mitochondrial Q biosynthesis (Johnson *et al.*, 2005).

No yeast enzyme has yet been characterized that catalyzes the second hydroxylation step, however in *E. coli* this reaction is catalyzed by UbiH (Gin *et al.*, 2003). The decarboxylase step in the yeast Q biosynthetic pathway is also uncharacterized, however in *E. coli* both UbiD and UbiX have been identified as required for this step, particularly during logarithmic growth (Gulmezian *et al.*, 2007). The crystal structure of *Pseudomonas aeruginosa* UbiX showed that the protein contained a typical Rossmann fold and bound FMN (Kopec *et al.*, 2011). The structure of an *E. coli* paralog of UbiX with 51% identity, Pad1, was determined and also found to contain a typical Rossmann fold with a bound FMN (Rangarajan *et al.*, 2004). The crystal structure of *P. aeruginosa* PA0254, a homolog of *E. coli* UbiD with 25% identity, was determined and found to have a similar tertiary structure to an unpublished UbiD structure and contains a domain similar to the FMN-binding split barrel from a family of flavoproteins, however no evidence of a bound flavin was found (Jacewicz *et al.*, 2013). Expression of yeast *PAD1* in an *E. coli ubiX* mutant restores Q_8 synthesis and yeast *YDR539W*, which is adjacent to *PAD1* on chromosome 4, is a homolog of *E. coli ubiD* (Gulmezian *et al.*, 2007). In spite of this yeast mutants lacking either *PAD1* or *YDR539W*, or both *PAD1* and *YDR539W* produce wild-type levels of Q_6 (Gulmezian, 2006). Yeast *PAD1* and *YDR539W*, designated *FDC1* (ferulic acid decarboxylase), were shown to be essential for decarboxylation of phenylacrylic acids (Mukai *et al.*, 2010).

The Q biosynthetic complex

Genetic and biochemical experiments have demonstrated the interdependence of several of the Coq proteins and have shown the existence of a high molecular mass Coq protein complex. Each of the *coq3-coq9* null mutants accumulate only the early intermediates HHB and HAB resulting from prenylation of 4HB and pABA respectively (Xie *et al.*, 2012). Steady-state levels of Coq3p, Coq4p, Coq6p, Coq7p, and Coq9p are reduced in several *coq1-coq9* null mutants, although levels of Coq3p were stabilized in *coq4-coq9* mutant samples prepared with phosphatase inhibitors (Hsieh *et al.*, 2007; Tauche *et al.*, 2008; Xie *et al.*, 2012). This has been observed in other mitochondrial complexes such as the cytochrome bc_1 complex and ATP synthase in which the absence or mutation of one component protein results in reduced steady-state levels of the other subunits (Tzagoloff *et al.*, 1994; Glerum *et al.*, 1997). Gel filtration chromatography and blue native-polyacrylamide gel electrophoresis have (BN-PAGE) have shown that several of the Coq proteins exist in high molecular mass complexes and the *O*-methyltransferase activity of Coq3p can be detected in these complexes (Marbois *et al.*, 2005; Tran *et al.*, 2006; Hsieh *et al.*, 2007; Marbois *et al.*, 2009). Analysis of gel filtration fractions has also shown the association of DMQ_6 with the complexes (Marbois *et al.*, 2005). Supplementation of exogenous Q_6 was shown to stabilize the steady-state levels of Coq3p and Coq4p in the *coq7* null mutant (Tran *et al.*, 2006), enhance DMQ_6 production in the *coq7* null mutant (Padilla *et al.*, 2009), and stabilize steady-state levels of Coq9p in the *coq3*, *coq4*, *coq6*, *coq7* null mutants and steady-state levels of Coq4p in the *coq3*, *coq6*, and *coq7* null mutants (He *et al.*, 2014), suggesting that associated Q_6 plays a role in stability of the complex.

Co-precipitation experiments have demonstrated the physical association of several of the Coq proteins; biotinylated Coq3p was shown to co-precipitate Coq4p, and HA-tagged Coq9p co-purified Coq4p, Coqp5, Coq6p, and Coq7p (Marbois *et al.*, 2005; Hsieh *et al.*, 2007). Although Coq8p has not been shown to be associated in a complex with the other Coq proteins its putative role as a kinase is important for the stability of several other Coq proteins as overexpression of *COQ8* in various *coq* null mutants stabilizes several of the other Coq proteins and leads to the accumulation of later stage Q biosynthetic intermediates diagnostic of the mutated step (Padilla *et al.*, 2009; Xie *et al.*, 2012; He *et al.*, 2014).

A putative Q binding protein

In addition to the biosynthetic Coq1p-Coq9p proteins, the Coq10p protein was identified as necessary for Q activity in the electron transport chain but not for Q biosynthesis (Barros *et al.*, 2005). Coq10p is located on the matrix-face of the mitochondrial inner membrane and *S. cerevisiae coq10* null mutants produce endogenous Q_6 but have impaired mitochondrial respiration that can be rescued upon addition of exogenous Q_2 to isolated mitochondria (Barros *et al.*, 2005). *S. pombe coq10* null mutants also produce endogenous Q_{10} but have severely reduced respiration (Cui and Kawamukai, 2009). The primary sequence of Coq10p does not share homology with any proteins of known function and is classified as part of the aromatic-rich protein family, Pfam03654 (Punta *et al.*, 2012). Steady-state levels of Coq10p are not decreased in other *coq* null mutants and co-precipitation experiments have not demonstrated a physical interaction between Coq10p and the other Coq proteins, however sucrose gradient sedimentation suggests that Coq10p exists in an oligomeric form (Barros *et al.*, 2005; Hsieh *et al.*, 2007).

The structure of the *Caulobacter crescentus* Coq10p homolog CC1736 was determined via NMR and was found to be similar to steroidogenic acute regulatory-related lipid transfer (START) domain proteins (Shen *et al.*, 2005). Members of the START domain family consist of a seven-stranded β–sheet with a C-terminal α–helix that forms a hydrophobic pocket often used in the binding lipids such as cholesterol, polyketides, and phospholipids (Ponting and Aravind, 1999; Lo Conte *et al.*, 2000; Miller, 2007). Coq10p purified from *S. cerevisiae* was found to contain bound Q_6 in substoichiometric amounts (Barros *et al.*, 2005), while purified recombinant *S. pombe* Coq10p was found to bind Q_8 in equimolar amounts (Cui and Kawamukai, 2009). *E. coli* contains a CC1736 homolog encoded by *ratA* (also known as *yfjG*) (Allan *et al.*, 2012)

which does not appear to have a role in Q metabolism and function, but was shown to associate with the 50S ribosome to prevent formation of the 70S ribosome (Zhang and Inouye, 2011).

Recently the *ubiJ* gene has been characterized in *E. coli* and *Salmonella enterica* serovar Typhimurium in which it exists in an operonic structure between *ubiE* and *ubiB* (Aussel *et al.*, 2014). The product of *ubiJ* was shown to be required for aerobic Q biosynthesis in these organisms and is postulated to function as a carrier or chaperone for isoprenoid-containing intermediates due to the homology of its C-terminal domain with the C-terminal domain of *E. coli* LpxD, which caps the hydrophobic pocket responsible for binding acyl chains (Bartling and Raetz, 2009; Aussel *et al.*, 2014).

Conclusions

The redox properties of Q are key to its function as an essential electron carrier in the respiratory electron transport chain as well as to other redox functions in metabolism. Q is synthesized in *S. cerevisiae* through the function of eleven nuclear encoded genes, *COQ1-COQ9, YAH1, and ARH1*, which are all localized to mitochondria. The functions of some of the Coq proteins in Q biosynthesis remains poorly understood and there are steps in the Q biosynthetic pathway for which no enzyme has yet been characterized. There is an additional protein, Coq10p, which is not essential for Q biosynthesis but is required for the function of Q in mitochondrial respiration. The structure of a bacterial Coq10p homolog, CC1736, has been solved and has been shown to be a START domain protein, suggesting a role in lipid binding. The function of Coq10p as a Q-binding protein as well as its role in *de novo* Q biosynthesis will be discussed in Chapter 2 of this dissertation. Several of the biosynthetic Coq proteins have been shown to exist in one or more macromolecular complexes on the matrix side of the inner mitochondrial membrane, however the organization and composition of these complexes has not been fully characterized. Investigation of the Q biosynthetic complex by proteomic analysis of tandem affinity-tagged proteins as well as functional characterization of potentially novel members of this complex will be discussed in Chapter 3.

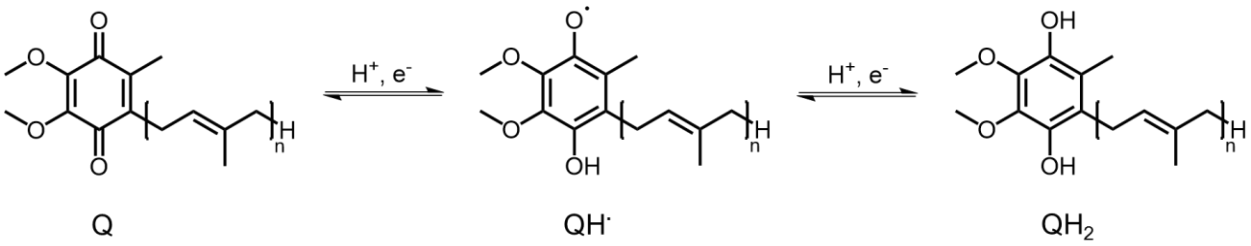

Figure 1. Structure and redox activity of Q. Fully oxidized Q, shown on the left can sequentially accept two electrons and two protons to form the radical semiquinone ($QH^{\cdot}$), middle, and the fully reduced ubiquinol (QH_2), shown on the right. This is a reversible process. 'n' denotes the number of isoprene units and is species-specific.

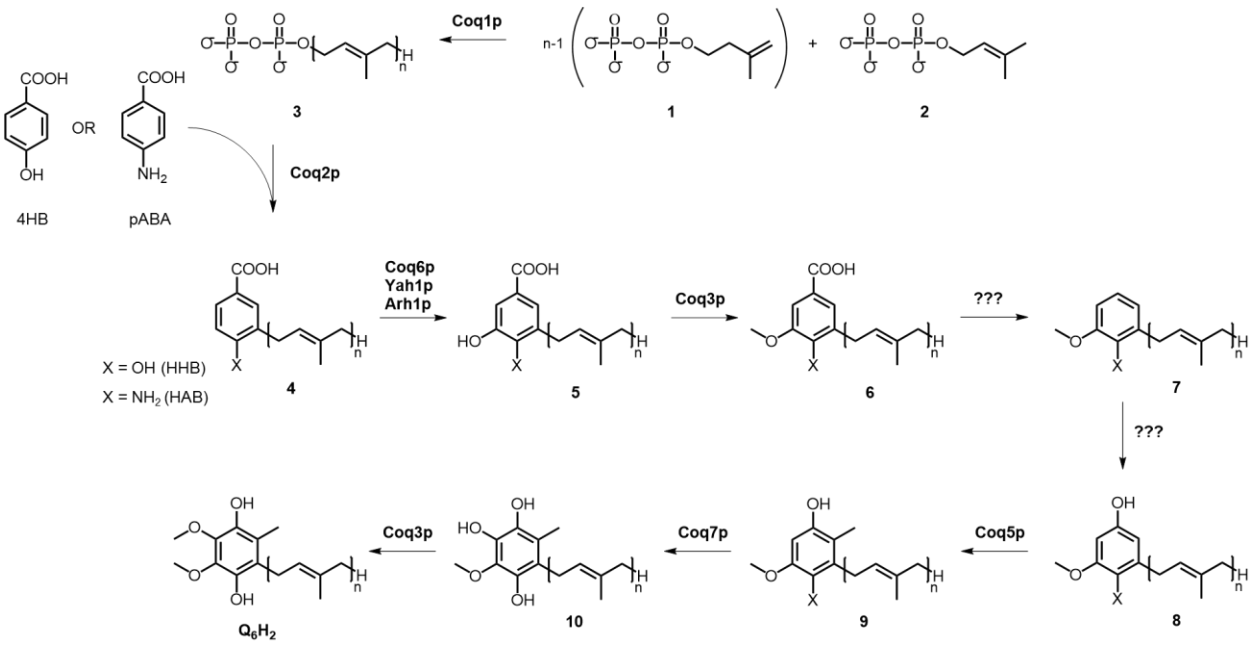

Figure 2. Q biosynthetic pathway in *Saccharomyces cerevisiae*. Coq1p catalyzes condensation of isopentenyl diphosphate (**1**) and dimethylallyl diphosphate (**2**), forming hexaprenyl diphosphate (**3**). Coq2p attaches hexaprenyl diphosphate to either 4HB or pABA, forming HHB and HAB respectively (**4**). Coq6p, facilitated by Yah1p and Arh1p, catalyzes a hydroxylation reaction producing either 5-hexaprenyl-3,4-dihydroxybenzoic acid or 4-amino-5-hexaprenyl-3-hydoxybenzoic acid (**5**). Coq3p then catalyzes an *O*-methyltransferase reaction, yielding either 5-hexaprenyl-4-hydroxy-3-methoxybenzoic acid or 4-amino-5-hexaprenyl-3-methoxybenzoic acid (**6**). Two consecutive steps facilitated by unknown enzymes, denoted by '???', catalyze consecutive decarboxylation and hydroxylation reactions, producing demethyldemethoxy-Q_6 or aminodemethyldemethoxy-Q_6 (**8**). Coq5p catalyzes a *C*-methyltransferase reaction to produce DMQ_6 or amino-DMQ_6 (**9**). Coq7p catalyzes the final hydroxylation step to produce demethyl-Q_6 (**10**), and Coq3p catalyzes a second *O*-methyltransferase reaction to produce $\mathbf{Q_6H_2}$. The amino group derived from pABA can take the place of the hydroxyl group, denoted as 'X', in several of the intermediates and it is not clear at

which point(s) it is removed. Ring positions on all compounds are numbered according to IUPAC convention.

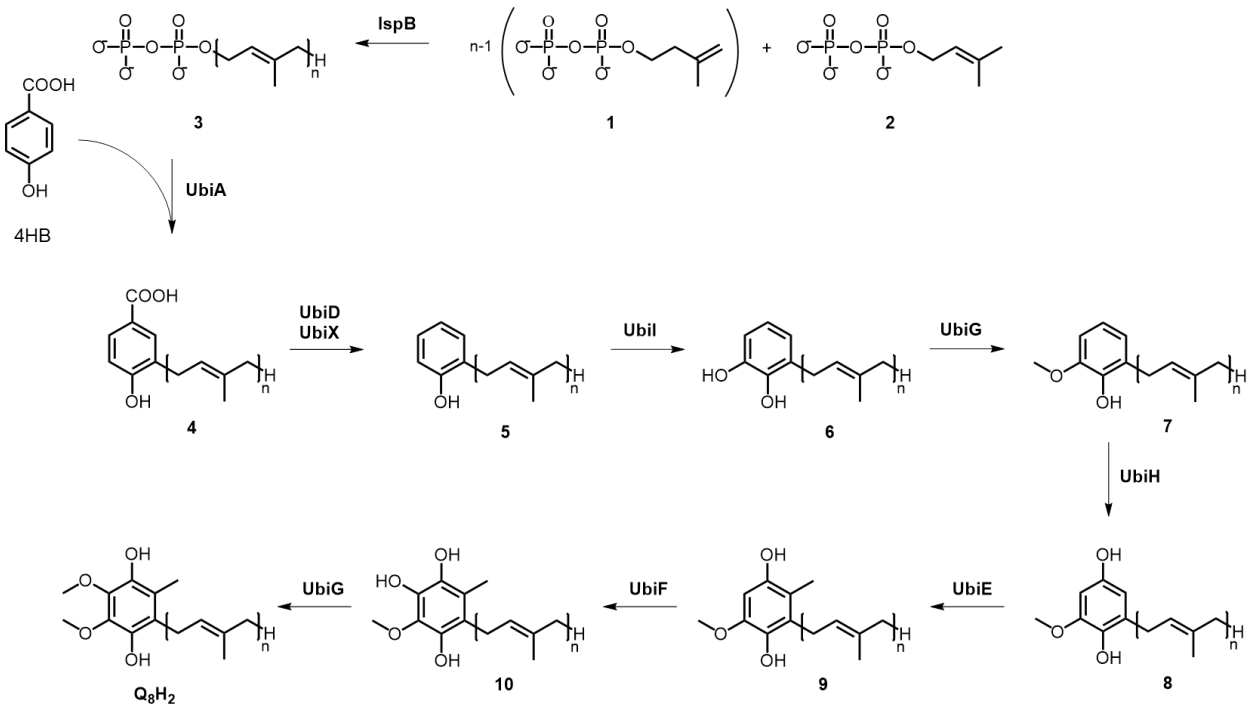

Figure 3. Q biosynthetic pathway in *Escherichia coli*. IspB catalyzes condensation of isopentenyl pyrophosphate (**1**) and dimethylallyl pyrophosphate (**2**), forming octaprenyl diphosphate (**3**). UbiA attaches octaprenyl diphosphate to 4HB, forming 4-hydroxy-3-octaprenylbenzoic acid (**4**). UbiD and UbiX catalyze a decarboxylation reaction to yield 2-octaprenylphenol (**5**). UbiI catalyzes a hydroxylation reaction, yielding 2-hydroxy-3-octaprenylphenol (**6**), followed by an *O*-methyltransferase reaction catalyzed by UbiG, yielding 2-methoxy-6-octaprenylphenol (**7**). UbiH catalyzes the second hydroxylation reaction, producing demethyldemethoxy-Q_8 (**8**). UbiE then catalyzes a *C*-methyltransferase reaction to produce DMQ_8 (**9**). UbiF catalyzes the third hydroxylation step to produce demethyl-Q_8 (**10**), and UbiG catalyzes a second *O*-methyltransferase reaction to produce $\mathbf{Q_8H_2}$. Ring positions on all compounds are numbered according to IUPAC convention.

References

Allan, C. M., Hill, S., Morvaridi, S., Saiki, R., Johnson, J. S., Liau, W. S., Hirano, K., Kawashima, T., Ji, Z., Loo, J. A., Shepherd, J. N. and Clarke, C. F. (2012). A conserved START domain coenzyme Q-binding polypeptide is required for efficient Q biosynthesis, respiratory electron transport, and antioxidant function in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1831**(4), 776-791.

Ashby, M. N. and Edwards, P. A. (1990). Elucidation of the deficiency in two yeast coenzyme Q mutants. Characterization of the structural gene encoding hexaprenyl pyrophosphate synthetase. *J Biol Chem* **265**(22), 13157-13164.

Ashby, M. N., Kutsunai, S. Y., Ackerman, S., Tzagoloff, A. and Edwards, P. A. (1992). *COQ2* is a candidate for the structural gene encoding para-hydroxybenzoate:polyprenyltransferase. *J Biol Chem* **267**(6), 4128-4136.

Aussel, L., Loiseau, L., Chehade, M. H., Pocachard, B., Fontecave, M., Pierrel, F. and Barras, F. (2014). ubiJ, a New Gene Required for Aerobic Growth and Proliferation in Macrophage, Is Involved in Coenzyme Q Biosynthesis in Escherichia coli and Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology* **196**(1), 70-79.

Baba, S. W., Belogrudov, G. I., Lee, J. C., Lee, P. T., Strahan, J., Shepherd, J. N. and Clarke, C. F. (2004). Yeast Coq5 *C*-methyltransferase is required for stability of other polypeptides involved in coenzyme Q biosynthesis. *J Biol Chem* **279**(11), 10052-10059.

Barkovich, R. J., Shtanko, A., Shepherd, J. A., Lee, P. T., Myles, D. C., Tzagoloff, A. and Clarke, C. F. (1997). Characterization of the *COQ5* gene from *Saccharomyces cerevisiae*. Evidence for a *C*-methyltransferase in ubiquinone biosynthesis. *J Biol Chem* **272**, 9182-9188.

Barros, M. H., Johnson, A., Gin, P., Marbois, B. N., Clarke, C. F. and Tzagoloff, A. (2005). The *Saccharomyces cerevisiae COQ10* gene encodes a START domain protein required for function of coenzyme Q in respiration. *J Biol Chem* **280**(52), 42627-42635.

Barros, M. H. and Nobrega, F. G. (1999). *YAH1* of *Saccharomyces cerevisiae*: a new essential gene that codes for a protein homologous to human adrenodoxin. *Gene* **233**(1-2), 197-203.

Barros, M. H., Nobrega, F. G. and Tzagoloff, A. (2002). Mitochondrial ferredoxin is required for heme A synthesis in *Saccharomyces cerevisiae*. *J Biol Chem* **277**(12), 9997-10002.

Bartling, C. M. and Raetz, C. R. H. (2009). Crystal Structure and Acyl Chain Selectivity of *Escherichia coli* LpxD, the N-Acyltransferase of Lipid A Biosynthesis. *Biochemistry* **48**(36), 8672-8683.

Belogrudov, G. I., Lee, P. T., Jonassen, T., Hsu, A. Y., Gin, P. and Clarke, C. F. (2001). Yeast *COQ4* encodes a mitochondrial protein required for coenzyme Q synthesis. *Arch Biochem Biophys* **392**, 48-58.

Bentinger, M., Tekle, M. and Dallner, G. (2010). Coenzyme Q - Biosynthesis and functions. *Biochem Biophys Res Commun* **396**(1), 74-79.

Bousquet, I., Dujardin, G. and Slonimski, P. P. (1991). *ABC1*, a novel yeast nuclear gene has a dual function in mitochondria: it suppresses a cytochrome *b* mRNA translation defect and is essential for the electron transfer in the bc_1 complex. *Embo J* **10**(8), 2023-2031.

Brandt, U. and Trumpower, B. (1994). The protonmotive Q cycle in mitochondria and bacteria. *Crit Rev Biochem Mol Biol* **29**(3), 165-197.

Casey, J. and Threlfall, D. R. (1978). Formation of 3-hexaprenyl-4-hydroxybenzoate by matrix-free mitochondrial membrane-rich preparations of yeast. *Biochimica Et Biophysica Acta* **530**(3), 487-502.

Chehade, M. H., Loiseau, L., Lombard, M., Pecqueur, L., Ismail, A., Smadja, M., Golinelli-Pimpaneau, B., Mellot-Draznieks, C., Hamelin, O., Aussel, L., Kieffer-Jaquinod, S., Labessan, N., Barras, F., Fontecave, M. and Pierrel, F. (2013). *ubiI*, a New Gene in *Escherichia coli* Coenzyme Q Biosynthesis, Is Involved in Aerobic C5-hydroxylation. *Journal of Biological Chemistry* **288**(27), 20085-20092.

Cheng, W. and Li, W. K. (2014). Structural Insights into Ubiquinone Biosynthesis in Membranes. *Science* **343**(6173), 878-881.

Clarke, C. F. (2000). New advances in coenzyme Q biosynthesis. *Protoplasma* **213**(3-4), 134-147.

Clarke, C. F., Williams, W. and Teruya, J. H. (1991). Ubiquinone biosynthesis in *Saccharomyces cerevisiae*. Isolation and sequence of *COQ3*, the 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase gene. *J Biol Chem* **266**(25), 16636-16644.

Crane, F. L. (1965). Distribution of ubiquinones. Biochemistry of quinones. R. A. Morton. London, Academic Press**:** 183-206.

Cui, T. Z. and Kawamukai, M. (2009). Coq10, a mitochondrial coenzyme Q binding protein, is required for proper respiration in *Schizosaccharomyces pombe*. *Febs J* **276**(3), 748-759.

Dai, Y. N., Zhou, K., Cao, D. D., Jiang, Y. L., Meng, F., Chi, C. B., Ren, Y. M., Chen, Y. X. and Zhou, C. Z. (2014). Crystal structures and catalytic mechanism of the C-methyltransferase Coq5 provide insights into a key step of the yeast coenzyme Q synthesis pathway. *Acta Crystallographica Section D-Biological Crystallography* **70**, 2085-2092.

Dibrov, E., Robinson, K. M. and Lemire, B. D. (1997). The *COQ5* gene encodes a yeast mitochondrial protein necessary for ubiquinone biosynthesis and the assembly of the respiratory chain. *J Biol Chem* **272**(14), 9175-9181.

Divakaruni, A. S. and Brand, M. D. (2011). The Regulation and Physiology of Mitochondrial Proton Leak. *Physiology* **26**(3), 192-205.

Do, T. Q., Hsu, A. Y., Jonassen, T., Lee, P. T. and Clarke, C. F. (2001). A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in *Saccharomyces cerevisiae abc1* mutants. *J Biol Chem* **276**(21), 18161-18168.

Do, T. Q., Schultz, J. R. and Clarke, C. F. (1996). Enhanced sensitivity of ubiquinone deficient mutants of *Saccharomyces cerevisiae* to products of autooxidized polyunsaturated fatty acid. *Proc Natl Acad Sci USA* **93**, 7534-7539.

Doimo, M., Trevisson, E., Airik, R., Bergdoll, M., Santos-Ocana, C., Hildebrandt, F., Navas, P., Pierrel, F. and Salviati, L. (2014). Effect of vanillic acid on COQ6 mutants identified in patients with coenzyme Q10 deficiency. *Biochimica Et Biophysica Acta-Molecular Basis of Disease* **1842**(1), 1-6.

Dosselaere, F. and Vanderleyden, J. (2001). A metabolic node in action: Chorismate-utilizing enzymes in microorganisms. *Critical Reviews in Microbiology* **27**(2), 75-131.

Eggink, G., Engel, H., Vriend, G., Terpstra, P. and Witholt, B. (1990). Rubredoxin reductase of *Pseudomonas oleovorans* - structural relationship to other flavoprotein oxidoreductases based on one NAD and two FAD fingerprints. *Journal of Molecular Biology* **212**(1), 135-142.

Eppink, M. H. M., Schreuder, H. A. and VanBerkel, W. J. H. (1997). Identification of a novel conserved sequence motif in flavoprotein hydroxylases with a putative dual function in FAD/NAD(P)H binding. *Protein Science* **6**(11), 2454-2458.

Frei, B., Kim, M. C. and Ames, B. N. (1990). Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc Natl Acad Sci U S A* **87**(12), 4879-4883.

Georgellis, D., Kwon, O. and Lin, E. C. C. (2001). Quinones as the redox signal for the Arc two-component system of bacteria. *Science* **292**(5525), 2314-2316.

Gin, P. and Clarke, C. F. (2005). Genetic Evidence for a Multi-subunit Complex in Coenzyme Q Biosynthesis in Yeast and the Role of the Coq1 Hexaprenyl Diphosphate Synthase. *J Biol Chem* **280**(4), 2676-2681.

Gin, P., Hsu, A. Y., Rothman, S. C., Jonassen, T., Lee, P. T., Tzagoloff, A. and Clarke, C. F. (2003). The *Saccharomyces cerevisiae COQ6* gene encodes a mitochondrial flavin-dependent monooxygenase required for coenzyme Q biosynthesis. *J Biol Chem* **278**(28), 25308-25316.

Glerum, D. M., Muroff, I., Jin, C. and Tzagoloff, A. (1997). *COX15* codes for a mitochondrial protein essential for the assembly of yeast cytochrome oxidase. *J Biol Chem* **272**(30), 19088-19094.

Goewert, R. R. (1981). Studies on the biosynthesis of ubiquinone: The identification of 3,4-dihydroxy-5-hexaprenylbenzoic acid, 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid and the regulation of the conversion of tyrosine and chorismic acid to ubiquinone in *Saccharomyces cerevisiae*. Ph.D. Dissertation, University of St. Louis.

Grandier-Vazeille, X., Bathany, K., Chaignepain, S., Camougrand, N., Manon, S. and Schmitter, J. M. (2001). Yeast mitochondrial dehydrogenases are associated in a supramolecular complex. *Biochemistry* **40**(33), 9758-9769.

Gulmezian, M. (2006). Characterization of *Escherichia coli ubiX*, *Saccharomyces cerevisiae PAD1* and *YDR539W*, and a complex of polypeptides involved in coenzyme Q biosynthesis, UCLA.

Gulmezian, M., Hyman, K. R., Marbois, B. N., Clarke, C. F. and Javor, G. T. (2007). The role of UbiX in *Escherichia coli* coenzyme Q biosynthesis. *Arch Biochem Biophys* **467**(2), 144-153.

He, C. H., Xie, L. X., Allan, C. M., Tran, U. C. and Clarke, C. F. (2014). Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast coq null mutants. *Biochim Biophys Acta* **1841**(4), 630-644.

Hildebrandt, T. M. and Grieshaber, M. K. (2008). Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria. *Febs Journal* **275**(13), 3352-3361.

Hill, S., Hirano, K., Shmanai, V. V., Marbois, B. N., Vidovic, D., Bekish, A. V., Kay, B., Tse, V., Fine, J., Clarke, C. F. and Shchepinov, M. S. (2011). Isotope-reinforced polyunsaturated fatty acids protect yeast cells from oxidative stress. *Free Radical Biology and Medicine* **50**(1), 130-138.

Hsieh, E. J., Dinoso, J. B. and Clarke, C. F. (2004). A tRNA(TRP) gene mediates the suppression of *cbs2-223* previously attributed to *ABC1/COQ8*. *Biochem Biophys Res Commun* **317**(2), 648-653.

Hsieh, E. J., Gin, P., Gulmezian, M., Tran, U. C., Saiki, R., Marbois, B. N. and Clarke, C. F. (2007). *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex. *Arch Biochem Biophys* **463**(1), 19-26.

Hsu, A. Y., Poon, W. W., Shepherd, J. A., Myles, D. C. and Clarke, C. F. (1996). Complementation of *coq3* mutant yeast by mitochondrial targeting of the *Escherichia coli* UbiG polypeptide: evidence that UbiG catalyzes both *O*-methylation steps in ubiquinone biosynthesis. *Biochemistry* **35**(30), 9797-9806.

Jacewicz, A., Izumi, A., Brunner, K., Schnell, R. and Schneider, G. (2013). Structural Insights into the UbiD Protein Family from the Crystal Structure of PA0254 from *Pseudomonas aeruginosa*. *Plos One* **8**(5), 10.

Jezek, P. and Hlavata, L. (2005). Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *International Journal of Biochemistry & Cell Biology* **37**(12), 2478-2503.

Johnson, A., Gin, P., Marbois, B. N., Hsieh, E. J., Wu, M., Barros, M. H., Clarke, C. F. and Tzagoloff, A. (2005). *COQ9*, a new gene required for the biosynthesis of coenzyme Q in *Saccharomyces cerevisiae*. *J Biol Chem* **280**(36), 31397-31404.

Jonassen, T., Proft, M., Randez-Gil, F., Schultz, J. R., Marbois, B. N., Entian, K. D. and Clarke, C. F. (1998). Yeast Clk-1 homologue (Coq7/Cat5) is a mitochondrial protein in coenzyme Q synthesis. *J Biol Chem* **273**, 3351-3357.

Kagan, R. M. and Clarke, S. (1994). Widespread Occurrence of Three Sequence Motifs in Diverse S-Adenosylmethionine-Dependent Methyltransferases Suggests a Common Structure for These Enzymes. *Archives of Biochemistry and Biophysics* **310**(2), 417-427.

Kainou, T., Okada, K., Suzuki, K., Nakagawa, T., Matsuda, H. and Kawamukai, M. (2001). Dimer formation of octaprenyl-diphosphate synthase (IspB) is essential for chain length determination of ubiquinone. *J Biol Chem* **276**(11), 7876-7883.

Katz, J. E., Dlakic, M. and Clarke, S. (2003). Automated identification of putative methyltransferases from genomic open reading frames. *Mol Cell Proteomics* **2**(8), 525-540.

Kelley, L. A. and Sternberg, M. J. E. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nature Protocols* **4**(3), 363-371.

Kopec, J., Schnell, R. and Schneider, G. (2011). Structure of PA4019, a putative aromatic acid decarboxylase from *Pseudomonas aeruginosa*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67**, 1184-1188.

Lagier-Tourenne, C., Tazir, M., Lopez, L. C., Quinzii, C. M., Assoum, M., Drouot, N., Busso, C., Makri, S., Ali-Pacha, L., Benhassine, T., Anheim, M., Lynch, D. R., Thibault, C., Plewniak, F., Bianchetti, L., Tranchant, C., Poch, O., DiMauro, S., Mandel, J. L., Barros, M. H., Hirano, M. and Koenig, M. (2008). ADCK3, an ancestral kinase, is mutated in a form of recessive ataxia associated with coenzyme Q10 deficiency. *Am J Hum Genet* **82**(3), 661-672.

Lambert, A. J. and Brand, M. D. (2004). Superoxide production by NADH: ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochemical Journal* **382**, 511-517.

Lange, H., Kaut, A., Kispal, G. and Lill, R. (2000). A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins. *Proc Natl Acad Sci U S A* **97**(3), 1050-1055.

Lenaz, G. and De Santis, A. (1985). A survey of the function and specificity of ubiquione in the mitochondrial respiratory chain. Coenzyme Q. G. Lenaz. Chichester, U.K., John Wiley & Sons**:** 165-199.

Leonard, C. J., Aravind, L. and Koonin, E. V. (1998). Novel families of putative protein kinases in bacteria and archaea: evolution of the "eukaryotic" protein kinase superfamily. *Genome Res* **8**(10), 1038-1047.

Li, J., Saxena, S., Pain, D. and Dancis, A. (2001). Adrenodoxin reductase homolog (Arh1p) of yeast mitochondria required for iron homeostasis. *J Biol Chem* **276**(2), 1503-1509.

Lichtenthaler, H. K. (1999). The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 47-65.

Lo Conte, L., Ailey, B., Hubbard, T. J. P., Brenner, S. E., Murzin, A. G. and Chothia, C. (2000). SCOP: a Structural Classification of Proteins database. *Nucleic Acids Research* **28**(1), 257-259.

Malpica, R., Sandoval, G. R., Rodriguez, C., Franco, B. and Georgellis, D. (2006). Signaling by the arc two-component system provides a link between the redox state of the quinone pool and gene expression. *Antioxid Redox Signal* **8**(5-6), 781-795.

Manzella, L., Barros, M. H. and Nobrega, F. G. (1998). *ARH1* of *Saccharomyces cerevisiae*: A new essential gene that codes for a protein homologous to the human adrenodoxin reductase. *Yeast* **14**(9), 839-846.

Marbois, B., Gin, P., Faull, K. F., Poon, W. W., Lee, P. T., Strahan, J., Shepherd, J. N. and Clarke, C. F. (2005). Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q. *J Biol Chem* **280**, 20231-20238.

Marbois, B., Gin, P., Gulmezian, M. and Clarke, C. F. (2009). The yeast Coq4 polypeptide organizes a mitochondrial protein complex essential for coenzyme Q biosynthesis. *Biochim Biophys Acta* **1791**, 69-75.

Marbois, B., Xie, L. X., Choi, S., Hirano, K., Hyman, K. and Clarke, C. F. (2010). para-Aminobenzoic Acid Is a Precursor in Coenzyme Q(6) Biosynthesis in *Saccharomyces cerevisiae*. *J Biol Chem* **285**(36), 27827-27838.

Marbois, B. N. and Clarke, C. F. (1996). The *COQ7* gene encodes a protein in *Saccharomyces cerevisiae* necessary for ubiquinone biosynthesis. *J Biol Chem* **271**, 2995-3004.

Martin-Montalvo, A., Gonzalez-Mariscal, I., Padilla, S., Ballesteros, M., Brautigan, D. L., Navas, P. and Santos-Ocana, C. (2011). Respiratory-induced coenzyme Q biosynthesis is regulated by a phosphorylation cycle of Cat5p/Coq7p. *Biochemical Journal* **440**, 107-114.

Martin-Montalvo, A., Gonzalez-Mariscal, I., Pomares-Viciana, T., Padilla-Lopez, S., Ballesteros, M., Vazquez-Fonseca, L., Gandolfo, P., Brautigan, D. L., Navas, P. and Santos-Ocana, C. (2013). The Phosphatase Ptc7 Induces Coenzyme Q Biosynthesis by Activating the Hydroxylase Coq7 in Yeast. *Journal of Biological Chemistry* **288**(39), 28126-28137.

Melzer, M. and Heide, L. (1994). Characterization of polyprenyldiphosphate: 4-hydroxybenzoate polyprenyltransferase from *Escherichia coli*. *Biochim Biophys Acta* **1212**(1), 93-102.

Miller, W. L. (2007). Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* **1771**(6), 663-676.

Mugoni, V., Postel, R., Catanzaro, V., De Luca, E., Turco, E., Digilio, G., Silengo, L., Murphy, M. P., Medana, C., Stainier, D. Y. R., Bakkers, J. and Santoro, M. M. (2013). Ubiad1 Is an Antioxidant Enzyme that Regulates eNOS Activity by CoQ10 Synthesis. *Cell* **152**(3), 504-518.

Mukai, N., Masaki, K., Fujii, T., Kawamukai, M. and Iefuji, H. (2010). *PAD1* and *FDC1* are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J Biosci Bioeng* **109**(6), 564-569.

Nakahigashi, K., Miyamoto, K., Nishimura, K. and Inokuchi, H. (1992). Isolation and characterization of a light-sensitive mutant of *Escherichia coli* K-12 with a mutation in a gene that is required for the biosynthesis of ubiquinone. *J Bacteriol* **174**(22), 7352-7359.

Niewmierzycka, A. and Clarke, S. (1999). S-adenosylmethionine-dependent methylation in *Saccharomyces cerevisiae* - Identification of a novel protein arginine methyltransferase. *Journal of Biological Chemistry* **274**(2), 814-824.

Nowicka, B. and Kruk, J. (2010). Occurrence, biosynthesis and function of isoprenoid quinones. *Biochimica Et Biophysica Acta-Bioenergetics* **1797**(8), 1587-1605.

Okada, K., Kainou, T., Matsuda, H. and Kawamukai, M. (1998). Biological significance of the side chain length of ubiquinone in *Saccharomyces cerevisiae*. *FEBS Lett* **431**(2), 241-244.

Olson, R. E. (1966). Biosynthesis of ubiquinones in animals. *Vitam Horm* **24**, 551-574.

Olson, R. E. and Rudney, H. (1983). Biosynthesis of ubiquinone. *Vitam Horm* **40**, 1-43.

Ozeir, M., Muhlenhoff, U., Webert, H., Lill, R., Fontecave, M. and Pierrel, F. (2011). Coenzyme Q Biosynthesis: Coq6 Is Required for the C5-Hydroxylation Reaction and Substrate Analogs Rescue Coq6 Deficiency. *Chemistry & Biology* **18**(9), 1134-1142.

Padilla, S., Tran, U. C., Jimenez-Hidalgo, M., Lopez-Martin, J. M., Martin-Montalvo, A., Clarke, C. F., Navas, P. and Santos-Ocana, C. (2009). Hydroxylation of demethoxy-Q6 constitutes a control point in yeast coenzyme Q6 biosynthesis. *Cell Mol Life Sci* **66**(1), 173-186.

Pennock, J. F. and Threlfall, D. R. (1983). Biosynthesis of Ubiquinone and Related Compounds. Biosynthesis of Isoprenoid Compounds. J. W. Porter and S. L. Spurgeon. New York, NY, John Wiley & Sons**:** 191-302.

Petrossian, T. C. and Clarke, S. G. (2009). Multiple Motif Scanning to Identify Methyltransferases from the Yeast Proteome. *Molecular & Cellular Proteomics* **8**(7), 1516-1526.

Pierrel, F., Hamelin, O., Douki, T., Kieffer-Jaquinod, S., Muhlenhoff, U., Ozeir, M., Lill, R. and Fontecave, M. (2010). Involvement of Mitochondrial Ferredoxin and Para-Aminobenzoic Acid in Yeast Coenzyme Q Biosynthesis. *Chem Biol* **17**(5), 449-459.

Ponting, C. P. and Aravind, L. (1999). START: a lipid binding domain in StAR, HD-ZIP and signalling proteins. *Trends in Biochemical Sciences* **24**(4), 130-132.

Poon, W. W., Barkovich, R. J., Hsu, A. Y., Frankel, A., Lee, P. T., Shepherd, J. N., Myles, D. C. and Clarke, C. F. (1999). Yeast and rat Coq3 and *Escherichia coli* UbiG polypeptides catalyze both O-methyltransferase steps in coenzyme Q biosynthesis. *J Biol Chem* **274**(31), 21665-21672.

Poon, W. W., Davis, D. E., Ha, H. T., Jonassen, T., Rather, P. N. and Clarke, C. F. (2000). Identification of *Escherichia coli ubiB*, a gene required for the first monooxygenase step in ubiquinone biosynthesis. *J Bacteriol* **182**(18), 5139-5146.

Proft, M., Kotter, P., Hedges, D., Bojunga, N. and Entian, K. D. (1995). *CAT5*, a new gene necessary for derepression of gluconeogenic enzymes in *Saccharomyces cerevisiae*. *Embo Journal* **14**(24), 6116-6126.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E. L. L., Eddy, S. R., Bateman, A. and Finn, R. D. (2012). The Pfam protein families database. *Nucleic Acids Research* **40**(D1), D290-D301.

Rangarajan, E. S., Li, Y., Iannuzzi, P., Tocilj, A., Hung, L. W., Matte, A. and Cygler, M. (2004). Crystal structure of a dodecameric FMN-dependent UbiX-like decarboxylase (Pad1) from *Escherichia coli* O157: H7. *Protein Sci* **13**(11), 3006-3016.

Rea, S. L., Graham, B. H., Nakamaru-Ogiso, E., Kar, A. and Falk, M. J. (2010). Bacteria, yeast, worms, and flies: exploiting simple model organisms to investigate human mitochondrial diseases. *Dev Disabil Res Rev* **16**(2), 200-218.

Rodel, G., Michaelis, U., Forsbach, V., Kreike, J. and Kaudewitz, F. (1986). Molecular cloning of the yeast nuclear genes *CBS1* and *CBS2*. *Current Genetics* **11**(1), 47-53.

Rohmer, M., Seemann, M., Horbach, S., BringerMeyer, S. and Sahm, H. (1996). Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate

pathway for terpenoid biosynthesis. *Journal of the American Chemical Society* **118**(11), 2564-2566.

Saiki, R., Lunceford, A. L., Shi, Y., Marbois, B., King, R., Pachuski, J., Kawamukai, M., Gasser, D. L. and Clarke, C. F. (2008). Coenzyme Q10 supplementation rescues renal disease in Pdss2kd/kd mice with mutations in prenyl diphosphate synthase subunit 2. *Am J Physiol Renal Physiol* **295**(5), F1535-1544.

Sharma, P., Stagge, S., Bekker, M., Bettenbrock, K. and Hellingwerf, K. J. (2013). Kinase Activity of ArcB from Escherichia coli Is Subject to Regulation by Both Ubiquinone and Demethylmenaquinone. *Plos One* **8**(10), 7.

Shen, Y., Goldsmith-Fischman, S., Atreya, H. S., Acton, T., Ma, L., Xiao, R., Honig, B., Montelione, G. T. and Szyperski, T. (2005). NMR structure of the 18 kDa protein CC1736 from *Caulobacter crescentus* identifies a member of the START domain superfamily and suggests residues mediating substrate specificity. *Proteins* **58**(3), 747-750.

Shepherd, J. A., Poon, W. W., Myles, D. C. and Clarke, C. F. (1996). The biosynthesis of ubiquinone: Synthesis and enzymatic modification of biosynthetic precursors. *Tetrahedron Lett* **37**, 2395-2398.

Siebert, M., Severin, K. and Heide, L. (1994). Formation of 4-hydroxybenzoate in *Escherichia coli*: characterization of the *ubiC* gene and its encoded enzyme chorismate pyruvate-lyase. *Microbiology* **140 (Pt 4)**, 897-904.

Stenmark, P., Grunler, J., Mattsson, J., Sindelar, P. J., Nordlund, P. and Berthold, D. A. (2001). A new member of the family of di-iron carboxylate proteins. Coq7 (clk-1), a membrane-bound hydroxylase involved in ubiquinone biosynthesis. *J Biol Chem* **276**(36), 33297-33300.

Suzuki, K., Ueda, M., Yuasa, M., Nakagawa, T., Kawamukai, M. and Matsuda, H. (1994). Evidence That *Escherichia coli ubiA* Product Is a Functional Homolog of Yeast *COQ2*, and the Regulation of *ubiA* Gene Expression. *Bioscience Biotechnology and Biochemistry* **58**(10), 1814-1819.

Tauche, A., Krause-Buchholz, U. and Rodel, G. (2008). Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of *O*-methylase Coq3p depends on Abc1p/Coq8p. *FEMS Yeast Res* **8**(8), 1263-1275.

Tran, U. C. and Clarke, C. F. (2007). Endogenous synthesis of coenzyme Q in eukaryotes. *Mitochondrion* **7S**, S62-S71.

Tran, U. C., Marbois, B., Gin, P., Gulmezian, M., Jonassen, T. and Clarke, C. F. (2006). Complementation of *Saccharomyces cerevisiae coq7* mutants by mitochondrial targeting of the *Escherichia coli* UbiF polypeptide: two functions of yeast Coq7 polypeptide in coenzyme Q biosynthesis. *J Biol Chem* **281**(24), 16401-16409.

Turunen, M., Olsson, J. and Dallner, G. (2004). Metabolism and function of coenzyme Q. *Biochim Biophys Acta* **1660**(1-2), 171-199.

Tzagoloff, A. and Dieckmann, C. L. (1990). *PET* genes of *Saccharomyces cerevisiae*. *Microbiol Rev* **54**, 211-225.

Tzagoloff, A., Yue, J., Jang, J. and Paul, M. F. (1994). A new member of a family of ATPases is essential for assembly of mitochondrial respiratory chain and ATP synthetase complexes in *Saccharomyces cerevisiae*. *J Biol Chem* **269**(42), 26144-26151.

UniProt, C. (2014). Activities at the Universal Protein Resource (UniProt). *Nucleic Acids Res* **42**(Database issue), D191-198.

Wang, K. C. and Ohnuma, S. (2000). Isoprenyl diphosphate synthases. *Biochim Biophys Acta* **1529**(1-3), 33-48.

Wierenga, R. K., Terpstra, P. and Hol, W. G. J. (1986). Prediction of the occurrence of the ADP-binding beta-alpha-beta-fold in proteins, using an amino acid sequence fingerprint. *Journal of Molecular Biology* **187**(1), 101-107.

Xie, L. T. X., Hsieh, E. J., Watanabe, S., Allan, C. M., Chen, J. Y., Tran, U. C. and Clarke, C. F. (2011). Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants. *Biochim Biophys Acta* **1811**(5), 348-360.

Xie, L. X., Ozeir, M., Tang, J. Y., Chen, J. Y., Kieffer-Jaquinod, S., Fontecave, M., Clarke, C. F. and Pierrel, F. (2012). Over-expression of the Coq8 kinase in S*accharomyces cerevisiae coq* null mutants allows for accumulation of diagnostic intermediates of the Coenzyme Q6 biosynthetic pathway. *J Biol Chem* **287**(28), 23571-23581.

Zhang, M., Luo, J., Ogiyama, Y., Saiki, R. and Kawamukai, M. (2008). Heteromer formation of a long-chain prenyl diphosphate synthase from fission yeast Dps1 and budding yeast Coq1. *Febs J* **275**, 3653-3668.

Zhang, Y. L. and Inouye, M. (2011). RatA (YfjG), an Escherichia coli toxin, inhibits 70S ribosome association to block translation initiation. *Molecular Microbiology* **79**(6), 1418-1429.

Chapter 2

A conserved START domain coenzyme Q-binding polypeptide is required for efficient Q biosynthesis, respiratory electron transport, and antioxidant function in *Saccharomyces cerevisiae*

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A conserved START domain coenzyme Q-binding polypeptide is required for efficient Q biosynthesis, respiratory electron transport, and antioxidant function in *Saccharomyces cerevisiae*

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ABSTRACT

Coenzyme Q_n (ubiquinone or Q_n) is a redox active lipid composed of a fully substituted benzoquinone ring and a polyisoprenoid tail of *n* isoprene units. *Saccharomyces cerevisiae coq1–coq9* mutants have defects in Q biosynthesis, lack Q_6, are respiratory defective, and sensitive to stress imposed by polyunsaturated fatty acids. The hallmark phenotype of the Q-less yeast *coq* mutants is that respiration in isolated mitochondria can be rescued by the addition of Q_2, a soluble Q analog. Yeast *coq10* mutants share each of these phenotypes, with the surprising exception that they continue to produce Q_6. Structure determination of the *Caulobacter crescentus* Coq10 homolog (CC1736) revealed a steroidogenic acute regulatory protein-related lipid transfer (START) domain, a hydrophobic tunnel known to bind specific lipids in other START domain family members. Here we show that purified CC1736 binds Q_2, Q_3, Q_{10}, or demethoxy-Q_3 in an equimolar ratio, but fails to bind 3-farnesyl-4-hydroxybenzoic acid, a farnesylated analog of an early Q-intermediate. Over-expression of *C. crescentus* CC1736 or *COQ8* restores respiratory electron transport and antioxidant function of Q_6 in the yeast *coq10* null mutant. Studies with stable isotope ring precursors of Q reveal that early Q-biosynthetic intermediates accumulate in the *coq10* mutant and *de novo* Q-biosynthesis is less efficient than in the wild-type yeast or rescued *coq10* mutant. The results suggest that the Coq10 polypeptide:Q (protein:ligand) complex may serve essential functions in facilitating *de novo* Q biosynthesis and in delivering newly synthesized Q to one or more complexes of the respiratory electron transport chain.

1. Introduction

Coenzyme Q (ubiquinone or Q) is a small lipophilic electron carrier found primarily in the inner mitochondrial membrane where it plays a key role in respiratory electron transport [1]. Q consists of a polyisoprenoid 'tail' whose length is species dependent and a fully substituted redox-active benzoquinone 'head' [2]. The reduced or hydroquinone form, QH_2, also serves as a lipid soluble chain-terminating antioxidant [3]. Yeast mutants lacking Q and QH_2 are sensitive to oxidative stress induced by treatment with polyunsaturated fatty acids [4].

Abbreviations: αLnn, α-linolenic acid (C18:3, $n-3$); BCA, bicinchoninic acid; BHT, butylated hydroxytoluene; BN-PAGE, blue native-polyacrylamide gel electrophoresis; DMQ, demethoxy-Q; DOD, drop out growth medium with dextrose; FHB, farnesyl-hydroxybenzoate; HAB, hexaprenyl-4-aminobenzoic acid; HHB, hexaprenyl-4-hydroxybenzoic acid; HPLC, high performance liquid chromatography; IDMQ, 4-imino-demethoxy-Q; pABA, *para*-aminobenzoic acid; PBS, phosphate buffered saline; PUFA, polyunsaturated fatty acid; Q, coenzyme Q or ubiquinone; QH_2, coenzyme QH_2 or ubiquinol; START, steroidogenic acute regulatory-related lipid transfer; YPD, rich growth medium with dextrose; YPG, rich growth medium with glycerol; YPGal, rich growth medium with galactose

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The toxicity of polyunsaturated fatty acid autoxidation products can be abrogated by substitution of the fatty acid bis-allylic hydrogen atoms with deuterium atoms [5].

In the yeast *Saccharomyces cerevisiae* Q_6 biosynthesis occurs in the mitochondria and is dependent on eleven known proteins, Coq1p–Coq9p, Yah1p, and Arh1p [6–8]. The yeast *coq1–coq9* mutants lack Q, are respiratory defective and unable to grow on non-fermentable carbon sources. *YAH1* and *ARH1* genes encode ferrodoxin and ferredoxin reductase, are essential for yeast viability, and play roles in iron-sulfur cluster biogenesis in addition to Q biosynthesis [7,9].

An additional protein, Coq10p, is required for Q_6 activity in the electron transport chain but is not essential for Q biosynthesis [10]. *S. cerevisiae coq10* null mutants contain wild-type levels of Q_6 but are nonetheless respiration defective. Mitochondria isolated from *coq10* mutants show greatly impaired oxidation of substrates of electron transport as measured by oxygen consumption unless supplemented with exogenous Q_2 [10]. This rescue of respiratory electron transport by addition of Q_2 is a hallmark phenotype of the *coq* mutants. Thus, while yeast *coq1–coq9* mutants are "Q-less", the *coq10* mutant contains Q_6 but its respiratory defects are nevertheless rescued when a soluble analog of Q (such as Q_2) is added. The *coq10* null mutant in the yeast *Schizosaccharomyces pombe* displays similar phenotypes as it produces endogenous Q_{10} but fails to respire as measured by oxygen consumption [11].

How Coq10p mediates Q-dependent respiratory electron transport is still mysterious. Stoichiometric considerations suggest that Coq10p is unlikely to play a direct role in shuttling Q between the respiratory complexes, because Coq10p content is three orders of magnitude less abundant than Q_6 and two orders of magnitude less abundant than other respiratory chain components, such as the bc_1 complex [10]. Yeast respiratory super complexes (as assayed by high molecular mass cytochrome *b*) were detectable but greatly decreased in the *coq10* mutant relative to that of wild-type yeast [12]. Thus, while it is possible that Coq10p transports or shuttles Q_6 or Q_{10} to the respiratory chain complexes [11], it may also serve to escort or chaperone Q to sites within the respiratory chain complexes that are critical for the Q cycle. It seems likely that Q_6 can access the P-site of the bc_1 complex without Coq10p because treatment of *coq10* mutant mitochondria with antimycin A induces H_2O_2 production [12]. While this response to antimycin suggests an active Q-cycle, the bc_1 complex is not functional since electrons are not transferred to cytochrome c_1. Treatment of *coq10* mutant mitochondria with myxothiazol failed to induce H_2O_2 production, consistent with a defect in residence and/or function of Q_6 at the bc_1 complex [12], perhaps at the N-site.

Coq10p homologs are present in a variety of organisms, from bacteria to humans [10]. Expression of the human *COQ10* homolog in *coq10* null mutants of *S. cerevisiae* and *S. pombe* restored growth on non-fermentable carbon sources [10,11]. The primary sequence of Coq10p does not share homology with any protein domains of known function and is classified as part of the aromatic-rich protein family Pfam03654 [13]. The structure of the *Caulobacter crescentus* Coq10p homolog CC1736 was determined by NMR [14] and revealed a structure similar to that of the steroidogenic acute regulatory-related lipid transfer (START) domain, which is known to bind lipids such as cholesterol or polyketides via a hydrophobic tunnel [15,16]. The START domain structure is classified as a helix-grip type, consisting of a seven-stranded anti-parallel β-sheet with a C-terminal α-helix [17]. Purification of *S. cerevisiae* Coq10p indicates that it binds endogenous Q_6, but as purified from yeast the content of Q_6 was substoichiometric [10]. Studies with *S. pombe* Coq10p indicate that this protein binds Q_{10} at an equimolar ratio of ligand to protein and that this binding depends on conserved hydrophobic amino acids [11], as shown via multiple sequence alignment (Fig. 1). Point mutation analyses and molecular modeling studies suggest that *S. cerevisiae* Coq10p likely contains a similar hydrophobic tunnel capable of binding lipid substrates [18]. One postulated function of Q binding by CC1736 and by extension Coq10p may be to chaperone Q to its proper locations in the respiratory chain complexes.

Most of the Coq proteins in *S. cerevisiae* including Coq10p are localized to the matrix side of the inner mitochondrial membrane [8]. Blue native-PAGE and co-precipitation experiments demonstrate that several of the Coq proteins exist in a high molecular weight complex [8,19–21]. Additionally, the steady-state levels of several of these Coq proteins are interdependent as levels decrease significantly in various *coq* null mutants [20]. In contrast, steady state levels of Coq10p are not affected by other *coq* gene deletions [20]. Coq10p has not been demonstrated to interact with the other Coq proteins by co-immunoprecipitation but it was suggested to exist in an oligomeric form via sucrose gradient sedimentation [10] and was recently shown to co-migrate via BN-PAGE with Coq2p and Coq8p [22]. Coq8p contains protein kinase motifs and is required for phosphorylation of Coq3p, Coq5p, and Coq7p [23]. Although Coq8p has not been shown to exist in a macromolecular complex with other Coq proteins, it is required for the stability of several Coq proteins [20]. Over-expression of Coq8p stabilizes the steady-state levels of several Coq proteins in various *coq* null mutants including the *coq10* null [10,24,25] and increases the accumulation of later stage coenzyme Q biosynthetic intermediates [25,26]. Over-expression of Coq2p and Coq7p in the *coq10* null mutant also restores growth on non-fermentable carbon sources, however the greatest effect is observed with over-expression of Coq8p [10]. Over-expression of Coq8p leads to increased levels of endogenous Q_6 [10], which is thought to overcome the defect in Coq10p and allow for functional respiration. In contrast, severe over-expression (300-fold compared to wild-type yeast) of Coq10p in *S. cerevisiae* impairs mitochondrial respiration as observed by decreased oxygen consumption and a decreased ability to utilize non-fermentable carbon sources [24]. It was hypothesized that the respiratory defect caused by over-expression of Coq10p in *S. cerevisiae* is due to sequestering of the endogenous Q_6 by the excess Coq10p [24]. Over-expression of Coq8p suppresses the respiratory deficiency resulting from severe over-expression of Coq10p.

Here we show that expression of the *C. crescentus* CC1736 START domain polypeptide in *S. cerevisiae* restores growth of the *coq10* null mutant on non-fermentable carbon sources and functional electron transport. The content of Q_6 and Q_6 biosynthetic intermediates were also examined in the *coq10* null mutant as well as wild type and the *coq10* null over-expressing CC1736 or Coq8p. Binding studies with Q of varying tail lengths as well as Q biosynthetic intermediates were performed with purified recombinant CC1736. The results suggest that CC1736 and Coq10p bind Q and late-stage Q-intermediates, and play conserved roles in facilitating *de novo* Q synthesis and respiratory electron transport.

2. Materials and methods

2.1. Yeast strains and growth media

Yeast strains used in this study are described in Table 1. Media were prepared as described [27], and included: YPD (2% glucose, 1% yeast extract, 2% peptone), YPGal (2% galactose, 1% yeast extract, 2% peptone, 0.20% glucose), and YPG (3% glycerol, 1% yeast extract, 2% peptone). Synthetic Dextrose/Minimal medium (SDC and SD–Ura) was prepared as described [28], and consisted of all components minus uracil. Drop out dextrose medium (DOD) was prepared as described [29] except that dextrose was used in place of galactose. Plate media contained 2% bacto agar.

2.2. Construction of multicopy yeast expression vector with the CYC1 promoter and a mitochondrial leader sequence from COQ3

Plasmids used and generated in this study are listed in Table 2. A 0.5 kb BamH1-Kpn1 fragment containing the yeast *CYC1* promoter and the amino terminal mitochondrial leader sequence (residues 1 to 35) of yeast *COQ3* was isolated from pQM [30]. This fragment

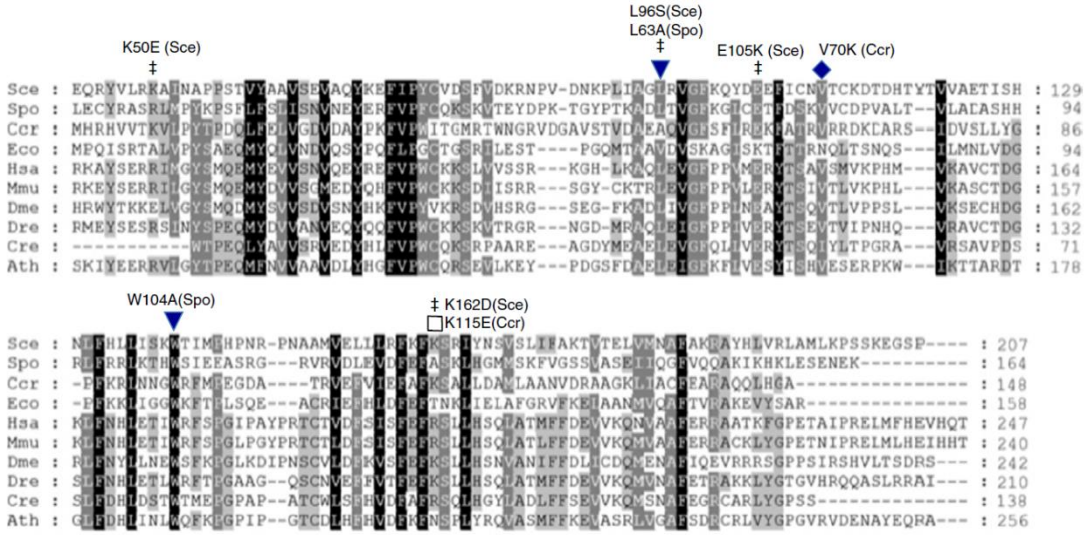


Fig. 1. Conserved amino acid residues in Coq10 polypeptide homologues. Protein sequences were aligned with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Ibis Biosciences, Carlsbad, CA) and shaded as described by Genedoc with three levels of shading (http://www.nrbsc.org/gfx/genedoc/) [54]. Residues conserved in all proteins are shaded black, in 80% dark grey, and in 60% light grey. Amino-terminal segments of eukaryotic polypeptides preceding the first methionine of the *C. crescentus* (Ccr) and *E. coli* (Eco) polypeptides are not conserved and were omitted from the alignment for clarity. Residues determined to be important for maintenance of respiration are designated with filled symbols and include *C. crescentus* V70K (this work), *S. pombe* L63A and W104A [11]. Additional mutations affecting *S. cerevisiae* Coq10p function are designated by ‡ and include K50E, L96S, E105K, and K162D [18]. Mutation of K115E in CC1736 (marked by an open square) did not impair rescue of the *S. cerevisiae coq10* null mutant (this work). The aligned sequences include: *S. cerevisiae* COQ10 (NCBI GeneID: 854154), *Schizosaccharomyces pombe* COQ10 (942096), *C. crescentus* CBL5 (942096), *E. coli* yfjG (945614), *Homo sapiens* COQ10A (93058), *M. musculus* COQ10B (67876), *Drosophila melanogaster* CG9410 (35568), *Danio rerio* Zgc:73324 (393762), *Chlamydomonas reinhardtii* COQ10 (5718019), and *Arabidopsis thaliana* AT4G17650 (827485).

was inserted into the BamH1 and Kpn1 sites of the multicopy yeast/*Escherichia coli* shuttle vector pRS426 [31] and named pRCM.

2.3. Cloning of C. crescentus CC1736 in yeast expression vectors

The source plasmid pCcR19-21.1 [32], encodes the full-length CC1736 gene from *C. crescentus* plus eight non-native C-terminal residues (LEHHHHHH) cloned into pET21d (Novagen derivative). A segment of DNA containing the CC1736 ORF was amplified from pCcR19-21.1 template DNA with the forward primer 5′-GG**GGTACC** ATGTTGCACCGTCACGTCGTTAC-3′ (−3 to +20 bp of CC1736 underlined, with Kpn1 site bold) and the reverse complement primer 5′-GG**GGTACC**TTTGTTAGCAGCCGGATCTCAGT-3′ (+489 to +467 underlined, Kpn1 site bold). The resulting PCR product was digested with Kpn1 and cloned into the Kpn1 site of the yeast expression plasmids pRCM and pCH (similar to pRCM but without the Coq3 mitochondrial leader) [30], to generate pRCM-CC1736 and pCH-CC1736, respectively.

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]
W303-1B	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303ΔCOQ10	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq10::HIS3*	[10]
CC303	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1coq3::LEU2*	[4]
W303ΔCOR1	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cor1::HIS3*	[50]
NM101, coq7Δ-1	NM101, *coq7Δ-1::LEU2*	[51]
W303ΔABC1	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 abc1/coq8::HIS3*	[52]
W303ΔCOQ9	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq9::URA3*	[53]
CC304.1	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp2::LEU2*	[4]

[a] Dr. Rodney Rothstein, Department of Human Genetics, Columbia University. The a of MAT a is in bold to distinguish it from MAT alpha.

2.4. Site directed mutagenesis

Site-directed mutagenesis was performed with a Quick-Change mutagenesis kit (Stratagene) according to manufacturer's protocol. The plasmid pCcR19-21.1 provided the template for the PCR reactions, which utilized the following primers for V70K and K115E codon replacement: V70Ksense, 5′-AGAAGTTCGCGACCCGCAAACGTC

Table 2
Plasmids used.

Plasmids	Relevant genes/markers	Source or reference
pRS426	Yeast shuttle vector; multicopy	[31]
pCH1	Yeast vector with *CYC1* promoter; multicopy	[30]
pRCM	pCH1 with *COQ3* mito leader; multicopy	This work
p4HN4	pRS426 with Yeast *ABC1/COQ8*; multicopy	[20]
PG140/ST3	Yeast *COQ10*; multicopy	[10]
pRCM-CC1736	pRCM with *C. crescentus* CC1736; multicopy	This work
pET21d	*E. coli* expression vector	EMD4Biosciences
pET21d-CC1736	pET21d with *C. crescentus* CC1736	This work
pRCM-K115E	pRCM with CC1736-K115E; multicopy	This work
pRCM-V70K	pRCM with CC1736-V70K; multicopy	This work

GTGACAAAGACGC-3′ (the lysine codon is underlined); V70Kantisense, 5′-GCGTCTTTGTCACGACGTTTGCGGGTCGCGAACTTCT-3′ (the antisense lysine codon is underlined); K115Esense, 5′-CATCGAGTTTGCGTTCGAA TCCGCGCTGCTAGACG-3′ (the glutamate codon is underlined); and K115Eantisense,5′-CGTCTAGCAGCGCGGATTCGAACGCAAACTCGATG-3′ (the glutamate anticodon is underlined). The substitution mutations in the resulting clones, pET-CC1736V70K and pET-CC1736K115E were confirmed by sequence analyses performed by the UCLA DNA sequencing facility. The Kpn1 fragments from pET-CC1736V70K and pET-CC1736K115E were cloned into the Kpn1 site of pRCM to generate V70K-CC1736 or K115E-CC1736, respectively.

2.5. Complementation of the yeast coq10 null mutant by C. crescentus CC1736

Yeast transformations were performed as described [33]. The *coq10* null mutant W303ΔCOQ10 was transformed with each of the following multi-copy plasmids: pRCM, (empty vector), p4HN4 (*COQ8*), pRCM-CC1736, pRCM-V70K, pRCM-K115E, or with the positive control plasmid, PG140/ST3 [10], containing yeast *COQ10*. Transformed yeast cells were selected on SD–Ura plate medium for 3 days at 30 °C. Colonies from these plates were grown in selective liquid medium to mid log phase (OD_{600nm} = 0.2–1.0). An aliquot was diluted with sterile water to OD_{600nm} = 0.2, and cells were serially diluted (1:5), and 2 μl of each sample were spotted onto SD or SD-Ura (fermentable) or YPG (non-fermentable) plate medium, and incubated at 30 °C.

2.6. Fatty acid sensitivity assays

A fatty acid sensitivity assay was used to assess relative sensitivities of different yeast mutants to oxidative stress [4]. Yeast strains were grown in YPD media at 30 °C and 250 rpm and harvested while in logarithmic phase (OD_{600nm} per ml = 0.2–1.0). The cells were washed twice with sterile water and resuspended in 0.10 M phosphate buffer (0.2% dextrose, pH 6.2) to an optical density of 0.20 OD_{600nm} per ml. Aliquots (20 ml) were placed in new sterile flasks (125 ml) and fatty acids were added to a final concentration of 200 μM (from stocks prepared in ethanol). Following incubation (30 °C and 250 rpm) aliquots were removed and viability was ascertained by plate dilution assays. Plate dilution assays were performed by spotting 2 μl of 1:5 serial dilutions (starting at 0.20 OD_{600nm}/ml) onto YPD plate medium.

2.7. Live cell lipid peroxidation assay

The live cell lipid peroxidation assay was performed as described in [34]. Aliquots (10 ml) were removed from incubator (250 rpm at 30 °C) at the designated time and cells were washed twice with sterile water to remove excess fatty acids. Cells (2 OD_{600nm}) were resuspended in 1 ml 0.10 M phosphate buffer, pH 6.2, 0.2% dextrose, and treated with a 5 μM final concentration of C11-BODIPY(581/591) (Molecular Probes). The C11-BODIPY was dissolved in ethanol and used as a 2 mM stock. After 30 min incubation at room temperature with shaking, cells were collected by centrifugation at 10,000 ×g for 30 s, washed and resuspended in 1 ml 0.10 M phosphate buffer, pH 6.2, 0.2% dextrose. Aliquots (100 μl) were placed into a black, flat-bottomed 96-well plate in quadruplicates. Fluorescence was measured with a 485 nm excitation and a 520 nm emission filter in a Perkin Elmer, 1420 Multi label Counter Victor3, and data was obtained using the Wallac workstation. Cells were visualized by fluorescent microscopy with an Olympus IX70 fluorescence microscope, a 100X oil objective, and using a 490 nm excitation with a 520 nm emission filter (FITC).

2.8. Preparation of mitochondria from S. cerevisiae

The yeast strains were grown in selective media overnight and 1 ml of this culture was transferred to 600 ml YPGal + 0.2% dextrose and incubated with shaking (250 rpm, 30 °C). The cells were harvested at OD_{600nm} between 2 and 3. The crude mitochondria were isolated as described [35], then flash frozen in liquid nitrogen and stored at −80 °C. The protein concentration was measured with a BCA assay (Thermo Scientific).

2.9. Enzymatic assays

NADH-cytochrome *c* reductase activity was measured as described in [36] with the following modifications. The assay was measured spectrophotometrically by monitoring the reduction of cytochrome *c* at an absorbance of 550 nm at 23 °C. The reaction cuvette contained phosphate buffer (6.2 mM K_2HPO_4/33.8 mM KH_2PO_4, pH 6.2), 0.9 mM KCN, 0.14 mM EDTA, 30 μM NADH, and 20.45 μM cytochrome *c*. Samples either contained ethanol as a vehicle control or 1 mM Q_2, added in from a stock in ethanol. The rate of cytochrome *c* oxidation by yeast mitochondria was determined by monitoring the decrease in absorbance at 550 nm at 30 °C. Crude mitochondria (20 μg) were resuspended in 100 μl 0.25 M sucrose and added to 1.5 ml cuvette containing 1 ml phosphate buffer (6.2 mM K_2HPO_4/33.8 mM KH_2PO_4, 0.244% Brij 30, pH 6.2) and 20.45 μM freshly reduced cytochrome *c* [36] in the presence or absence of 0.9 mM KCN. The sample mixture was inverted and the decrease in absorbance at 550 nm was recorded to determine the enzymatic activity using an extinction coefficient of 19.6 mM^{-1} cm^{-1} [36].

2.10. Western blot analysis of His-tagged proteins

Proteins were separated by SDS gel electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a PVDF membrane and blocked with PBS (37 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) containing 3% skimmed milk for 1 h at room temperature. His_6-tagged proteins were detected with chicken polyclonal antisera directed against the His tag and conjugated to horseradish peroxidase (Abcam, ab3553). Prior to use antisera were diluted to 1:1500 in PBS containing 3% skimmed milk and 0.1% Tween 20. Rabbit polyclonal antisera raised against yeast Atp2 were diluted 1:10,000 in PBS containing 3% skimmed milk and 0.1% Tween 20 was used as a loading control. The secondary goat anti-rabbit antibody conjugated to peroxidase (Calbiochem) was diluted 1:10,000 in the same solution as the primary antibody. Binding was visualized with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

2.11. Analyses of Q_6 and Q_6-intermediates in wild-type and coq10 null mutant yeast

The content of Q_6 and detection of Q_6 intermediates was determined as described [29] with the following modifications. To monitor Q_6 and Q_6 intermediates during log phase growth, early-, mid-, and late-log phase growth was first determined from growth curves of W303-1A and W303ΔCOQ10 in DOD medium. Wild-type and *coq10* null mutant strains showed similar values of OD_{600} during early-, mid-, and late-log phase culture. For labeling, yeast strains were grown overnight in SD-complete pre-cultures, which were used to inoculate 50 ml DOD medium to an OD_{600} of 0.05. For early-log phase, cells were labeled at an OD_{600} of 0.5 with 5 μg/ml $^{13}C_6$-pABA, 5 μg/ml $^{13}C_6$-4HB, or ethanol as a vehicle control for 5 h (250 rpm at 30 °C). Mid-log cells were labeled at an OD_{600} of 1.5 and late-log cells at an OD_{600} of 3.0 under the same conditions. Alternatively, Q_6 and Q_6 intermediates were monitored in W303-1A and W303Δ*COQ10* yeast transformants at early-log phase. For these experiments, yeast

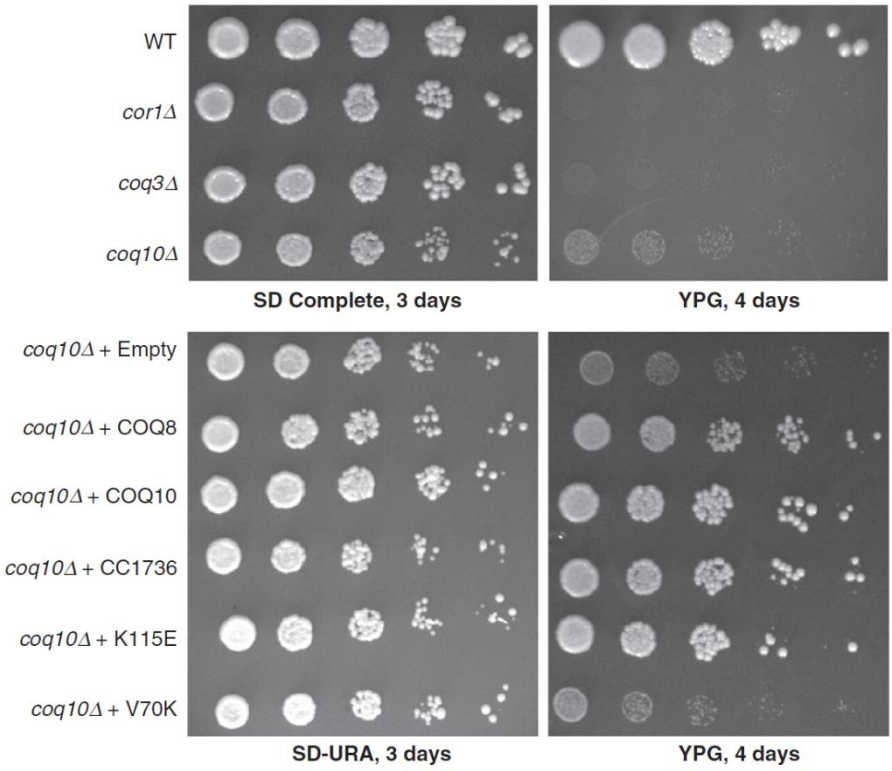


Fig. 2. Complementation of a yeast *coq10* null mutant by expression of *C. crescentus* CC1736 requires the amino acid residue V70 for respiratory function. Wild-type, respiratory deficient *cor1*, yeast Q-less *coq3* null mutant, and the *coq10* null mutant were grown in SD complete medium and harvested during mid-log phase (0.2–1.0 OD_{600nm}). The *coq10* null mutant W303ΔCOQ10 was transformed with each of the following multi-copy plasmids: Empty (pRS426), *COQ8* (p4HN4), *COQ10* (pG140/ST3), CC1736 (pRCM-CC1736), or with plasmids encoding CC1736 with amino acid substitutions K115E (pRCM-K115E) or V70K (pRCM-V70K). Yeast transformants were grown in selective media and harvested during mid-log phase. Cells were washed twice with sterile water and diluted to a final OD_{600nm} of 0.2. Serial dilutions (1:5) were prepared and 2 μl of each sample was spotted onto SD-Complete or SD-Ura, and rich glycerol (YPG) plate medium and incubated at 30 °C for 3 or 4 days, as indicated.

strains were grown overnight in SD − Ura and diluted into 50 ml DOD − Ura media to an OD_{600} of 0.05. Once yeast cultures reached an OD_{600} of 0.5, cells were labeled with either 5 μg/ml $^{13}C_6$-4HB or 5 μg/ml $^{13}C_6$-pABA for 3 h (250 rpm at 30 °C). Q_6 and Q_6 intermediates were also monitored in concentrated cultures. For these experiments, yeast strains were inoculated overnight in selective media and diluted in 100 ml YPD media for a second overnight inoculation. Yeast cells (100 ODs) were harvested at log phase (2.0–4.0 OD_{600nm}) and resuspended in 4 ml DOD media. Cells were incubated with either 40 μg/ml $^{13}C_6$-pABA or $^{13}C_6$-4HB for 2 h (250 rpm at 30 °C).

After labeling, cells were collected by centrifugation at 1000 ×*g* for 5 min, washed twice with sterile water, and lipid extracts were prepared with a 2:1 petroleum ether:methanol extraction as described [29]. The organic phase was transferred to a new borosilicate tube, the extraction with petroleum ether repeated two times, and the pooled organic phase was concentrated under a stream of N_2 gas. A Q_6 standard curve was prepared concurrently and extracted alongside the yeast cell pellet samples. Lipids were resuspended in either 100 or 200 μl of ethanol.

HPLC-MS/MS analyses were performed as described [29] with the following modifications; 20 μl of each lipid extract were injected onto a Luna Phenyl-Hexyl column (100×4.6 mm, 5 μm) and the HPLC mobile phase consisted of Solvent A (methanol:isopropanol; 95:5, with 2.5 mM ammonium formate) and Solvent B (isopropanol, with 2.5 mM ammonium formate). Initial conditions were 100% Solvent A and linearly decreasing to 95% at 8 min with a flow rate of 0.8 ml/min. In each sample the amount of analyte was corrected for recovery of the Q_4 internal standard. Samples were analyzed using a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA). Analyst 1.4.2 software (Applied Biosystems) was used for data acquisition and analysis. Multiple reaction monitoring (MRM) mode was used for detection of Q_6 and Q_6 intermediates.

2.12. Induction and purification of C. crescentus CC1736 polypeptide

E. coli strain BL21 (DE3) pMgK was transformed with pCcR19-21.1 or pET-CC1736K115E. Transformants were recovered on LB + Amp + Kan plate media and then transferred to 5 ml of LB Broth Miller medium containing 100 μg/ml ampicillin and 60 μg/ml kanamycin and grown overnight with aeration (250 rpm at 37 °C). The cells were diluted in 2 l of the same medium and grown for 2–3 h (250 rpm, 21 °C). Following incubation 1 mM IPTG was added to the culture and incubation proceeded for 16 h. Cells were collected by centrifugation at 5000 ×*g*

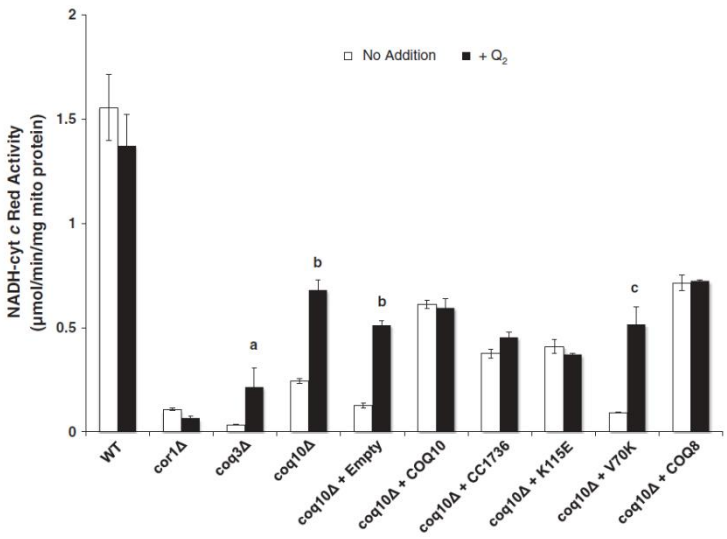


Fig. 3. Mitochondria were isolated from wild type, respiratory deficient *cor1* null mutant, Q-less *coq3* null mutant, Q-replete *coq10* null mutant and *coq10* null mutant harboring plasmids expressing the designated proteins. NADH-cytochrome *c* reductase activity was determined in the absence (*white bars*) or presence (*black bars*) of 1 μM coenzyme Q_2 (performed in triplicate for each sample). NADH-cytochrome *c* activity of the yeast *coq3* null mutant, *coq10* null mutant and *coq10* null mutant expressing empty vector and V70K is significantly rescued by the addition of 1 μM coenzyme Q_2 as determined by the Student's one-tailed t-test; a, $p<0.0175$; b, $p<6.1\ E-04$; c, $p<3.3\ E-03$. Values are given as the average ± standard deviation.

for 10 min, and then washed twice and resuspended in 50 ml of lysis buffer (50 mM NaH_2PO_4, pH 8, 10 mM imidazole, 500 mM NaCl, 5% glycerol, 0.1% Triton X-100, 5 mM BME, 1 mM protease inhibitor) at 4 °C with stirring for 45 min. The cells were then lysed by sonication (Fisher Sonic Dismembrator, Model 300; 6 cycles, 45 s bursts, 45 s on ice) at 35% duty cycle. Unbroken cells and inclusion bodies were removed by centrifugation (12,400 ×g, 15 min, 4 °C) and the supernatant was applied to 8 ml Ni-NTA superflow resin column (Qiagen, Valencia, CA) at 4 °C. The column was washed with 12 column volumes of buffer A (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 20 mM imidazole, 5% glycerol), proceeded by a second wash with 5 column volumes of 50 mM imidazole in buffer A, and a third wash with 3 column volumes of 100 mM imidazole in buffer A. The His-tagged CC1736 polypeptide was eluted with 500 mM imidazole in buffer A (Thermo Scientific, Rockford, IL). The protein was concentrated to 1.5 ml with an Amicon Ultra centrifugal filter device (10,000 MWCO, Millipore). The concentrated protein was then applied to a Superdex 75 (GE Healthcare), size exclusion column (75 × 1.5 cm), equilibrated in the mobile phase (20 mM MES, pH 6.5, 100 mM NaCl, 5.0 mM $CaCl_2$, 1.0 mM TCEP, 0.1 mM dodecyl maltoside, 0.02% NaN_3, pre-filtered through a 0.2 μm filter membrane) to separate the CC1736 polypeptide from other contaminating proteins.

Table 3
Cytochrome *c* oxidase activity assay.

	Cytochrome *c* oxidase (μmol/min/mg protein)
W3031B	0.652 (±0.184)
cor1Δ	0.572 (±0.045)
coq3Δ	0.172 (±0.019)
coq10Δ	0.230 (±0.016)
coq10Δ+empty	0.288 (±0.051)
coq10Δ+COQ10	0.403 (±0.073)
coq10Δ+CC1736	0.470 (±0.066)
coq10Δ+K115E	0.372 (±0.073)
coq10Δ+V70K	0.184 (±0.124)
coq10Δ+COQ8	0.603 (±0.036)

2.13. In vitro Q-binding assay

Each binding assay contained 2–3 nmol purified *C. crescentus* CC1736 and 8–10 nmol cytochrome *c* polypeptide. The ligands tested included: Q_2, Q_{10}, and ergosterol (all from Sigma), Q_3 (prepared as described [37]), demethoxy-Q3 (DMQ_3, prepared as described [28]), and 3-farnesyl-4-hydroxybenzoate (FHB, prepared as described [38]). Each compound (concentration range from 0.005 to 0.27 mM) was mixed well with either CC1736 or cytochrome *c* in 20 mM MES buffer, pH 6.4, containing 100 mM NaCl, 5.0 mM $CaCl_2$, 0.1 mM dodecyl maltoside, 1.0 mM TCEP, and 2% ethanol. Samples were incubated for 45 min at 30 °C and centrifuged (1300 ×*g*, one min). The supernatant was applied to a protein-desalting–spin-column (PIERCE, catalog number 89862), pre-equilibrated with binding buffer to separate protein-bound-ligand from unbound ligand. Samples were subjected to centrifugation (1300 ×*g*, one min) and the protein concentration was determined by the Lowry assay [39]. Prior to lipid extraction, 3 nmol of Q_4 (for samples testing binding of Q_2, Q_3, DMQ_3, or FHB) or 3 nmol Q_9 (for samples testing binding of Q_{10} and ergosterol) was added as an internal standard to each sample (65 μl). Six calibration standards were prepared concurrently and contained the internal standard and experimental ligand over a range from 0.2 to 2.2 nmol to generate a calibration curve. Saturated sodium chloride (1 ml) and hexanes:2-propanol (v:v, 3:2) (2 ml) was added to each sample and vortex-mixed for 45 s at top speed. The organic phase was transferred to a new borosilicate tube and concentrated down under a stream of N_2 gas. Lipids were resuspended in 90 μl of ethanol, and 70 μl of each lipid extract was manually injected into a reversed-

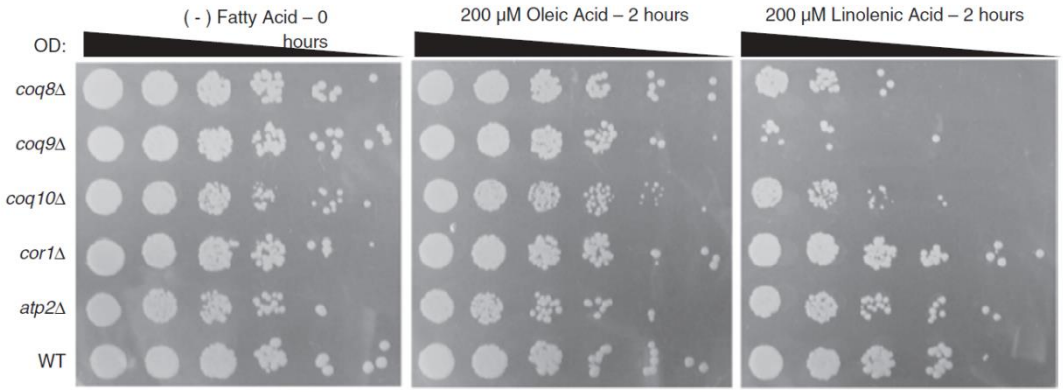


Fig. 4. Yeast *coq8*, *coq9*, and *coq10* mutants are hypersensitive to treatment with αLnn. Yeast strains were grown in YPD media and harvested during mid-log phase (0.2–1.0 OD_{600nm}). The *cor1* and *atp2* null mutants serve as respiratory deficient controls. Cells were washed twice with sterile water and resuspended in phosphate buffer to a final OD_{600nm} of 0.2. The designated fatty acids (final concentration of 200 μM) were added to a flask containing 20 ml of yeast/phosphate buffer as described in Section 2.6. Samples were removed either before addition of fatty acids (0 h control) or after 2 h of incubation at 30 °C in the presence of the designated fatty acid. Serial dilutions (1:5) were prepared and 2 μl were spotted onto YPD plate medium. Images were taken after two days of growth at 30 °C.

phase HPLC system [40]. Samples were applied to a Luna Hexa-phenyl column (100 × 4.6 mm, 5 μm) and separated with an isocratic mobile phase (1 ml/min; 88:24:10 MeOH/EtOH/2-propanol), followed by UV detection at the designated wavelength. Lipid ligands were detected by UV absorption: 275 nm for Q_2, Q_3, and Q_{10}; 271 nm for DMQ_3; 260 nm for FHB; and 272 nm for ergosterol. In each sample the amount of analyte was corrected for recovery of the Q_n internal standard. The number of mol ligand bound: mol polypeptide, and dissociation constant of ligand was determined with the Km calculator from Graph Pad Prism.

3. Results

3.1. Expression of C. crescentus CC1736 START domain polypeptide restores respiration in the yeast coq10 mutant

The yeast *coq10* null mutant shows very slow growth on YPG medium (containing glycerol as the sole non-fermentable carbon source) (Fig. 2). This phenotype is less severe than that of other respiratory deficient mutants, such as *cor1* or *coq3* null yeast mutants [10]. Over-expression of the *C. crescentus* CC1736 START domain

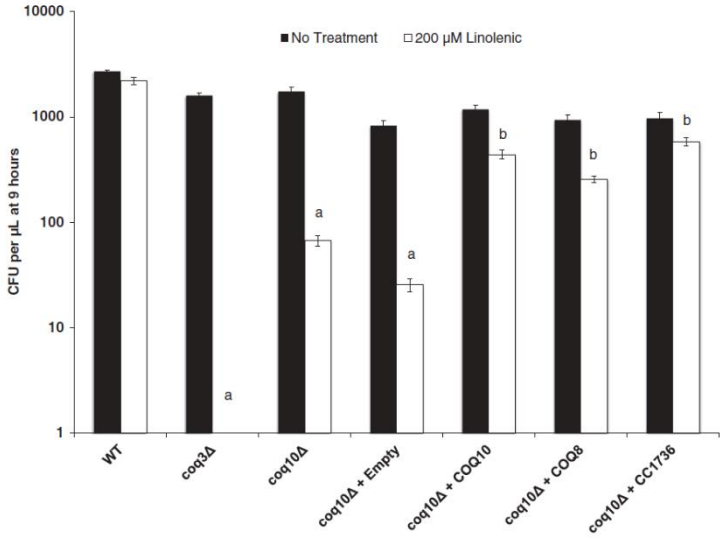


Fig. 5. Yeast *coq10* null mutants expressing Coq8p, Coq10p, or CC1736 are resistant to treatment with αLnn. The fatty acid sensitivity assay was performed as described in Fig. 4 except three 100 μl aliquots were removed at 9 h of either no treatment or 200 μM αLnn. Dilutions were prepared, and then spread onto SD-complete or SD − Ura plate medium. The chart shows the number of colony forming units (CFU) of untreated (black) and αLnn-treated (white) yeast cells. Yeast *coq3Δ*, *coq10Δ*, and *coq10Δ* null mutants harboring empty vector are profoundly sensitivity to PUFA treatment as compared to PUFA treated wild-type yeast as determined by the Student's two-tailed *t*-test; a, $p<4.3\ E-07$. Yeast *coq10* null mutants expressing Coq8p, Coq10p, or CC1736 are resistant to PUFA treatment as compared to *coq10* null mutants expressing empty vector; b, $p<2.6\ E-04$. For all analyses the significance level α was adjusted to 0.00357 according to the Bonferroni correction.

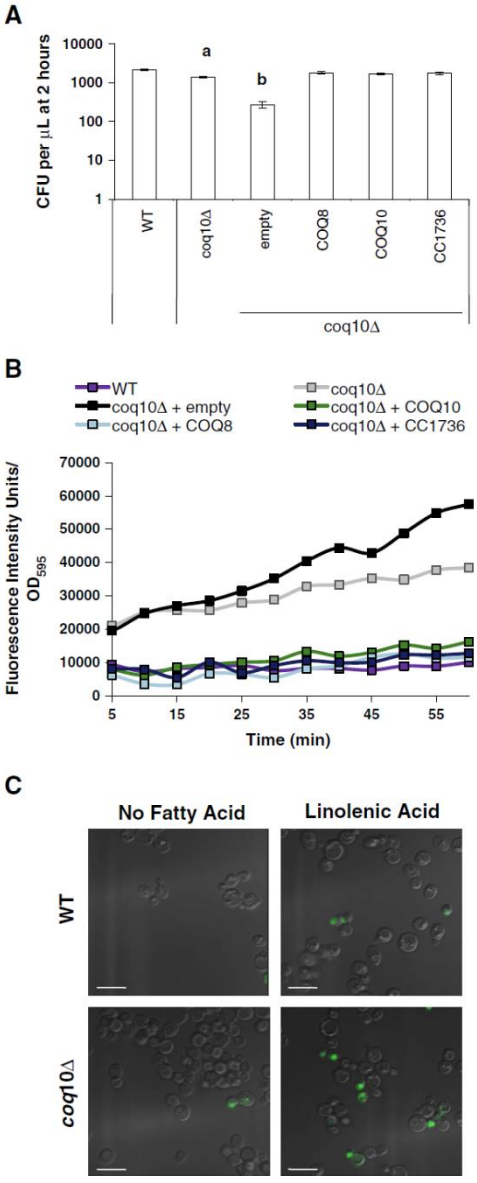


Fig. 6. Sensitivity of *coq10* null mutant to αLnn treatment is due to increased levels of lipid peroxidation. (*A*) Wild-type cells were treated with αLnn for 2 h and three 100 µl aliquots were removed and spread onto YPD plates after dilution. The chart shows the CFU per µl. Yeast *coq10*, and *coq10* null mutants harboring empty vector are sensitive to PUFA treatment as compared to PUFA treated wild-type yeast as determined by the Student's two-tailed *t*-test; **a**, p<6.8E-04, **b**, p<1.3E-05. The significance level α was adjusted to 0.01 according to the Bonferroni correction. (*B*) Following αLnn treatment, yeast cells were treated with 5 µM C11-Bodipy 581/591 for 30 min at room temperature. Four 100 µl aliquots were plated in a 96-well plate and the fluorescence was measured by fluorimetry. (*C*) Lipid peroxidation within cells was examined as described in (*B*) except cells were visualized by fluorescent microscopy. Green fluorescence indicates increased levels of lipid peroxidation. Scale bar = 6.6 µm.

polypeptide (Fig. 1) harboring the 35-residue amino-terminal mitochondrial targeting sequence from Coq3 [30], restores growth of the *coq10* null mutant on YPG medium, comparable to that mediated by expression of yeast Coq10p or Coq8p (Fig. 2). This result identifies CC1736 polypeptide as a functional ortholog of yeast Coq10p.

To identify residues important for function of CC1736, two amino acid substitutions were introduced, V70K and K115E. These residues are conserved among many of the Coq10 homologues (Fig. 1) and were predicted to be important for ligand binding by the START domain of CC1736 [14]. These mutations were introduced into the pRCM-CC1736 construct as described in Section 2.4. Expression of the CC1736-V70K polypeptide failed to rescue the yeast *coq10* null mutant growth on glycerol, while expression of CC1736-K115E retained ability to rescue (Fig. 2).

The defect in NADH-cytochrome *c* reductase activity in *coq10* or *coq3* mutant mitochondria can be partially rescued by addition of Q_2 (a soluble Q analog with a tail of two isoprene units) (Fig. 3) [10]. In contrast, Q_2 does not augment NADH-cytochrome *c* reductase activity in either wild-type yeast or the rescued *coq10* strains (Fig. 3). Over-expression of Coq8p, Coq10p, or CC1736 in the *coq10* null mutant provided a significant rescue of the NADH-cytochrome *c* reductase activity in isolated crude mitochondria (Fig. 3). Mitochondria isolated from *coq10* null yeast harboring V70K, also exhibited profound defects in NADH-cytochrome *c* reductase activity that were rescued by Q_2 (Fig. 3). Although the addition of Q_2 did not restore NADH-cytochrome *c* reductase activity levels to that of wild type, this is likely due to the tendency of the *coq10* mutant to lose mitochondrial DNA [10], and is evident from a decrease in the cytochrome *c* oxidase activity (Table 3).

The inability of the V70K polypeptide to rescue is not due to failure of expression but may be due to the inability of being processed correctly. A western blot (Fig. S1) shows that the V70K polypeptide is not processed in the same manner as either the CC1736 wild type or K115E polypeptides. These results suggest that the V70K mutation interferes or prevents the processing of the amino terminal mitochondrial leader sequence fused to CC1736.

3.2. The coq10 mutant is sensitive to PUFA treatment

The yeast *coq* mutants are very sensitive to treatment with PUFAs, due to the lack of Q_6/Q_6H_2 antioxidant protection [4,41]. As shown in Fig. 4, the yeast *coq8*, *coq9*, and *coq10* mutants treated with linolenic acid (αLnn) for 2 h showed higher sensitivity as compared to wild-type yeast or yeast treated with oleic acid. While the sensitivity of the *coq8* and *coq9* mutants to PUFA stress is predicted due to their lack of Q_6, the *coq10* null mutant has near normal amounts of Q_6 in mitochondria [10]. The sensitivity of the *coq10* null cannot be attributed to a lack of respiration per se, because the *cor1* and *atp2* mutants, with defects in complex III or V, respectively, remain resistant to PUFA treatment (Fig. 4). To understand the requirement of Coq10p for the antioxidant function of Q/QH_2, we decided to further characterize the nature of the PUFA sensitivity of the *coq10* mutant.

3.3. Over-expression of Coq10p, Coq8p, or CC1736 rescues the sensitivity of the yeast coq10 null mutant to PUFA stress and suppresses accumulation of lipid peroxidation products

As shown in Fig. 5, αLnn stress causes a ten-fold decrease in the *coq10* mutant cell viability (colony forming units or CFU) as compared to either αLnn-treated wild-type yeast or to untreated control. The CFU assay is more quantitative than the plate dilution assay, and shows that although the *coq10* mutant is sensitive to PUFA treatment, it is less sensitive than the Q-less *coq3* mutant. The sensitivity of the *coq10* mutant to PUFA treatment is rescued by over-expression of Coq10p, Coq8p, or *C. crescentus* CC1736 (Fig. 5).

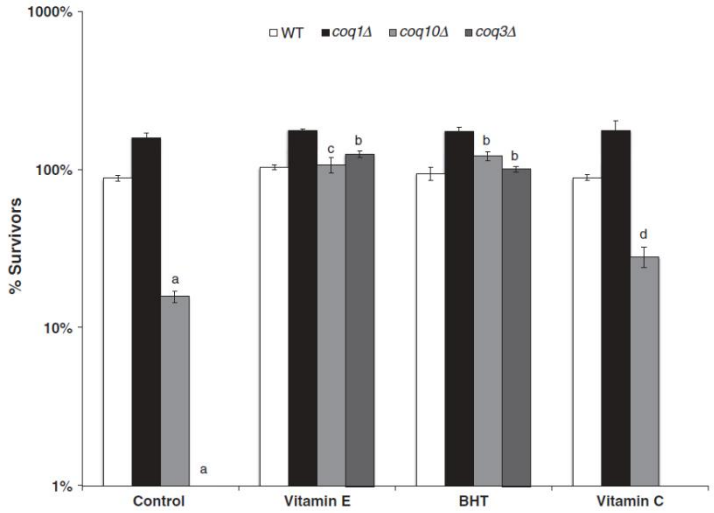


Fig. 7. Treatment of yeast *coq10* null mutants with BHT, vitamin E, or vitamin C rescues the αLnn toxicity. The fatty acid sensitivity assay and statistical analyses were as described for Fig. 3, except yeast were treated with designated antioxidant compounds prior to the addition of αLnn. Three 100 μl aliquots were removed at 4 h, and following dilution, spread onto YPD plates. Cell viability was assessed by colony count. Yeast strains include wild type (*white*), *cor1* (*black*), *coq10* (*light gray*), or *coq3* (*dark gray*). In the absence of antioxidants, the number of surviving αLnn treated *coq3* and *coq10* null mutant cells is significantly lower as compared to wild type (a, $p<1.6\ E-05$). In the presence of lipid soluble antioxidants, the number of surviving αLnn treated *coq3* and *coq10* null mutant cells is significantly higher as compared to no antioxidant treatment (b, $p<2\ E-05$, c, $p<1.1\ E-04$). The water-soluble antioxidant, vitamin C failed to rescue *coq3* mutant cells hypersensitivity to αLnn treatment. In contrast, vitamin C afforded partial protection of the *coq10* mutant cells hypersensitivity to αLnn treatment (d, $p<3.9\ E-03$). For all analyses the significance level α was adjusted to 0.0071 according to the Bonferroni correction.

To assess the level of lipid peroxidation, yeast *coq10* null mutants were treated with C11-BODIPY(581/591), a lipophilic dye that fluoresces upon oxidation by lipid peroxidation products. The oxidation of C11-BODIPY(581/591) causes a fluorescent shift from red to green and is a qualitative indicator of lipid peroxidation in living cells [42]. αLnn-treated yeast *coq10* mutants showed a dramatic increase in fluorescent intensity as compared to αLnn-treated wild-type cells (Fig. 6B and C). The increased levels of lipid peroxidation in the *coq10* null mutant are not due to complete loss of cell viability because after 2 h of incubation with PUFA roughly 70% remain viable (Fig. 6A). The lipid peroxidation levels in the yeast *coq10* null mutant treated with PUFA are decreased by the over-expression of Coq8p, Coq10p, or *C. crescentus* CC1736. These results indicate that Coq10p is required for the ability of Q to function as a lipid soluble chain-terminating antioxidant.

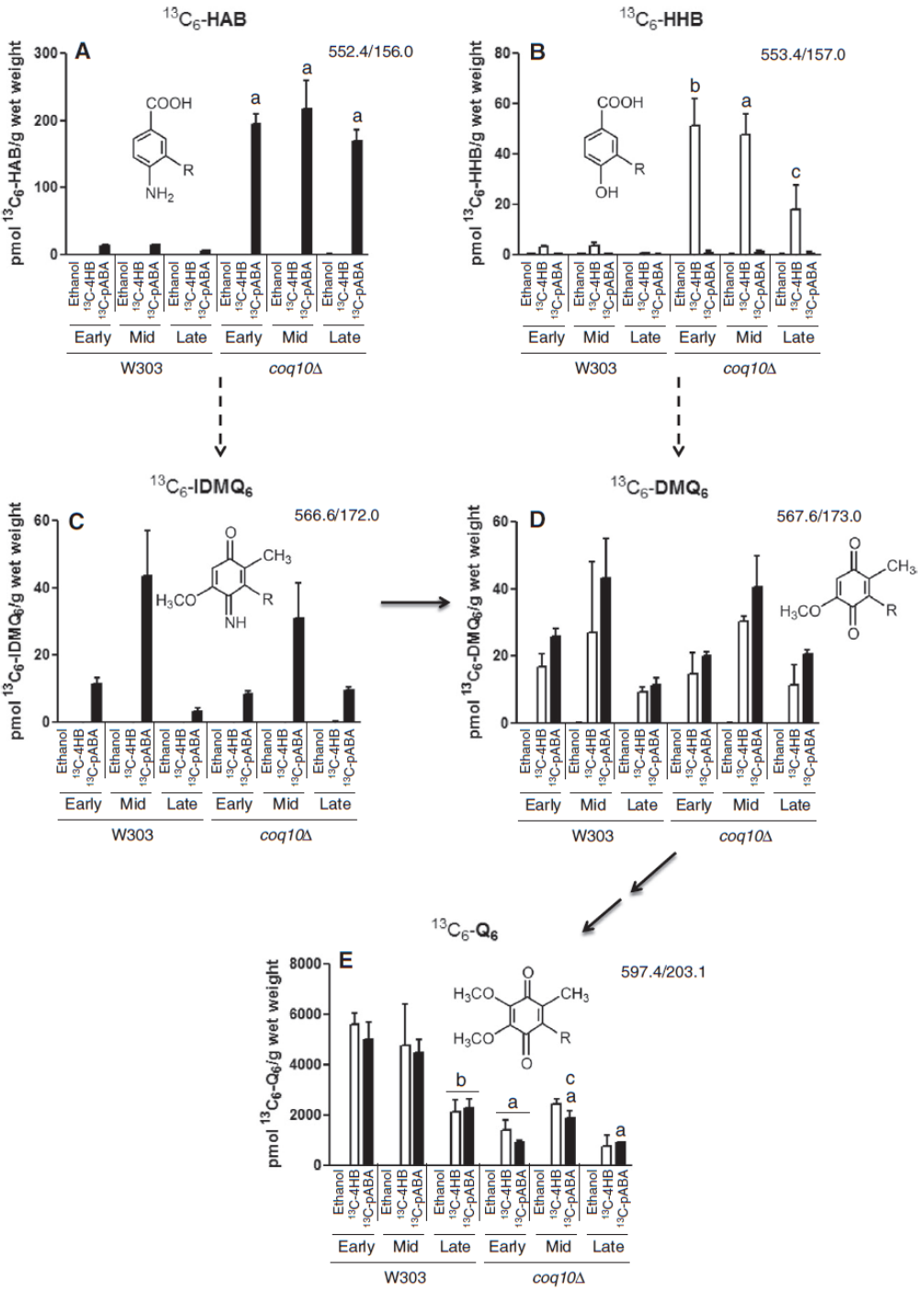
3.4. The coq10 mutant sensitivity to PUFA treatment is rescued by antioxidants

Previous studies have shown that the PUFA sensitivity of the Q-less *coq* mutants can be rescued by the addition of lipid soluble chain terminating antioxidants, such as vitamin E and BHT [4,5]. The sensitivity of the yeast *coq10* null mutants is also fully rescued by the addition of these lipid soluble antioxidants (Fig. 7). This rescue of PUFA sensitivity in the *coq10* mutant demonstrates that the function of Q as a lipid chain-terminating antioxidant depends on the presence of the Coq10p polypeptide. Interestingly, the water-soluble antioxidant vitamin C failed to rescue the αLnn hypersensitivity of the *coq3* null mutant (Fig. 7). However, vitamin C provides partial but significant rescue to *coq10* null mutant αLnn sensitivity. These results suggest that vitamin C may function to regenerate the Q_6H_2 hydroquinone in the *coq10* mutant and partially restore the ability of Q to function as an antioxidant.

3.5. Yeast coq10 null mutants accumulate coenzyme Q intermediates and show decreased de novo synthesis of Q_6

Previous studies have shown that the yeast *coq10* null mutant mitochondria, when isolated from early stationary phase cultures in YPGal medium, contain near normal levels of Q_6 relative to wild-type yeast [10]. To determine the efficiency of *de novo* Q_6 biosynthesis, yeast *coq10* null mutants were labeled with either $^{13}C_6$-pABA or $^{13}C_6$-4HB, two aromatic ring precursors of Q biosynthesis (Fig. 8). Incorporation

Fig. 8. Yeast *coq10* null mutants have decreased *de novo* synthesis of $^{13}C_6$-labeled Q_6 compared to wild type, most notably during early-log phase growth. Wild-type and *coq10* null yeast strains were cultured in SD-complete medium overnight and diluted to an OD_{600nm} of 0.05 in DOD-complete medium. Ethanol (vehicle-control), $^{13}C_6$-4HB (*white bars*), or $^{13}C_6$-pABA (*black bars*) were added to yeast cultures at an OD_{600nm} of 0.5 (early-log phase), 1.5 (mid-log phase), or 3.0 (late-log phase). Prior to lipid extraction a known amount of Q_4 was added as an internal standard to each sample and to the Q_6 calibration standards. $^{13}C_6$-labeled precursor-to-product ion transitions are as follows: (*A*) $^{13}C_6$-HAB, 552.4/156.0; (*B*) $^{13}C_6$-HHB, 553.4/157.0; (*C*) $^{13}C_6$-IDMQ$_6$, 566.6/172.0; (*D*) $^{13}C_6$-DMQ$_6$, 567.6/173.0; (*E*) $^{13}C_6$-Q$_6$, 597.4/203.1. Dashed arrows leading from HAB to IDMQ$_6$ and from HHB to DMQ$_6$ indicate multiple steps of Q biosynthesis (requiring Coq3, Coq4, Coq5, and Coq6). Solid arrows indicate the deimination of IDMQ$_6$ to DMQ$_6$ (requiring Coq9), and the final two steps converting DMQ$_6$ to Q$_6$ (requiring Coq7 and Coq3). Error bars depict the average ± standard deviation ($n=4$). (The total content of Q_6 and Q$_6$-intermediates ($^{13}C_6$-labeled + unlabeled), is depicted in Fig. S2). Statistical significance was determined with the two-tailed Student's t-test and lower-case letters above bars are indicative of statistical significance. In (*A*) and (*B*) the relative content of $^{13}C_6$-labeled HAB or HHB in the *coq10* null during early-, mid-, or late-log phase growth were compared to the corresponding growth phase of the wild type (a, $p<0.0001$; b, $p=0.0001$; c, $p=0.0112$). The significance level α was adjusted to 0.0167 according to the Bonferroni correction for both (*A*) and (*B*). In (*E*) the content of $^{13}C_6$-labeled Q_6 in the *coq10* null during early-, mid-, or late-log phase growth was compared to amounts present in the corresponding growth phase of the wild type (a, $p\leq0.0004$). Additionally $^{13}C_6$-labeled Q_6 in mid- and late-log phase was compared to early-log phase for both wild type and the *coq10* null. Labeled $^{13}C_6$-Q_6 in wild type at late-log phase was significantly different compared to early-log phase (b, $p\leq0.0004$), and labeled Q_6 in the *coq10* null in mid-log phase was significantly different compared to early-log phase (c, $p=0.0008$ for $^{13}C_6$-pABA). For all analyses in (*E*) the significance level α was adjusted to 0.0033 according to the Bonferroni correction.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A
552.4/156.0
pmol $^{13}C_6$-HAB/g wet weight
COOH
R
NH_2
$^{13}C_6$-HHB
B
553.4/157.0
pmol $^{13}C_6$-HHB/g wet weight
COOH
R
OH
$^{13}C_6$-IDMQ$_6$
C
566.6/172.0
pmol $^{13}C_6$-IDMQ$_6$/g wet weight
CH_3
H_3CO
R
NH
$^{13}C_6$-DMQ$_6$
D
567.6/173.0
pmol $^{13}C_6$-DMQ$_6$/g wet weight
CH_3
H_3CO
R
$^{13}C_6$-Q$_6$
E
597.4/203.1
pmol $^{13}C_6$-Q$_6$/g wet weight
H_3CO
CH_3
H_3CO
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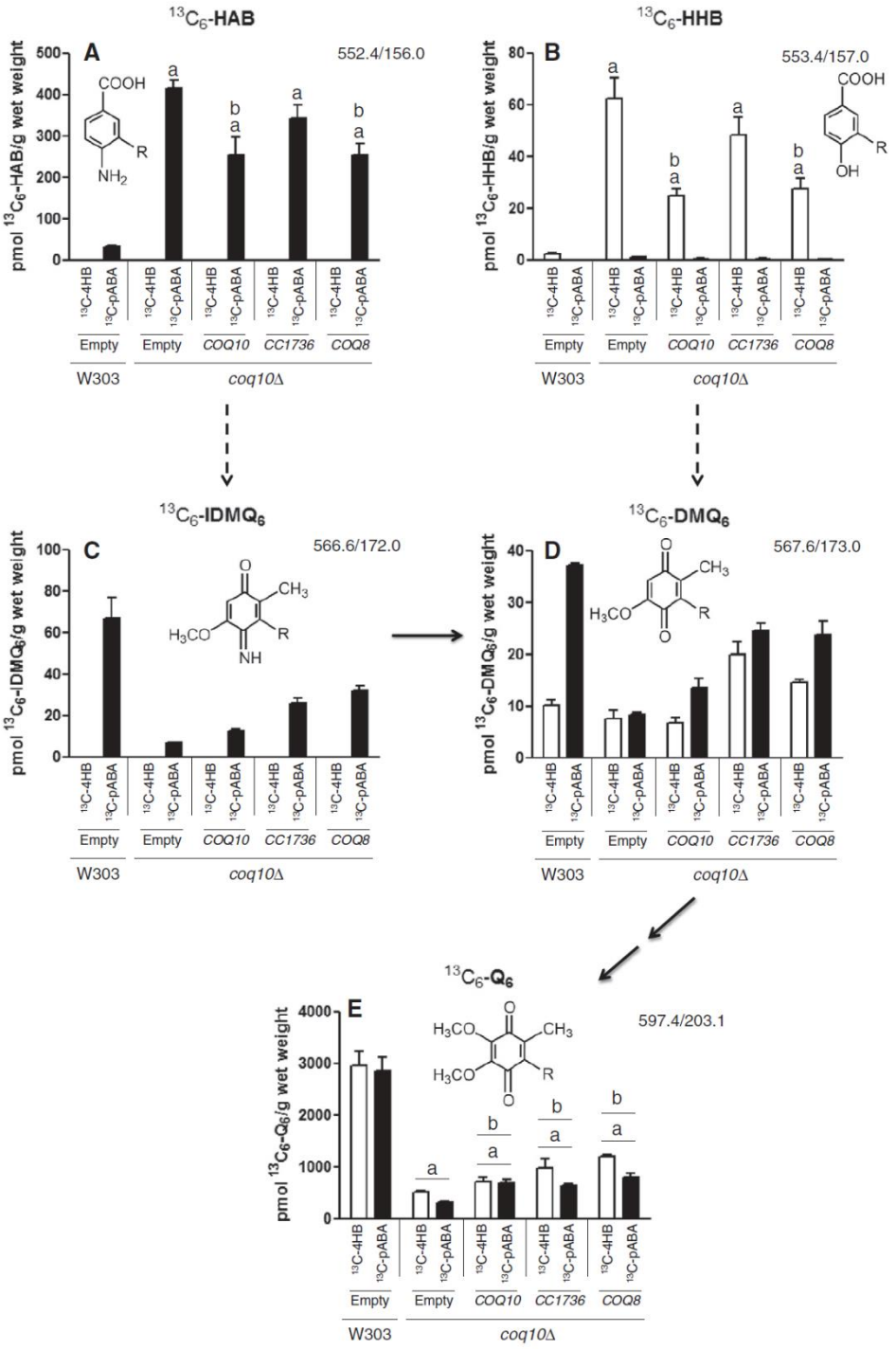
Ethanol
^{13}C-4HB
^{13}C-pABA
Early
Mid
Late
W303
coq10Δ


$^{13}C_6$-HAB
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$^{13}C_6$-IDMQ$_6$
C
566.6/172.0
pmol $^{13}C_6$-IDMQ$_6$/g wet weight
$^{13}C_6$-DMQ$_6$
D
567.6/173.0
pmol $^{13}C_6$-DMQ$_6$/g wet weight
$^{13}C_6$-Q$_6$
E
597.4/203.1
pmol $^{13}C_6$-Q$_6$/g wet weight
^{13}C-4HB
^{13}C-pABA
Empty
COQ10
CC1736
COQ8
W303
coq10Δ

of $^{13}C_6$-ring precursors into *de novo* Q_6 decreased as a function of growth phase in the wild type, with the greatest incorporation occurring at early-log phase and the lowest at late-log phase. In contrast *de novo* Q_6 synthesis in the *coq10* null mutant, while lower than wild type at early-log phase, did not further decrease as cells progressed to mid- or late-log phase (Fig. 8E). At early log phase, the yeast *coq10* null mutant incorporates nearly 80% less of either of the aromatic ring precursors into $^{13}C_6$–Q_6 as compared to wild type, indicating that Q-biosynthesis in the *coq10* null mutant is impaired (Fig. 8E). At late log phase, the difference between the *coq10* null and wild-type cells is less dramatic, but the trend of impaired *de novo* Q_6 biosynthesis in the *coq10* null mutant is still readily apparent. Unlabeled ^{12}C-Q_6 content displayed a similar trend, decreasing in wild type as cells progressed from early- to late-log phase, while remaining relatively constant in the *coq10* null mutant independent of growth phase (Fig. S2E). The addition of ring precursors boosted the total content of Q_6, an effect that was more dramatic in the wild-type as compared to the *coq10* mutant cells. These results indicate that the *coq10* mutant has impaired Q_6 biosynthesis and a lower content of Q_6, and these defects are most obvious when the measurements are performed on cells during early log phase growth.

The low content of newly synthesized Q_6 in the *coq10* null mutant suggested that Q_6-intermediates might accumulate in this mutant. To determine this, the lipid extracts were examined for the presence of $^{13}C_6$-Q_6 intermediates. The relative amounts of early (HAB and HHB), and late stage (IDMQ$_6$ and DMQ$_6$) intermediates are shown in Fig. 8 (panels A–D). These values are an approximation because the chemical standards required to quantify their detection by mass spectrometry are not available. However, the relative amounts can be compared. Relative to wild-type cells, the yeast *coq10* null mutant accumulates high levels of early Q_6-intermediates ($^{13}C_6$-HHB and $^{13}C_6$-HAB), and both the ^{12}C- (Fig. S2) and $^{13}C_6$-compounds (Fig. 8A and B) are detected.

The respiratory deficiency and PUFA sensitivity of the *coq10* null mutant is rescued by over-expression of Coq8p, CC1736, or Coq10p. To examine whether such over-expression rescues the defect in *de novo* Q synthesis, *coq10* transformants were incubated with the $^{13}C_6$–4HB and $^{13}C_6$-pABA ring precursors for 3 h during early log phase growth. Over-expression of Coq8p in the *coq10* null mutant increased the amount of *de novo* synthesized $^{13}C_6$-Q_6 by more than 130% as compared to the *coq10* null mutant harboring empty vector (Fig. 9E and Fig. S3). Similarly, over-expression of CC1736 in the *coq10* null mutant increased the content of *de novo* synthesized $^{13}C_6$-Q_6 by more than 90%. While over-expression of Coq10p increased the content of newly synthesized $^{13}C_6$-Q_6 from $^{13}C_6$-pABA by 110%, the stimulation of synthesis in the $^{13}C_6$–4HB-labeled cells was only 39% as compared to the *coq10* null. While these increases in *de novo* Q_6 content were significant, it was surprising that none of the *coq10* null transformants showed restoration of newly synthesized $^{13}C_6$-Q_6 to wild-type levels.

Thus, we performed similar labeling analyses during late log phase (Fig. S4). During late log phase over-expression of yeast Coq10p in the *coq10* null mutant nearly doubles the amount of $^{13}C_6$-ring precursor incorporated into $^{13}C_6$-Q_6 as compared to wild-type yeast, and over-expression of Coq8p in the *coq10* null mutant restores the amount of *de novo* synthesized $^{13}C_6$-Q_6 to that of wild type. It is important to note that at this stage, the wild-type cells showed a five-fold decrease in total Q_6 content as compared to wild-type cells assayed during early log phase (compare Figs. S2 and S4). It is evident that Q_6 content in wild-type yeast cells varies dramatically as a function of the growth phase and culture conditions. Thus, depending on the culture conditions, *coq10* null mutant yeast can appear to have Q content that is not significantly different from wild-type cells (Fig. S4).

Although over-expression of Coq10p, CC1736, and Coq8p increased the amount of *de novo* $^{13}C_6$-Q_6, all *coq10* null transformants continued to accumulate the early Q_6-intermediates HAB and HHB (Figs. 9 and S3). This was also true for the late log phase cells (data not shown). Thus the presence of each of these multi-copy plasmids in the *coq10* null mutant appears to impede the normal progression of Q biosynthetic steps, resulting in the accumulation of Q_6-intermediates.

3.6. Q-binding by the purified CC1736 START domain polypeptide

Because *S. cerevisiae* Coq10p is prone to aggregation [10], we took advantage of the previously described purification of the *C. crescentus* CC1736 protein with carboxyl-terminal six-His tag (Section 2.12 and [32]). The molecular mass of the isolated CC1736 and K115E polypeptides and their tryptic peptide fragments were in agreement with the theoretical masses predicted from the respective amino acid sequences (Fig. S5). Despite many attempts, we were not able to over-express or purify the CC1736-V70K polypeptide.

We developed an *in vitro* binding assay (as described in Section 2.13) to determine whether purified CC1736-His8 polypeptide has Q_n binding activity (Fig. 10). Because of the very low solubility of Q_{10}, it was not possible to determine B_{max} or K_d for this ligand (Table 4). However, CC1736 binding of Q_3 and Q_2 saturates at a molar ratio close to 1:1 (Table 4). CC1736 does not bind ergosterol (Fig. 10B), and an unrelated mitochondrial polypeptide (horse heart cytochrome *c*) lacks Q-binding, indicating specificity for Q in this binding assay. To examine whether CC1736 can bind intermediates of Q biosynthesis, DMQ$_3$ and FHB were tested as ligands in the *in vitro* binding assay. The results indicate that CC1736 can bind DMQ$_3$, a farnesylated analog of DMQ$_6$, but is unable to bind to FHB, a farnesylated analog of an early intermediate of Q biosynthesis (Fig. 10B).

4. Discussion

Previous studies showed the yeast *coq10* mutant to be Q_6-replete, yet its respiratory defect was rescued by the addition of Q_2, a soluble analog of Q_6. This is a hallmark phenotype of the *coq1–coq9* mutants that lack Q_6 [10]. Thus, the role of Coq10p in facilitating the function of Q_6 in respiratory electron transport poses an important and intriguing question. In this study we take advantage of the structurally characterized *C. crescentus* CC1736 START domain polypeptide [14]. We show that expression of CC1736 rescues the respiratory defects of the *coq10* mutant, and hence functions as an ortholog of yeast Coq10p. Since human Coq10p also functions to restore respiration of the *coq10* yeast mutant [10], Coq10p/CC1736 plays a conserved and essential role in facilitating the function of Q in respiration, from prokaryotes to eukaryotes.

C. crescentus CC1736 and the eukaryotic homologs of Coq10p belong to a family of lipid transfer proteins that contain a START domain. To date, Coq10p is the only identified START domain protein

Fig. 9. *De novo* synthesis of $^{13}C_6$-Q_6 in yeast *coq10* null mutants is rescued upon transformation with *COQ10, CC1736* or *COQ8*. The designated yeast strains were cultured in SD − Ura medium overnight, and diluted to an OD_{600nm} of 0.05 in DOD − Ura medium. $^{13}C_6$-4HB (white bars) or $^{13}C_6$-pABA (black bars) was added to yeast cultures at an OD_{600nm} of 0.5, corresponding to early-log phase. Lipid extraction of samples and analysis of precursor-to-product transitions was performed as described in the Fig. 8 legend. $^{13}C_6$-Q_6 and $^{13}C_6$-labeled Q_6-intermediates are shown as in Fig. 8. The bars depict the average ± standard deviation (n = 4). (The total content of Q_6 and Q_6-intermediates ($^{13}C_6$-ring-labeled + unlabeled) is depicted in Fig. S3). Statistical significance was determined with the two-tailed Student's t-test and lower-case letters above bars are indicative of statistical significance. In (*A*) and (*B*) the relative content of $^{13}C_6$-labeled HAB or HHB in each of the *coq10* null transformants was compared to wild type (a, $p < 0.0001$). The relative content of $^{13}C_6$-labeled HAB or HHB between the *coq10* null with empty vector and the other *coq10* null transformants was also compared (b, $p \leq 0.0003$). In (*A*) and (*B*), the significance level α was adjusted to 0.005 according to the Bonferroni correction. In (*E*) the content of $^{13}C_6$-labeled Q_6 in each of the *coq10* null transformants was compared to wild type (a, $p < 0.0001$). The relative content of $^{13}C_6$-labeled Q_6 between the *coq10* null transformed with empty vector and the three other *coq10* null transformants was also compared (b, $p \leq 0.0024$). For all analyses in (*E*) the significance level α was adjusted to 0.005 according to the Bonferroni correction.

in *S. cerevisiae* [10,43]. There are 15 identified members of the START domain protein family in mammals [44]. Members of the START domain superfamily function to transfer lipids between sub-cellular compartments, regulate lipid cell signaling events, and serve important roles in lipid metabolism [43]. Here we developed an *in vitro* binding assay and showed that CC1736 binds Q_2 or Q_{10} in an equimolar ratio (Fig. 10). The absence of specificity for the length of the polyisoprenoid tail suggests that the binding site of CC1736 primarily recognizes the benzoquinone head group of Q. Our findings that CC1736 fails to bind either ergosterol or FHB, a farnesylated analog of an early Q-intermediate, are consistent with this idea. We also determined that CC1736 binds Q containing a farnesyl tail (Q_3), and DMQ_3, a farnesylated analog of demethoxy-Q, the penultimate Q-intermediate. Because CC1736 is amenable to NMR structural analyses [14], our results indicate that further study with CC1736 and Q analogs could identify the residues responsible for Q-binding.

Mutational analyses of yeast Coq10p have identified residues that are important for respiration and/or growth on non-fermentable sources [11,18]. Here we showed that the V70K mutation in CC1736 prevented rescue of the *coq10* null yeast (Figs. 2 and 3). However, the V70K mutation interfered with mitochondrial processing of the CC1736 polypeptide (Fig. S1). Therefore it is not certain whether the loss of function due to this mutation can be attributed to loss of binding or loss of correct targeting to mitochondria. Other studies testing functionality of Coq10 mutant polypeptides did not determine whether the mutant polypeptides were directed to mitochondria and correctly processed.

The yeast *coq10* mutant is also rescued by over-expression of Coq8p. Over-expression of Coq8p in each of the *coq* null mutants (*coq3–coq9*) was recently shown to restore the steady-state levels of Coq4, Coq6, Coq7, and Coq9 polypeptides [24,25], and results in the accumulation of novel late-stage Q intermediates [25]. Conserved kinase sequence motifs present in Coq8 are essential for this stabilization [23,25], consistent with the hypothesis that a phosphorylated, multi-subunit Coq polypeptide complex is essential for Q biosynthesis [22,23]. While these studies implicate Coq8 as a kinase responsible for mediating the phosphorylation of several of the Coq polypeptides, direct experimental evidence for Coq8 kinase activity is still lacking. The story is likely more complicated, since the phosphorylation state of two serine residues and one threonine residue identified in Coq7 appear to regulate Q biosynthesis [45]. Expression of Coq7 phosphomimetic forms decreased Q content, and while alanine substitution of these same residues increased Q content. The identity of the kinase(s) mediating such phosphorylation remains to be determined.

The multi-subunit Coq polypeptide complex is likely to be important for the catalytic efficiency of Q biosynthesis, minimizing the release of Q-intermediates, including unsubstituted quinones, which are potentially reactive electrophiles, and catechols, which are prone to oxidation. In mitochondria isolated from the *coq10* null mutant, steady state levels of Coq4p, Coq6p, Coq7p, and Coq9p are significantly decreased as shown by western blot analysis [20]. The imbalance of Coq proteins suggests that the Coq complex is unstable. Despite the disruption of the steady state levels of these Coq proteins, the *coq10* null mutant continues to produce Q_6 [10], and it was concluded that Coq10p was not essential for Q_6 biosynthesis. However, previous studies did not address whether the yeast *coq10* null mutants might have subtle impairments in *de novo* Q synthesis.

In this study we traced *de novo* Q_6 synthesis with the $^{13}C_6$-ring-labeled precursors 4HB and pABA. Our analyses show that the *coq10* null mutant synthesizes Q_6 less efficiently and accumulates high levels of the early Q-intermediates HHB and HAB as compared to wild-type yeast (Figs. 8–9 and S2–S3). The decreased *de novo* synthesis of Q_6 is particularly obvious in early log phase cultures (Figs. 8 and S2). The inefficient Q biosynthesis observed in the *coq10* mutant is rescued by the over-expression of Coq10p, Coq8p, and in part by *C. crescentus* CC1736 (Figs. 9 and S4). The decreased *de novo* synthesis of Q_6 in the *coq10* null is particularly obvious in early log phase cultures. This is primarily due to the higher content of Q_6 in early log phase wild-type cells. In fact, wild-type yeast showed a profound decrease in Q_6 biosynthesis and content during the progression from early- to late-log phase (Figs. 8 and S2). This decrease in Q_6 content in wild-type cells accounts for an apparent near normal content of Q_6 when *coq10* null and wild-type cells harvested at late log (Fig. S4), or near stationary phase [10].

It seems likely that the decreased content and biosynthesis of Q_6 in *coq10* null cells during early log phase may account for their sensitivity to PUFA treatment (Figs. 4 - 7). PUFA sensitivity assays are routinely performed on early log-phase cultures, and over-expression of Coq10p, Coq8p, and CC1736 rescued the sensitivity of the *coq10* mutant to PUFA treatment. Stress imposed by PUFA treatment is due to the presence of vulnerable bis-allylic hydrogen atoms [5,34]. In the absence of chain-terminating antioxidants, such as QH_2 or vitamin E, toxic PUFA autoxidation products accumulate and result in cell death. The sensitivity of the *coq10* mutant to PUFA treatment is fully rescued by the addition of lipid soluble chain-terminating antioxidants, such as BHT or vitamin E. While addition of vitamin C fails to rescue the PUFA sensitivity of the Q-less *coq* mutants (such as *coq3*), vitamin C partially rescued the PUFA sensitivity of the *coq10* mutant. These results indicate that in the absence of Coq10p, Q_6 content in log phase cells may be inadequate. Hence the *coq10* null cells are sensitive to PUFA stress, yet not as sensitive at the Q-less *coq* mutants. In the *coq10* null mutant cells, vitamin C may act to restore this essential redox function of QH_2.

Although over-expression of Coq8p, CC1736, or Coq10p in the *coq10* null mutant results in a more efficient rate of *de novo* Q_6 biosynthesis, high levels of the early Q_6-intermediates HAB and HHB persist, suggesting that the stoichiometry of Coq8p and Coq10p is important for optimal Q_6 synthesis. These results, together with our *in vitro* binding assays that show Q_3 and DMQ_3 bind to CC1736, suggest that Coq10:Q_6 may stabilize the Coq polypeptide complex and/or enhance the efficiency of Q_6 biosynthesis.

Future studies should aim to elucidate the relationship between Coq8p, Coq10p and the Coq polypeptide complex. We propose that Coq10p:Q_6 may be important for the delivery of Q_6 to the Coq polypeptide complex, generating *de novo* Q_6, which is delivered to the respiratory complexes (Fig. 11A). The *coq10* null mutant is known to contain lower steady state levels of the Coq4, Coq6, Coq7 and Coq9 polypeptides [20], decreased *de novo* Q_6 biosynthesis (Figs. 8 and 9), and hence a less efficient delivery of "new Q" to the respiratory complexes (Fig. 11B). Over-expression of Coq8p restores *de novo* Q_6 synthesis, and hence efficient delivery of Q_6 to the respiratory complexes, even in the absence of Coq10p (Fig. 11C).

The presence of Q_6 or a Q_6-intermediate is likely to be an essential component of the Coq polypeptide complex. Padilla et al., [26] showed that addition of Q_6 to cultures of the *coq7* null mutant re-establishes synthesis of DMQ_6. In the absence of exogenous Q_6 (or over-expression of Coq8p) the *coq7* null mutant accumulates just the early intermediates HHB and HAB. We note that addition of exogenous Q_6 may act directly to stabilize the Coq polypeptide multi-subunit complex [46], and via its interaction with Coq10p, may also be delivered to respiratory chain complexes. This model accounts for the observation that exogenously added Q_6 failed to rescue a *coq2*/*coq10* double mutant, but did rescue each of the single mutants, as well as the *coq2*/*coq3* and *coq2*/*coq4* double mutants [24].

The inefficiency in *de novo* Q_6 biosynthesis reported here for the *coq10* mutant does not result in a severe decrease in the total Q_6 content. Thus, the severe respiratory defect and sensitivity to PUFA treatment manifested by the *coq10* mutant remain to be explained. We propose that much of the Q_6 pool in yeast may not be readily available for respiration, but may be sequestered or aggregated at some non-functional site. Addition of Q_2 rescues the *coq10* mutant because Q_2 is a small soluble analog, less prone to aggregation/sequestration. We further propose that newly synthesized Q_6 is accessible to the respiratory chain complexes, and can function as an antioxidant. A

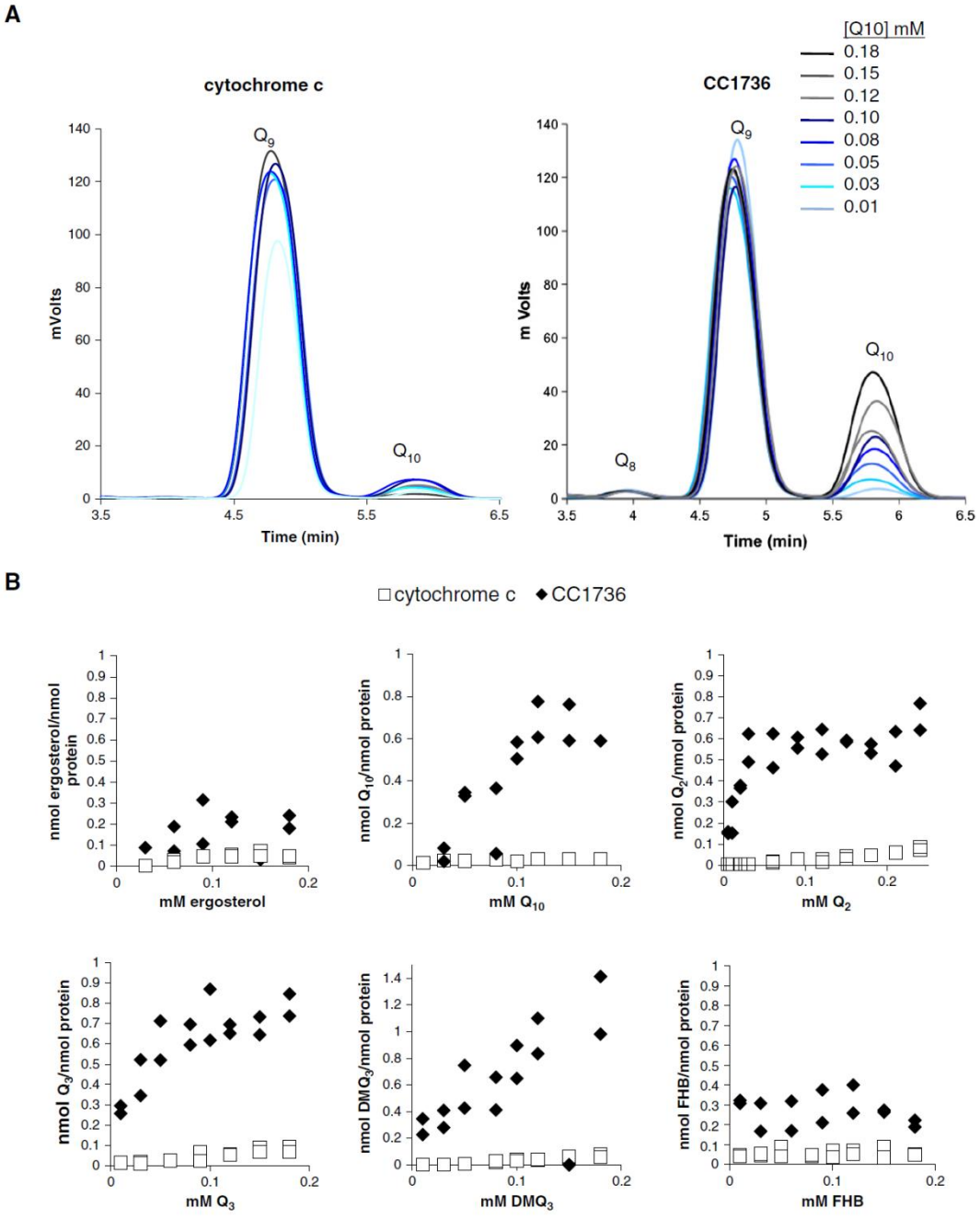

Fig. 10. *C. crescentus* CC1736 binds Q_{10}, Q_2, Q_3, and DMQ_3. (*A*) Purified CC1736 (*right panel*) or cytochrome *c* (*left panel*) were added to binding buffer containing Q_{10} at the eight concentrations designated (0.01–0.18 mM). Samples were incubated for 45 min at 30 °C and unbound ligands were separated from the protein by application to a desalting column, and lipid extracts of the eluate were subjected to reversed-phase HPLC and the ligands were detected by UV absorption as described in Section 2.13. (*B*) shows the amount of each ligand recovered in association with either CC1736 (black diamonds) or cytochrome *c* (open squares): ergosterol, Q_{10}, Q_2, Q_3, DMQ_3, or FHB in ascending concentration. One representative assay is shown of at least two assays performed for each ligand. Each concentration of ligand was tested in duplicate per binding assay.

Table 4
Determination of binding constants for CC1736.

CC1736	Q_2	Q_{10}	Q_3	DMQ_3
Bmax	1.11 ± 0.31	2.77 ± 2.90	1.56 ± 1.00	3.00 ± 0.82
K_d (mM ligand)	0.018 ± 0.002	0.259 ± 0.356	0.035 ± 0.016	0.14 ± 0.077

Bmax, mol ligand bound: mol protein at saturation.
K_d, dissociation constant of ligand.
Average ± standard deviation for two independent binding assays (see Fig. 10).

recent report indicates that the submitochondrial distribution of Q (inner versus outer membrane) impacts respiratory efficiency in mice harboring just one copy of the Coq7p ortholog Mclk1 [47]. It seems plausible that in turn, the content of Q in the inner membrane may be a direct function of Coq7p (and Coq10p:Q) in stabilizing the Coq multisubunit complex, and hence delivery of new Q to the respiratory complexes of the inner membrane. It is significant that restoration of small amounts of *de novo* Q synthesis affords profound rescue of Q-less yeast [23,46] and *C. elegans* mutants [48,49]. In this scenario, the provision of a small amount of newly synthesized Q may prime the assembly of Q into one or more sites of the respiratory chain complexes.

5. Conclusions

Expression of the CC1736 START domain polypeptide rescues the respiration defect and the PUFA sensitivity of the *coq10* yeast mutant.

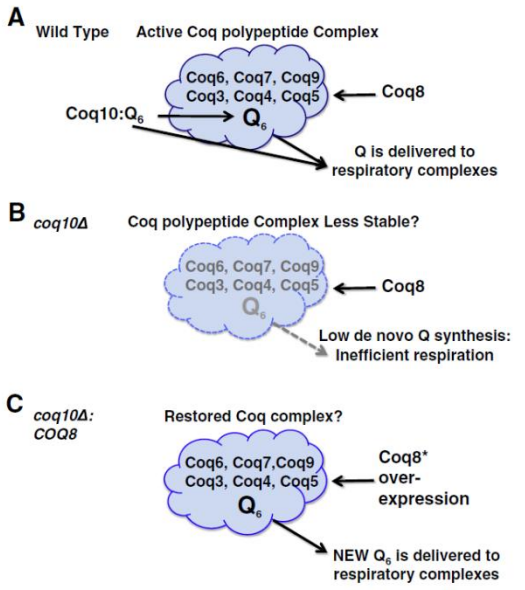

Fig. 11. A model for Coq10/START domain polypeptide function in *de novo* Q_6 biosynthesis and in delivery of Q_6 to respiratory chain complexes. (*A*) In wild-type cells, the Coq10:Q protein:ligand complex is postulated to deliver Q_6 to the multisubunit Coq polypeptide complex and so enhance stability of the Coq polypeptides and *de novo* synthesis of Q_6. Newly synthesized Q_6 in the mitochondrial inner membrane is delivered to respiratory chain complexes and can function as an antioxidant. We postulate that Coq10:Q_6 may also deliver Q_6 to respiratory complexes. (*B*) The *coq10* null mutant contains lower steady state levels of Coq polypeptides [20] and is shown with a less stable multisubunit Coq complex. Impaired *de novo* synthesis of Q_6 and lack of Coq10:Q_6 delivery to respiratory complexes accounts for the inefficient respiration observed in the *coq10* null mutant. (*C*) The *coq10* null mutant can be rescued by over-expression of *COQ8*, via its ability to restore the Coq polypeptide complex [25]. We postulate that the enhanced *de novo* Q_6 biosynthesis formed by the Coq multisubunit complex is efficiently delivered to respiratory complexes, despite the absence of Coq10p.

In vitro binding assays show that CC1736 binds Q and the penultimate Q-biosynthetic intermediate DMQ. Although the yeast *coq10* null mutant is replete in Q_6, it is respiratory defective. In this study we use stable isotope ring precursors and show that *de novo* Q_6 biosynthesis in the *coq10* mutant is inefficient. Over-expression of Coq8p restores newly synthesized Q_6 to wild-type levels, and rescues the respiratory deficiency and the sensitivity of the *coq10* null mutant to PUFA stress. The results suggest that efficient Q *de novo* biosynthesis is important for the function of Q as a mobile electron carrier in the respiratory electron transport chain and as a chain-terminating antioxidant.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2012.12.007.

References

[1] Y. Hatefi, The mitochondrial electron transport and oxidative phosphorylation system, Annu. Rev. Biochem. 54 (1985) 1015–1069.
[2] F.L. Crane, Distribution of ubiquinones, in: R.A. Morton (Ed.), Biochemistry of quinones, Academic Press, London, 1965, pp. 183–206.
[3] M. Turunen, J. Olsson, G. Dallner, Metabolism and function of coenzyme Q, Biochim. Biophys. Acta 1660 (2004) 171–199.
[4] T.Q. Do, J.R. Schultz, C.F. Clarke, Enhanced sensitivity of ubiquinone deficient mutants of *Saccharomyces cerevisiae* to products of autooxidized polyunsaturated fatty acid, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7534–7539.
[5] S. Hill, K. Hirano, V.V. Shmanai, B.N. Marbois, D. Vidovic, A.V. Bekish, B. Kay, V. Tse, J. Fine, C.F. Clarke, M.S. Shchepinov, Isotope-reinforced polyunsaturated fatty acids protect yeast cells from oxidative stress, Free Radic. Biol. Med. 50 (2011) 130–138.
[6] A. Tzagoloff, C.L. Dieckmann, *PET* genes of *Saccharomyces cerevisiae*, Microbiol. Rev. 54 (1990) 211–225.
[7] F. Pierrel, O. Hamelin, T. Douki, S. Kieffer-Jaquinod, U. Muhlenhoff, M. Ozeir, R. Lill, M. Fontecave, Involvement of mitochondrial ferredoxin and para-aminobenzoic acid in yeast coenzyme Q biosynthesis, Chem. Biol. 17 (2010) 449–459.
[8] U.C. Tran, C.F. Clarke, Endogenous synthesis of coenzyme Q in eukaryotes, Mitochondrion 7S (2007) S62–S71.
[9] M. Ozeir, U. Muhlenhoff, H. Webert, R. Lill, M. Fontecave, F. Pierrel, Coenzyme Q biosynthesis: Coq6 is required for the C5-hydroxylation reaction and substrate analogs rescue Coq6 deficiency, Chem. Biol. 18 (2011) 1134–1142.
[10] M.H. Barros, A. Johnson, P. Gin, B.N. Marbois, C.F. Clarke, A. Tzagoloff, The *Saccharomyces cerevisiae COQ10* gene encodes a START domain protein required for function of coenzyme Q in respiration, J. Biol. Chem. 280 (2005) 42627–42635.
[11] T.Z. Cui, M. Kawamukai, Coq10, a mitochondrial coenzyme Q binding protein, is required for proper respiration in *Schizosaccharomyces pombe*, FEBS J. 276 (2009) 748–759.
[12] C. Busso, E.B. Tahara, R. Ogusucu, O. Augusto, J.R. Ferreira-Junior, A. Tzagoloff, A.J. Kowaltowski, M.H. Barros, *Saccharomyces cerevisiae coq10* null mutants are responsive to antimycin A, FEBS J. 277 (2010) 4530–4538.
[13] M. Punta, P.C. Coggill, R.Y. Eberhardt, J. Mistry, J. Tate, C. Boursnell, N. Pang, K. Forslund, G. Ceric, J. Clements, A. Heger, L. Holm, E.L. Sonnhammer, S.R. Eddy, A. Bateman, R.D. Finn, The Pfam protein families database, Nucleic Acids Res. 40 (2012) D290–301.
[14] Y. Shen, S. Goldsmith-Fischman, H.S. Atreya, T. Acton, L. Ma, R. Xiao, B. Honig, G.T. Montelione, T. Szyperski, NMR structure of the 18 kDa protein CC1736 from *Caulobacter crescentus* identifies a member of the START domain superfamily and suggests residues mediating substrate specificity, Proteins 58 (2005) 747–750.

[15] C.P. Ponting, L. Aravind, START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins, Trends Biochem. Sci. 24 (1999) 130–132.
[16] W.L. Miller, Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter, Biochim. Biophys. Acta 1771 (2007) 663–676.
[17] L. Lo Conte, B. Ailey, T.J. Hubbard, S.E. Brenner, A.G. Murzin, C. Chothia, SCOP: a structural classification of proteins database, Nucleic Acids Res. 28 (2000) 257–259.
[18] C. Busso, L. Bleicher, J.R. Ferreira-Junior, M.H. Barros, Site-directed mutagenesis and structural modeling of Coq10p indicate the presence of a tunnel for coenzyme Q6 binding, FEBS Lett. 584 (2010) 1609–1614.
[19] B. Marbois, P. Gin, K.F. Faull, W.W. Poon, P.T. Lee, J. Strahan, J.N. Shepherd, C.F. Clarke, Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q, J. Biol. Chem. 280 (2005) 20231–20238.
[20] E.J. Hsieh, P. Gin, M. Gulmezian, U.C. Tran, R. Saiki, B.N. Marbois, C.F. Clarke, *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex, Arch. Biochem. Biophys. 463 (2007) 19–26.
[21] B. Marbois, P. Gin, M. Gulmezian, C.F. Clarke, The yeast Coq4 polypeptide organizes a mitochondrial protein complex essential for coenzyme Q biosynthesis, Biochim. Biophys. Acta 1791 (2009) 69–75.
[22] A. Tauche, U. Krause-Buchholz, G. Rodel, Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of *O*-methylase Coq3p depends on Abc1p/Coq8p, FEMS Yeast Res. 8 (2008) 1263–1275.
[23] L.X. Xie, E.J. Hsieh, S. Watanabe, C.M. Allan, J.Y. Chen, U.C. Tran, C.F. Clarke, Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants, Biochim. Biophys. Acta 1811 (2011) 348–360.
[24] M.A. Zampol, C. Busso, F. Gomes, J.R. Ferreira-Junior, A. Tzagoloff, M.H. Barros, Over-expression of *COQ10* in *Saccharomyces cerevisiae* inhibits mitochondrial respiration, Biochem. Biophys. Res. Commun. 402 (2010) 82–87.
[25] L.X. Xie, M. Ozeir, J.Y. Tang, J.Y. Chen, S. Kieffer-Jaquinod, M. Fontecave, C.F. Clarke, F. Pierrel, Over-expression of the Coq8 kinase in *Saccharomyces cerevisiae coq* null mutants allows for accumulation of diagnostic intermediates of the Coenzyme Q6 biosynthetic pathway, J. Biol. Chem. 287 (2012) 23571–23581.
[26] S. Padilla, U.C. Tran, M. Jimenez-Hidalgo, J.M. Lopez-Martin, A. Martin-Montalvo, C.F. Clarke, P. Navas, C. Santos-Ocana, Hydroxylation of demethoxy-Q6 constitutes a control point in yeast coenzyme Q6 biosynthesis, Cell Mol. Life Sci. 66 (2009) 173–186.
[27] D. Burke, D. Dawson, T. Stearns, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Plainview, NY, 2000.
[28] R.J. Barkovich, A. Shtanko, J.A. Shepherd, P.T. Lee, D.C. Myles, A. Tzagoloff, C.F. Clarke, Characterization of the *COQ5* gene from *Saccharomyces cerevisiae*. Evidence for a *C*-methyltransferase in ubiquinone biosynthesis, J. Biol. Chem. 272 (1997) 9182–9188.
[29] B. Marbois, L.X. Xie, S. Choi, K. Hirano, K. Hyman, C.F. Clarke, para-Aminobenzoic acid is a precursor in coenzyme Q6 biosynthesis in *Saccharomyces cerevisiae*, J. Biol. Chem. 285 (2010) 27827–27838.
[30] A.Y. Hsu, W.W. Poon, J.A. Shepherd, D.C. Myles, C.F. Clarke, Complementation of *coq3* mutant yeast by mitochondrial targeting of the *Escherichia coli* UbiG polypeptide: evidence that UbiG catalyzes both *O*-methylation steps in ubiquinone biosynthesis, Biochemistry 35 (1996) 9797–9806.
[31] T.W. Christianson, R.S. Sikorski, M. Dante, J.H. Shero, P. Hieter, Multifunctional yeast high-copy-number shuttle vectors, Gene 110 (1992) 119–122.
[32] Y. Shen, H.S. Atreya, R. Xiao, T.B. Acton, R. Shastry, L. Ma, G.T. Montelione, T. Szyperski, Resonance assignments for the 18 kDa protein CC1736 from *Caulobacter crescentus*, J. Biomol. NMR 29 (2004) 549–550.
[33] R. Elble, A simple and efficient procedure for transformation of yeasts, Biotechniques 13 (1992) 18–20.
[34] S. Hill, C.R. Lamberson, L. Xu, R. To, H.S. Tsui, V.V. Shmanai, A.V. Bekish, A.M. Awad, B.N. Marbois, C.R. Cantor, N.A. Porter, C.F. Clarke, M.S. Shchepinov, Small amounts of isotope-reinforced polyunsaturated fatty acids suppress lipid autoxidation, Free Radic. Biol. Med. 53 (2012) 893–906.
[35] B.S. Glick, L.A. Pon, Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*, Methods Enzymol. 260 (1995) 213–223.
[36] C. Lemaire, G. Dujardin, Preparation of respiratory chain complexes from *Saccharomyces cerevisiae* wild-type and mutant mitochondria: activity measurement and subunit composition analysis, Methods Mol. Biol. 432 (2008) 65–81.
[37] W.W. Poon, R.J. Barkovich, A.Y. Hsu, A. Frankel, P.T. Lee, J.N. Shepherd, D.C. Myles, C.F. Clarke, Yeast and rat Coq3 and *Escherichia coli* UbiG polypeptides catalyze both O-methyltransferase steps in coenzyme Q biosynthesis, J. Biol. Chem. 274 (1999) 21665–21672.
[38] J.A. Shepherd, W.W. Poon, D.C. Myles, C.F. Clarke, The biosynthesis of ubiquinone: synthesis and enzymatic modification of biosynthetic precursors, Tetrahedron Lett. 37 (1996) 2395–2398.
[39] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[40] R. Saiki, A.L. Lunceford, Y. Shi, B. Marbois, R. King, J. Pachuski, M. Kawamukai, D.L. Gasser, C.F. Clarke, Coenzyme Q10 supplementation rescues renal disease in *Pdss2kd/kd* mice with mutations in prenyl diphosphate synthase subunit 2, Am. J. Physiol. Renal Physiol. 295 (2008) F1535–1544.
[41] W.W. Poon, T.Q. Do, B.N. Marbois, C.F. Clarke, Sensitivity to treatment with polyunsaturated fatty acids is a general characteristic of the ubiquinone-deficient yeast *coq* mutants, Mol. Aspects Med. 18 (1997) s121–s127.
[42] G.P. Drummen, L.C. van Liebergen, J.A. Op den Kamp, J.A. Post, C11-BODIPY(581/591), an oxidation-sensitive fluorescent lipid peroxidation probe: (micro)spectroscopic characterization and validation of methodology, Free Radic. Biol. Med. 33 (2002) 473–490.
[43] B.J. Clark, The mammalian START domain protein family in lipid transport in health and disease, J. Endocrinol. 212 (2012) 257–275.
[44] R.E. Soccio, J.L. Breslow, StAR-related lipid transfer (START) proteins: mediators of intracellular lipid metabolism, J. Biol. Chem. 278 (2003) 22183–22186.
[45] A. Martin-Montalvo, I. Gonzalez-Mariscal, S. Padilla, M. Ballesteros, D.L. Brautigan, P. Navas, C. Santos-Ocana, Respiratory-induced coenzyme Q biosynthesis is regulated by a phosphorylation cycle of Cat5p/Coq7p, Biochem. J. 440 (2011) 107–114.
[46] U.C. Tran, B. Marbois, P. Gin, M. Gulmezian, T. Jonassen, C.F. Clarke, Complementation of *Saccharomyces cerevisiae coq7* mutants by mitochondrial targeting of the *Escherichia coli* UbiF polypeptide: two functions of yeast Coq7 polypeptide in coenzyme Q biosynthesis, J. Biol. Chem. 281 (2006) 16401–16409.
[47] J. Lapointe, Y. Wang, E. Bigras, S. Hekimi, The submitochondrial distribution of ubiquinone affects respiration in long-lived Mclk1+/− mice, J. Cell Biol. 199 (2012) 215–224.
[48] F. Gomez, R. Saiki, R. Chin, C. Srinivasan, C.F. Clarke, Restoring *de novo* coenzyme Q biosynthesis in *Caenorhabditis elegans coq-3* mutants yields profound rescue compared to exogenous coenzyme Q supplementation, Gene 506 (2012) 106–116.
[49] Y.Y. Yang, V. Vasta, S. Hahn, J.A. Gangoiti, E. Opheim, M.M. Sedensky, P.G. Morgan, The role of DMQ(9) in the long-lived mutant clk-1, Mech. Ageing Dev. 132 (2011) 331–339.
[50] A. Tzagoloff, M.A. Wu, M. Crivellone, Assembly of the mitochondrial membrane system. Characterization of COR1, the structural gene for the 44-kilodalton core protein of yeast coenzyme QH2-cytochrome c reductase, J. Biol. Chem. 261 (1986) 17163–17169.
[51] B.N. Marbois, C.F. Clarke, The *COQ7* gene encodes a protein in *Saccharomyces cerevisiae* necessary for ubiquinone biosynthesis, J. Biol. Chem. 271 (1996) 2995–3004.
[52] T.Q. Do, A.Y. Hsu, T. Jonassen, P.T. Lee, C.F. Clarke, A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in *Saccharomyces cerevisiae abc1* mutants, J. Biol. Chem. 276 (2001) 18161–18168.
[53] A. Johnson, P. Gin, B.N. Marbois, E.J. Hsieh, M. Wu, M.H. Barros, C.F. Clarke, A. Tzagoloff, *COQ9*, a new gene required for the biosynthesis of coenzyme Q in *Saccharomyces cerevisiae*, J. Biol. Chem. 280 (2005) 31397–31404.
[54] K.B. Nicholas, H. B. Nicholas Jr., D. W. Deerfield II, GeneDoc: Analysis and visualization of genetic variation, EMBNEW NEWS, 4 (1997) 14.

Chapter 3

Characterization of the mitochondrial coenzyme Q biosynthetic complex in *Saccharomyces cerevisiae*

Abstract

Coenzyme Q (Q or ubiquinone) is a redox active lipid composed of a fully-substituted benzoquinone ring and a polyisoprenoid tail and is required for mitochondrial electron transport. In the yeast *Saccharomyces cerevisiae* Q is synthesized by the products of eleven known genes, *COQ1-COQ9*, *YAH1*, and *ARH1*. The function of some of the Coq proteins remains unknown and there are steps in the Q biosynthetic pathway which are not fully characterized. Several of the Coq proteins are associated in a macromolecular complex on the matrix-face of the inner mitochondrial membrane and this complex is required for efficient Q synthesis. Here we further characterize this complex via Western blotting and proteomic analysis of tandem affinity purified CNAP (consecutive non-denaturing affinity purification)-tagged Coq proteins. We show that Coq8p, a putative kinase required for the stability of the Q biosynthetic complex, is associated with a Coq6p-containing complex. Additionally Q_6 and late-stage Q biosynthetic intermediates were also found to co-purify with the complex. The protein of unknown function YLR290C is also identified as a constituent of the complex and is shown to be required for efficient *de novo* Q biosynthesis. Given its effect on Q synthesis and its association with the biosynthetic complex we propose that YLR290C be renamed Coq11p.

Introduction

Coenzyme Q (Q or ubiquinone) is small lipophilic molecule consisting of a hydrophobic polyisoprenoid chain and a fully-substituted benzoquinone ring. The length of the polyisoprenoid group is species specific and contains 6, 8, 9, and 10 isoprenyl units in *Saccharomyces cerevisiae*, *Escherichia coli*, mice, and humans respectively (Crane, 1965) and localizes Q to biological membranes. The primary function of Q is to serve as a two electron carrier in respiratory electron transport, where it accepts electrons from NADH at complex I or succinate at complex II and donates them to cytochrome *c* at complex III (Brandt and Trumpower, 1994). *S. cerevisiae* lacks complex I and instead uses the internal or external NADH:Q oxidoreductases on the inner mitochondrial membrane which unlike complex I do not facilitate proton transport across the inner membrane (Grandier-Vazeille *et al.*, 2001). Q also functions as an electron carrier in other aspects of metabolism including dihydroorotate dehydrogenase (pyrimidine synthesis), fatty acyl-CoA dehydrogenase (fatty acid β-oxidation), and other dehydrogenases that oxidize various substrates including choline, sarcosine, sulfide, and glycerol-3-phosphate (Lenaz and De Santis, 1985; Hildebrandt and Grieshaber, 2008; Bentinger *et al.*, 2010). QH_2 has also been demonstrated to function as a lipid-soluble antioxidant capable of mitigating lipid peroxidation and regenerating alpha-tocopherol (vitamin E) (Frei *et al.*, 1990; Turunen *et al.*, 2004). Primary deficiencies in Q biosynthesis have been identified in several human patients and manifest a variety of symptoms including encephalomyopathy, ataxia, cerebellar atrophy, myopathy, and steroid-resistant nephrotic syndrome (Laredj *et al.*, 2014).

In *S. cerevisiae* Q synthesis is dependent on the product of elven known genes: *COQ1-COQ9*, *YAH1*, and *ARH1*. *COQ1-COQ9* were identified as complementation groups of Q-deficient yeast mutants (Tzagoloff and Dieckmann, 1990; Johnson *et al.*, 2005), while *YAH1* and *ARH1* were identified through lipid analysis of Yah1p- and Arh1p-depleted strains under control of galactose-inducible promoters (Pierrel *et al.*, 2010). Deletion of any of these *COQ* genes results in a loss of Q synthesis and consequently a failure to respire and grow on a non-fermentable carbon source (Tran and Clarke, 2007; Pierrel *et al.*, 2010). Deletion of either *YAH1* or *ARH1* results in a loss in viability as the biosynthesis of iron-sulfur clusters and heme A also requires these gene products (Manzella *et al.*, 1998; Barros and Nobrega, 1999; Lange *et al.*, 2000; Li *et al.*, 2001; Barros *et al.*, 2002). While required for Q biosynthesis the functions of Coq4p, Coq8p, and Coq9p are not fully understood, although Coq8p has been proposed to function as a protein kinase (Xie *et al.*, 2011) and Coq9p is required for the replacement of the ring amino group with a hydroxyl group (Xie *et al.*, 2012). There are currently steps in the yeast Q biosynthetic pathway, such as the decarboxylation reaction and subsequent ring hydroxylation, for which no enzyme has yet been characterized.

Genetic and biochemical experiments have demonstrated the interdependence of several of the Coq proteins and have shown the existence of a high molecular mass Coq protein complex. Each of the *coq3-coq9* null mutants accumulate only the early intermediates 3-hexaprenyl-4-hydroxybenzoic acid (HHB) and 3-hexaprenyl-4-aminobenzoic acid (HAB) resulting from prenylation of 4HB and pABA respectively (Xie *et al.*, 2012). Steady-state levels of Coq3p, Coq4p, Coq6p, Coq7p, and Coq9p are reduced in several *coq1-coq9* null mutants, although levels of Coq3p were stabilized in *coq4-coq9* mutant samples prepared with phosphatase inhibitors (Hsieh *et al.*, 2007; Tauche *et al.*, 2008; Xie *et al.*, 2012). This has been

observed in other mitochondrial complexes such as the cytochrome *bc₁* complex and ATP synthase in which the absence or mutation of one component protein results in reduced steady-state levels of the other subunits (Tzagoloff *et al.*, 1994; Glerum *et al.*, 1997). Gel filtration chromatography and blue native-polyacrylamide gel electrophoresis (BN-PAGE) have shown that several of the Coq proteins exist in high molecular mass complexes and the *O*-methyltransferase activity of Coq3p can be detected in these complexes (Marbois *et al.*, 2005; Tran *et al.*, 2006; Hsieh *et al.*, 2007; Marbois *et al.*, 2009). Analysis of gel filtration fractions has also shown the association of DMQ_6 with the complexes (Marbois *et al.*, 2005). Supplementation of exogenous Q_6 was shown to stabilize the steady-state levels of Coq3p and Coq4p in the *coq7* null mutant (Tran *et al.*, 2006), enhance DMQ_6 production in the *coq7* null mutant (Padilla *et al.*, 2009), and stabilize steady-state levels of Coq9p in the *coq3*, *coq4*, *coq6*, *coq7* null mutants and steady-state levels of Coq4p in the *coq3*, *coq6*, and *coq7* null mutants (He *et al.*, 2014), suggesting that associated Q_6 plays a role in stability of the complex.

Co-precipitation experiments have demonstrated the physical association of several of the Coq proteins; biotinylated Coq3p was shown to co-precipitate Coq4p, and HA-tagged Coq9p co-purified Coq4p, Coqp5, Coq6p, and Coq7p (Marbois *et al.*, 2005; Hsieh *et al.*, 2007). Although Coq8p has not been shown to be associated in a complex with the other Coq proteins its putative role as a kinase is important for the stability of several other Coq proteins as overexpression of *COQ8* in various *coq* null mutants stabilizes several of the other Coq proteins as well as multi-subunit Coq polypeptide complexes, and leads to the accumulation of later stage Q biosynthetic intermediates diagnostic of the mutated step (Padilla *et al.*, 2009; Xie *et al.*, 2012; He *et al.*, 2014). Overexpression of *COQ8* in the *coq1* or *coq2* null mutants, which do not produce any polyisoprenylated intermediates, fails to stabilize steady-state levels of sensitive Coq

polypeptides (Xie *et al.*, 2012). When diverse polyprenyl diphosphate synthases from prokaryotic species that do not synthesize Q are expressed in yeast *coq1* null mutants Q biosynthesis and steady-state levels of sensitive Coq polypeptides is restored (Gin and Clarke, 2005), suggesting that a polyisoprenylated compound is essential for complex stability.

The functions of certain Coq polypeptides in Q biosynthesis remains unclear and there are reactions in the Q biosynthetic pathway for which an enzyme has not been assigned. In this study the composition of the Q biosynthetic complex is investigated through the use of C-terminal tandem affinity purification tags coupled to proteomic analysis. The presence of associated Q_6 and Q_6 intermediates with the complex is also assessed. Two new candidate proteins associated with the complex are characterized via analysis of their null mutants as well as tandem affinity purification of one of these proteins.

Materials and Methods

Yeast strains and growth media

S. cerevisiae strains used in this study are described in Table 1. Media was prepared as described (Burke *et al.*, 2000), and included YPD (2% glucose, 1% yeast extract, 2% peptone), YPgal (2% galactose, 0.1% glucose, 1% yeast extract, 2% peptone), and YPG (3% glycerol, 1% yeast extract, 2% peptone). Synthetic Dextrose/Minimal medium was prepared as described (Barkovich *et al.*, 1997), and consisted of all components (SD-complete) or all components minus histidine (SD−his). Drop out dextrose medium (DOD) was prepared as described (Marbois *et al.*, 2010), except that galactose was replaced with 2% dextrose. Plate media was prepared with 2% bacto agar.

Construction of integrated CNAP-tagged yeast strains

The coding sequence for the CNAP (consecutive non-denaturing affinity purification) tag, **HHHHHHHHHH**GGAGGEDQVDPRLIDGK (His_{10} tag in bold, protein C epitope underlined) (Claypool *et al.*, 2008) and *HIS3* ORF were amplified from pFACNAPHISMX (Dr. C.M. Koehler, Department of Chemistry and Biochemistry, UCLA) using primers with 5' flanking regions corresponding to 50 bp upstream of the stop codon and 50 bp downstream of the stop codon of the gene of interest. Following PCR the product was purified using the PureLink Quick PCR Purification Kit (Life Technologies) and used to transform W303-1A yeast via the lithium acetate/PEG method (Gietz and Woods, 2006) to allow integration of the CNAP and *HIS3* coding sequence into the endogenous stop codon of the target gene. Integrants were selected on SD−his and screened by colony PCR to verify the presence of the *CNAP-HIS3* insert

at the correct locus. Sequences of primes used to generate CNAP-tagged constructs are listed in Table 2.

Mitochondrial purification from yeast

Yeast strains were grown in 5 mL YPgal pre-cultures and inoculated into 600 mL YPgal cultures for overnight growth in a shaking incubator (30 °C, 250 rpm). Cells were harvested at an OD_{600} of 3.5-4.0 and mitochondria were purified as described (Glick and Pon, 1995), with the addition of Complete EDTA-free protease inhibitor cocktail (Roche) and Phosphatase inhibitor cocktails I and II (Calbiochem). Purified mitochondria were flash frozen in liquid nitrogen and stored at −80 °C. The protein concentration was measured with a BCA assay using bovine serum albumin as standards (Thermo Scientific).

Tandem affinity purification of CNAP-tagged proteins

Purified mitochondria (15 mg protein) were pelleted by centrifugation at 12,000 × *g* for 10 min and solubilized at 2 mg/mL with solubilization buffer (20 mM HEPES-KOH pH 7.4, 100 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM $CaCl_2$, Complete EDTA-free protease inhibitor cocktail (Roche), Phosphatase inhibitor cocktails I and II (Calbiochem), 6 mg/mL digitonin (Biosynth)) for 1 h on ice with mixing every 10 min. The soluble supernatant fraction was then separated from the insoluble pellet by centrifugation at 100,000 × *g* for 10 min in a Beckman Airfuge. The soluble fraction was then mixed with 8 mL lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole) and 800 µL bed volume Ni-NTA resin (Qiagen) pre-equilibrated with lysis buffer and incubated at 4 °C for 90 min with mixing by rotation. The Ni-NTA slurry was then applied to a flowthrough column and washed twice with

12 mL Ni-NTA wash buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mg/mL digitonin), followed by elution of bound protein with 8 mL Ni-NTA elution buffer (20 mM HEPES-KOH pH 7.4, 100 mM NaCl, 300 mM imidazole, 10% glycerol, 1 mM $CaCl_2$, 1 mg/mL digitonin). The Ni-NTA eluate was applied directly to 1 mL anti-PC resin (Roche) pre-equilibrated with anti-PC equilibration buffer (20 mM HEPES-KOH pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$) and incubated overnight at 4°C with mixing by rotation. The anti-PC slurry was then applied to a flowthrough column and washed twice with 15 mL anti-PC wash buffer (20 mM HEPES-KOH pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mg/mL digitonin). Bound protein was eluted twice with 1 mL anti-PC elution buffer (20 mM HEPES-KOH pH 7.4, 100 mM NaCl, 5 mM EDTA, 1 mg/mL digitonin). In the first elution (E1) the anti-PC resin was incubated at 4°C for 15 min followed by incubation at room temperature for 15 minutes. In the second elution (E2) the anti-PC resin was incubated for 15 min at room temperature. Eluates were stored at -20°C.

SDS-PAGE and Western blot analysis

Protein samples incubated with SDS sample buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 1.33% beta-mercaptoethanol) were separated on 1 mm 12% Tris-glycine SDS-polyacrylamide gels by electrophoresis (Laemmli, 1970) followed by transfer to Immobilon-P PVDF membranes (Millipore) at 100 V for 1.5 h. Membranes were then blocked overnight in 3% non-fat milk, phosphate-buffered saline (140.7 mM NaCl, 9.3 mM Na_2HPO_4 pH 7.4), 0.1% Tween-20. Membranes were then probed with primary antibodies (Table 3) in 2% non-fat milk, phosphate-buffered saline, 0.1% Tween-20 at the following dilutions: Coq1p, 1:10,000; Coq2p, 1:1000; Coq3p, 1:1000; Coq4p, 1:1000; Coq5p, 1:5000;

Coq6p, 1:250; Coq7p, 1:500; affinity purified Coq8p, 1:30; Coq9p, 1:1000; affinity purified Coq10p, 1:15; Atp2p, 1:2000; Mdh1p, 1:10,000; and Protein C tag (HPC4), 0.7 µg/mL. Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Calbiochem) was used at 1:10,000 dilutions. Blots were visualized using Supersignal West Pico Chemiluminescent Substrate (Thermo).

SYPRO Ruby staining

Protein samples incubated with SDS sample buffer were separated on an 8-16% Criterion SDS-PAGE gel (Bio-Rad). Following electrophoresis the gel was washed twice with water and fixed for 1 h with 100 mL 10% methanol/7% acetic acid. The gel was washed twice with water and then stained overnight at room temperature in the dark with 80 mL with a 1:1 mixture of fresh and used SYPRO Ruby (Life Technologies). Following staining the gel was washed twice with water and destained for 4 h with 10% methanol/7% acetic acid. Proteins were visualized with an FX Pro Plus Molecular Imager (Bio-Rad) at 532 nm excitation and emission was measured with a 555 nm long pass filter.

In-gel trypsin digestion and proteomic analysis

Sample lanes from a SYPRO Ruby-stained gel were cut into 5 mm slices and subjected to in-gel trypsin digestion as described (Shirasaki *et al.*, 2012), with a few modifications. Gel slices were washed with 50 mM NH_4HCO_3/50% acetonitrile followed by 100% acetonitrile, three alternating cycles total. Samples were then incubated with 10 mM dithiothreitol (MP Biomedicals) at 60°C for one hour to reduce disulfide bonds, followed by treatment with 50 mM iodoacetamide (Calbiochem) at 45°C for 45 min in the dark to alkylate free sulfhydryl groups.

Gel slices were then washed once with 50 mM HN_4HCO_3, followed by three alternating washes with 100 mM NH_4HCO_3 and 100% acetonitrile. Gel slices were then dried by speedvac at 60°C for 10 min. Gel-entrapped proteins were then digested overnight at 37°C using 20 ng/μL trypsin (Promega) in 50 mM NH_4HCO_3. Following digestion tryptic peptides were extracted from the gel slices with 50% acetonitrile/0.1% TFA and dried by speedvac at 30°C for 2 h.

LC-MS/MS analysis of the trypsin digested peptides was performed as follows: Peptides were resuspended in 30 μL of 3% acetonitrile/0.1% formic acid. The peptides were analyzed using a nanoACQUITY UPLC system coupled to a Xevo QTof (quadrupole time-of-flight) mass spectrometer (Waters). Peptides were eluted from the UPLC to the electrospray ionization mass spectrometer using a 5 μm 2G-VM Symmetry C18 180 μm x 20 mm trap column in-line with a 1.8 μm HSS T3, 75 μm x 150 mm C18 analytical column (Waters). The digested peptides were separated using a 0 to 60 min gradient, beginning at 3% acetonitrile/0.1% formic acid to 40% acetonitrile/0.1% formic acid, followed by a 40-95% gradient between 60-62 mins at a flow rate of 0.3 μL min^{-1} (total run time 90 min). [Glu^1]-Fibrinopeptide B was used as a mass calibration standard (100 fmol/μL) and was infused via a separate electrospray ionization sprayer, and the standard peptide was measured every 45 s during data collection.

The mass spectrometer was operated in the MS^E data-independent acquisition mode (Silva *et al.*, 2006; Silva *et al.*, 2006; Geromanos *et al.*, 2009). ProteinLynx Global Server (PLGS version 3, Waters) was used for protein identification. The MS data were searched against the Uniprot *Saccharomyces cerevisiae* (strain ATCC 204508/S288c) reference proteome dataset. The database was further supplemented with sequences for keratin and trypsin. The MS data files were searched using the PLGS algorithm that includes precursor and product ion

tolerance, minimum number of peptide matches (1), minimum number of product ions per peptide (3), minimum number of product ions per protein (7), maximum false positive rate (4%). Searches were limited to trypsin proteolysis fragments and peptide precursors, and product ion mass tolerances were set to automatic detection. Carbamidomethylation of Cys residues was set as a fixed modification, and Met oxidation, Ser, Thr and Tyr phosphorylation, and Cys propionamidation resulting from iodoacetamide treatment were set as variable modifications.

Metabolic labeling of Q_6 with $^{13}C_6$-labeled precursors

Yeast strains were grown overnight in 5 mL SD-complete and diluted to an OD_{600} of 0.05 in 50 mL DOD-complete. Cultures were grown to an OD_{600} of 0.5, corresponding to early-log phase, and then labeled with either $^{13}C_6$-4HB or $^{13}C_6$-pABA at a concentration of 5 µg/mL (250 µg total). Cells were grown an additional three hours with label, harvested by centrifugation at 2000 × *g* for 10 min, and stored at −20 °C. Wet weights were determined for yeast cell pellets.

Analysis of Q_6 and Q_6 intermediates

Lipid extraction of eluate samples and cells grown in DOD-complete was performed with methanol/petroleum ether and Q_4 as an internal standard, and LC-MS/MS analysis of extracts was performed as described (Marbois *et al.*, 2010). Briefly, a 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (Foster City, CA) was used, and Analyst version 1.4.2 software (Applied Biosystems) was used for data acquisition and processing. A binary HPLC delivery system was used with a phenyl-hexyl column (Luna 5u, 100 × 4.60 mm, 5-µm, Phenomenex), with a mobile phase consisting of Solvent A (methanol/2-propanol, 95:5, 2.5 mM ammonium formate) and Solvent B (2-propanol, 2.5 mM ammonium formate). The percentage

of Solvent B was increased linearly from 0 to 5% over 6 min, and the flow rate was increased from 600 to 800 µL/min. The flow rate and mobile phase were changed back to initial conditions linearly by 7 min. All samples were analyzed in multiple reaction monitoring mode (MRM).

Results

Effects of CNAP tag integration on respiration and Q biosynthesis

The CNAP tag was integrated into the 3' end of the *COQ3*, *COQ6*, and *COQ9* ORFs, generating the CNAP3, CNAP6, and CNAP9 strains respectively. The ability of these strains to respire as measured by growth on a non-fermentable carbon sources was assessed by plate dilution assay, using W303 as the wild-type control and Δ*COQ8* as the Q-less, respiratory defective control (Fig. 1A). All three tagged strains grew as well as wild type on the non-fermentable carbon source, YPG, while the Q-less mutant failed to grow. The three tagged strains as well as the Q-less mutant were generated by chromosomal integration of the *HIS3* ORF and grew on SD−his, while the wild type did not. All five strains were able to grow on the permissible carbon source YPD.

De novo Q biosynthesis was measured by LC-MS/MS of cells grown in DOD-complete media labeled with the stable isotopically-labeled ring precursors $^{13}C_6$-4HB or $^{13}C_6$-pABA (Fig. 1B). Levels of *de novo* $^{13}C_6$-Q in CNAP3 synthesized from either precursor were not significantly different compared to wild type, while *de novo* $^{13}C_6$-Q synthesized from $^{13}C_6$-4HB in CNAP6 and $^{13}C_6$-pABA in CNAP9 was not significantly different compared to wild type. *De novo* $^{13}C_6$-Q synthesized from $^{13}C_6$-pABA in CNAP6 was 120% of wild type, while *de novo* $^{13}C_6$-Q synthesized from $^{13}C_6$-4HB in CNAP9 was 70% of wild type. The Δ*COQ8* strain did not produce detectable *de novo* $^{13}C_6$-Q_6. Total Q accumulation was also measured by analyzing unlabeled, ^{12}C-Q_6 by LC-MS/MS (Fig. 1C). Levels of accumulated Q_6 in CNAP3 grown with both precursors was not significantly different compared to wild type, while accumulated Q_6 in CNAP9 grown with $^{13}C_6$-pABA was not significantly different compared to wild type.

Accumulated Q_6 in CNAP6 was 80% of wild type under $^{13}C_6$-4HB labeling, while it was 120% under $^{13}C_6$-pABA compared to wild type. Accumulated Q_6 in CNAP9 labeled with $^{13}C_6$-4HB was 80% of wild type. The Δ*COQ8* strain did not accumulate detectable Q_6. The results indicate that CNAP-tagged Coq3, Coq6, or Coq9 polypeptides expressed from the integrated loci remain functional.

Co-precipitation of Coq proteins with CNAP-tagged Coq proteins

Solubilized mitochondria (15 mg protein) from W303, CNAP3, CNAP6, and CNAP9 were subject to tandem affinity purification of the CNAP tag. Purified mitochondria (25 µg protein) and fractions of the first anti-PC eluate (E1) were analyzed by Western blot to identify interacting Coq proteins (Fig. 2). Membranes were probed with antibodies to Coq1p-Coq10p, the protein C epitope to identify the tagged protein, and Atp2p and Mdh1p to assess the purity of the eluates. CNAP3 co-precipitated Coq4p, Coq5p, Coq6p, Coq7p, and Coq9p; CNAP6 co-precipitated Coq4p, Coq5p, Coq7p, Coq8p, and Coq9p; and CNAP9 co-precipitated Coq4p, Coq5p, Coq6p, and Coq7p. The Coq5p and Coq9p signal was much weaker in CNAP3 and CNAP6 compared to CNAP9. The predominant band in the Coq3p blot is a non-specific band at the same molecular mass as Coq3p, accounting for the non-shifted band in CNAP3 M. All three tagged proteins are present in their respective blots at shifted molecular masses due to the tag and are denoted by arrows. The three tagged proteins are also present on the anti-PC blot at their appropriate molecular masses. No signal is visible in the eluate fractions for the non-specific mitochondrial proteins Atp2p and Mdh1p.

Identification of associated lipids and proteins by mass spectrometry

Aliquots of anti-PC eluates (29% E1 and 29% E2) were combined for each strain and subject to lipid extraction (Fig. 3). Extracts were then analyzed by LC-MS/MS for Q_6 and Q_6 biosynthetic intermediates. The CNAP3, CNAP6, and CNAP9 eluates were all found to have 11.7-fold, 8.6-fold, and 22.6-fold higher levels respectively of associated Q_6 compared to wild type (Fig. 3A). The late stage intermediate DMQ_6 was also measured and found to be 30.0-fold higher in CNAP3, 24.0-fold higher in CNAP6, and 97.0-fold in CNAP9 compared to wild type (Fig. 3C). The late stage intermediate $IDMQ_6$ was also found in measurable quantities in the CNAP3, CNAP6, and CNAP9 eluates, while no $IDMQ_6$ was detected in the wild type eluate (Fig. 3E). Representative HPLC traces of Q_6, DMQ_6, and $IDMQ_6$ are shown (Figs. 3B, 3D, and 3F). The early intermediates HHB and HAB were not detectable in any of the analyzed eluates (data not shown).

Aliquots of the anti-PC eluates (20% E1 and 20% E2) were combined for each strain, dried by speedvac, and resuspended in 42 µL SDS sample buffer. Protein was then separated on an 8-16% Criterion SDS-PAGE gel (Bio-Rad) and stained with SYPRO Ruby (Life Technologies) to visualize total protein (Fig. 4). CNAP3, CNAP6, and CNAP9 each contain several unique protein bands compared to wild type, specifically at 25-37 kDa and 50 kDa. Each sample lane was cut into 5 mm slices and subjected to in-gel trypsin digestion. Tryptic peptides were analyzed by LC-MS and the results from several trials are shown in Table 4. Several of the Coq proteins observed by Western blotting were detected by mass spectrometry of the CNAP3, CNAP6, and CNAP9 eluates.

Analyses of the ILV6 and YLR290C null mutants

The *ILV6* and *YLR290C* null mutants in the BY4741 background were purchased from the GE Healthcare yeast knockout collection for phenotypic analysis due to their presence in the CNAP-tagged Coq protein eluates (Table 4). The ability of these null mutants to grow on a non-fermentable carbon source was assessed by plate dilution assay using BY4741 as the wild-type control and Δ*COQ3* as the Q-less, mutant control (Fig. 5). Both the *ILV6* and *YLR290C* null mutants grew as well as wild type on the non-fermentable carbon source YPG, while the *coq3* null mutant failed to grow. All strains grew on the permissible carbon source YPD.

The levels of *de novo* $^{13}C_6$-Q_6 biosynthesis as well as accumulation of Q_6 and Q_6 intermediates were measured by LC-MS/MS of yeast cultures grown in DOD-complete medium labeled with the stable isotopically-labeled precursors $^{13}C_6$-4HB or $^{13}C_6$-pABA (Fig. 6). The *ilv6* and *ylr290c* null mutants both produced high levels of *de novo* $^{13}C_6$-HAB and $^{13}C_6$-HHB, similar to the *coq3* null mutant (Fig. 6A and 6C). The null mutants also accumulated higher levels of ^{12}C-HAB and ^{12}C-HHB compared to wild type, although the *ilv6* null accumulated ^{12}C-HAB to a greater extent under $^{13}C_6$-4HB and $^{13}C_6$-pABA labeling (370% and 420% respectively) than the *ylr290c* null (170% and 150% respectively), and relative levels of ^{12}C-HHB were only modestly greater than wild type (Fig. 6B and 6D). The late stage intermediate $^{13}C_6$- or ^{12}C-IDMQ$_6$ was not detected in this analysis (data not shown), however the *ylr290c* null mutant labeled with $^{13}C_6$-pABA was found to have reduced levels of $^{13}C_6$-DMQ$_6$ relative to the wild type, while the *ilv6* null mutant had increased levels when labeled with $^{13}C_6$-pABA (Fig. 6E). In contrast the *ilv6* null mutant labeled with $^{13}C_6$-pABA and the *ylr290c* null mutant both accumulated higher levels of ^{12}C-DMQ$_6$ relative to wild type (Fig. 6F). The *ilv6* null mutant produced 60% *de novo* $^{13}C_6$-Q_6 compared to wild type when labeled with $^{13}C_6$-4HB, while the *ylr290c* null mutant produced severely reduced *de novo* $^{13}C_6$-Q_6 when labeled with either $^{13}C_6$-4HB or $^{13}C_6$-pABA (6.1% and

4.5% of wild type respectively) (Fig. 6G). The *ylr290c* null mutant also accumulated significantly less ^{12}C-Q_6 when labeled with $^{13}C_6$-4HB and $^{13}C_6$-pABA compared to the wild type (17.5% and 21.8% of wild type respectively) (Fig. 6H). Interesting the *ylr290c* null mutant appeared to synthesize wild-type levels of Q_6 when grown in rich media (unpublished observations). Representative traces of *de novo* $^{13}C_6$-Q_6 comparing the wild type to the *ylr290c* null labeled with $^{13}C_6$-4HB are shown (Fig. 6I). These results indicate that both *ylr290c* and *ilv6* null mutants accumulate higher levels of Q_6 intermediates, and that the *ylr290c* null mutant has defective Q synthesis compared to wild type.

Analysis of CNAP-tagged YLR290C

The CNAP tag was integrated into the 3' end of the *YLR290C* open reading frame in W303-1A to yield the strain designated CA-1. The levels of *de novo* and accumulated Q_6 were measured in this strain to assess the impact of the tag on Q_6 biosynthesis (Fig. 7). The CA-1 strain produced modestly reduced levels of *de novo* $^{13}C_6$-Q_6 with both $^{13}C_6$-4HB (70% of wild type) and $^{13}C_6$-pABA (80% of wild type). Levels of accumulated ^{12}C-Q_6 were also decreased in CA-1 when labeled with either $^{13}C_6$-4HB (70% of wild type) or $^{13}C_6$-pABA (70% of wild type).

Solubilized CA-1 mitochondria (15 mg protein) were subjected to tandem affinity purification of the CNAP tag (Fig. 8). Purified mitochondria (25 µg protein) and fractions of the first anti-PC eluate (E1) were analyzed with by Western blotting to identify interacting Coq proteins. Membranes were probed with antibodies to Coq1p-Coq9p, the protein C epitope to identify the tagged protein, and Atp2p and Mdh1p to assess the purity of the eluates. Coq4p, Coq5p, and Coq7p were observed to co-precipitate with YLR290C-CNAP in the CA-1 strain,

while the non-specific mitochondrial proteins Atp2p and Mdh1p were not present in the eluate. The anti-PC blot verifies the presence of the CNAP-tagged protein.

Aliquots of anti-PC eluates (29% E1 and 29% E2) were combined for the wild type and CA-1 strains and subject to lipid extraction (Fig. 9). Extracts were then analyzed by LC-MS/MS for Q_6 and Q_6 biosynthetic intermediates. The CA-1 eluate was found to have 5.4-fold higher levels of associated Q_6 compared to wild type (Fig. 9A). The late stage intermediate DMQ_6 was also measured and found to be 6.7-fold higher in CA-1 relative to wild type (Fig. 9C). The late stage intermediate $IDMQ_6$ was also found in measurable quantities in the CA-1 eluate, while no $IDMQ_6$ was detected in the wild type eluate (Fig. 9E). Representative traces of Q_6, DMQ_6, and $IDMQ_6$ are shown (Fig. 9B, 9D, and 9F). The early intermediates HHB and HAB were not detectable in any of the analyzed eluates (data not shown).

Discussion

This study characterized the composition of the Q biosynthetic complex in yeast mitochondria using tandem affinity purification of C-terminally tagged Coq proteins. The CNAP tag was used to purify the complex from digitonin-solubilized mitochondria under gentle conditions to preserve the noncovalent associations between protein constituents of the complex to allow for identification by both Western blotting and mass spectrometry analysis. The CNAP tag did not disrupt the function of the tagged Coq proteins as assayed by growth on a non-fermentable carbon source and *de novo* Q biosynthesis (Fig. 1), making it a suitable system to study the *in vivo* Q biosynthetic complex. Tandem affinity purification of tagged Coq3p, Coq6p, and Coq9p confirmed the association of Coq3p, Coq4p, Coq5p, Coq6p, Coq7p, and Coq9p in one or more complexes, and also demonstrated the association of Coq8p with a Coq6p-containing complex (Fig. 2). Coq3p, Coq5p, and Coq7p are phosphorylated in a Coq8p-dependent manner (Tauche *et al.*, 2008; Xie *et al.*, 2011) and overexpression of *COQ8* in various *coq* null mutants stabilizes several of the other Coq polypeptides and leads to accumulation of later stage Q biosynthetic intermediates (Padilla *et al.*, 2009; Xie *et al.*, 2012; He *et al.*, 2014), however in this study we show the first direct evidence of association of Coq8p with the Q biosynthetic complex.

The eluates from tandem affinity purification were also subjected to proteomic analysis to identify potentially novel binding partners beyond the known Coq proteins, the results of which are shown in Table 4. Several of the Coq proteins were observed in the eluates for the CNAP-tagged Coq proteins, consistent with results observed by Western blotting in Figure 2. In addition to the Coq proteins a few other proteins were detected at levels comparable to the Coq

proteins: YLR290C in the CNAP3 eluate and Ilv6p in the CNAP9 eluate. Ilv6p was also detected in the wild type control and the CNAP3 and CNAP6 eluates, but was most strongly detected with the CNAP9 eluate. The potential function of these two proteins in Q biosynthesis was assessed by monitoring growth on a non-fermentable carbon source and measuring *de novo* Q synthesis using stable isotopically-labeled ring precursors for the corresponding null mutants. The *ylr290c* and *ilv6* null mutants did not display impaired growth on a non-fermentable carbon source (Fig. 5), however the *ylr290c* null was found to have severely decreased *de novo* Q synthesis as well as decreased total Q as measured by $^{13}C_6$-Q_6 and ^{12}C-Q_6 respectively (Fig. 6). The ability of the *ylr290c* null mutant to grow on a non-fermentable carbon source in spite of its decreased Q synthesis is not surprising as other mutants with decreased Q synthesis have been characterized and retain the ability to grow on a non-fermentable carbon source (Allan *et al.*, 2012; Nguyen *et al.*, 2014).

The CNAP tag was integrated into the 3'-end of the *YLR290C* ORF to generate a C-terminal fusion protein which was subject to tandem affinity purification to determine whether there were any co-purifying proteins. Western blotting showed that YLR290C co-purified with Coq4p, Coq5p, and Coq7p, confirming the results of the proteomic analysis and demonstrating association of YLR290C with the Q biosynthetic complex (Fig. 8). The levels of co-purified proteins were less than observed for CNAP-tagged Coq3p, Coq6p, and Coq9p, which is consistent with the observation that proteomic analysis of the CA-1 eluate did not identify additional binding partners (not shown). It may be that YLR290C is more transiently associated with the Q biosynthetic complex or is less stable in digitonin extracts of mitochondria, leading to a reduced yield of associated proteins.

Previous studies have shown that the late stage Q intermediate DMQ_6 co-purifies with the Q biosynthetic complex (Marbois *et al.*, 2005), and exogenous Q_6 has been shown to stabilize particular Coq proteins and lead to production of later stage biosynthetic intermediates in certain *coq* mutants (Tran *et al.*, 2006; Padilla *et al.*, 2009; He *et al.*, 2014), suggesting a function for Q or a Q intermediate in the stability of the biosynthetic complex. To further explore the association of associated lipids with the Q biosynthetic complex eluates from tandem affinity purification were subjected to lipid extraction and LC-MS/MS to measure Q_6 and Q_6 intermediates. In the CNAP3, CNAP6, and CNAP9 eluates Q_6, DMQ_6, and $IDMQ_6$ were all detected at levels significantly greater than the wild-type control (Fig. 3), while the early intermediates HHB and HAB were not detected (data not shown). The CA-1 eluate was also analyzed to measure the association of Q_6 and Q_6 intermediates with a YLR290C-containing complex and was found to also contain Q_6, DMQ_6, and $IDMQ_6$ (Fig. 9), while the early intermediates HHB and HAB were not detected (data not shown). These results are consistent with the previous observations of associated DMQ_6 with the Q biosynthetic complex and ability of exogenous Q_6 to stabilize several Coq proteins in certain *coq* mutants. Coq4p was previously hypothesized to serve as a scaffolding protein for the complex and the crystal structure of the Coq4p homolog Alr8543 from *Nostoc sp. PCC7120* revealed a bound geranylgeranyl monophosphate, suggesting that Coq4p functions to stabilize the Q biosynthetic complex through its interactions with other Coq proteins and a polyisoprenoid lipid (Marbois *et al.*, 2009; Rea *et al.*, 2010). Coq10p was shown to be required for efficient *de novo* Q biosynthesis and was demonstrated to bind Q and a late-stage Q intermediate via a START domain, leading to the hypothesis that Coq10p acts a Q chaperone and is necessary for delivery of Q to the biosynthetic complex for efficient de novo Q synthesis (Allan *et al.*, 2012). The observation that YLR290C

co-purified with Q and late-stage Q intermediates is consistent with the Western blot results showing the association of YLR290C with Coq4p, Coq5p, and Coq7p, and supports the role of YLR290C as a component of the Q biosynthetic complex.

The function of YLR290C is unknown however it is known to be a mitochondrial protein through a high-throughput study (Perocchi *et al.*, 2006) and does not appear to have any significant non-fungal homologs among well-studied model organisms, with the exception of the uncharacterized *Arabidopsis thaliana* protein At1g32220, which has 26% identity with YLR290C and is predicted to be imported into the chloroplast (Boratyn *et al.*, 2013; UniProt, 2014). Primary sequence analysis identified YLR290C as part of the short-chain dehydrogenase/reductase (SDR) superfamily, which include a diverse family of oxidoreductases and catalyze reactions such as isomerization, decarboxylation, epimerization, imine reduction, and carbonyl-alcohol oxidoreduction (Marchler-Bauer *et al.*, 2013). SDR proteins contain a conserved Rossmann fold, a structural motif found in proteins that bind nucleotide cofactors such as NAD(P), FAD, and FMN (Rossmann *et al.*, 1974). The crystal structure of *Pseudomonas aeruginosa* UbiX, which catalyzes the decarboxylation step in the Q biosynthetic pathway in that organism, was shown to contain a Rossmann fold with a bound FMN (Kopec *et al.*, 2011). Similarly the structure of *E. coli* Pad1, a paralog with 51% identity to E. coli UbiX which catalyzes the decarboxylation reaction in the Q biosynthetic pathway, was shown to contain a typical Rossmann fold with a bound FMN (Rangarajan *et al.*, 2004). While the function of the Rossmann fold in YLR290C remains unclear it is tempting to speculate that it may also function to catalyze an FMN-dependent decarboxylation in the yeast Q biosynthetic pathway. The observation that the *ylr290c* null mutant still produces small amounts of Q_6 is not fully consistent this hypothesis, however there may be redundant decarboxylases capable of bypassing the

ylr290c null such as Pad1p and Fdc1p, two phenylacrylic acid decarboxylases with homology to the *E. coli* Q biosynthetic decarboxylases UbiD and UbiX (Gulmezian *et al.*, 2007; Mukai *et al.*, 2010). Expression of yeast *PAD1* in an *E. coli ubiX* mutant restored Q_8 synthesis, however yeast mutants lacking either *PAD1* or *FDC1*, or both *PAD1* and *FDC1* produce wild type levels of Q_6 (Gulmezian, 2006; Gulmezian *et al.*, 2007), potentially due to their functions as redundant decarboxylases.

ILV6 encodes the regulatory subunit of acetolactate synthase; *ILV2* catalyzes this committed step of branched-chain amino acid biosynthesis in yeast mitochondria (Cullin *et al.*, 1996). Ilv6p is not an essential protein and is not required for the catalytic activity of Ilv2p, but regulates Ilv2p through feedback inhibition by valine, a final product of the branched-chain amino acid biosynthetic pathway, an effect reversed by binding of ATP to Ilv6p (Cullin *et al.*, 1996; Pang and Duggleby, 2001). An association of Ilv6p with the Q biosynthetic complex may suggest a metabolic regulatory mechanism on Q synthesis, with both ATP and valine potentially serving an allosteric role. The regulatory effects of these two molecules on the Q biosynthetic complex through Ilv6p would likely be reversed with respect to their effects on Ilv2p, given the catabolic function of Q in energy metabolism versus the anabolic nature of amino acid biosynthesis. Although Q_6 intermediates were higher, the *ilv6* null mutant had near normal Q_6 content and *de novo* Q_6 biosynthesis.

In this study we show that tandem affinity tagged Coq3p, Coq6p, and Coq9p all co-purify with Coq polypeptide biosynthetic complex and recapitulate the results from previous affinity purification experiments (Marbois *et al.*, 2005; Hsieh *et al.*, 2007), with the additional observation that Coq3p is now shown to be associated the entire complex and not just Coq4p. In

addition Coq8p was observed to co-purify with a Coq6p-containing complex, but not with tagged Coq3p or Coq9p, suggesting a closer association with Coq6p. While Coq8p is required for the stability of the Coq polypeptides and the biosynthetic complex (Xie *et al.*, 2012; He *et al.*, 2014) this is the first evidence showing direct association of Coq8p with the complex. Consistent with this observation a recent study reported that in a mouse *Coq9*^{R239X} mutant steady-state levels of the mouse Coq8p homolog Adck3 were significantly decreased relative to wild type in heart and kidney tissues (Lohman *et al.*, 2014). Proteomic analysis revealed two primary candidate proteins as potential members of the Coq complex, YLR290C and Ilv6p, and analysis of the corresponding null mutants revealed that loss of YLR290C leads to significantly decreased synthesis of Q, consistent with a role in Q biosynthesis.

Tandem affinity purification of tagged YLR290C confirmed its association with the Q biosynthetic complex and lipid analysis demonstrated that a YLR290C-containing complex was also associated with Q, DMQ_6, and $IDMQ_6$. Lipid analysis of the Coq complex also revealed the association of Q_6 and the two late-stage biosynthetic intermediates DMQ_6 and $IDMQ_6$, but not the early-stage intermediates HHB and HAB, suggesting that an associated polyisoprenoid quinone is necessary for the stability of the complex. The crystal structure of human COQ9 was recently solved and shown to have a lipid-binding site, and mass spectrometry analysis of purified human COQ9 demonstrated that it is associated with several lipids, including Q (Lohman *et al.*, 2014). These observations strongly indicate YLR290C performs a definitive function in Q biosynthesis and we propose that this protein be designated Coq11p. These results have been incorporated into an updated model of the Q biosynthetic complex (Fig. 10), which now depicts Coq8p in association with Coq6p and YLR290C, designated Coq11p, in association with Coq4p, Coq5p, and Coq7p.

While an *ilv6* null mutant did not appear to affect Q synthesis it may still function in a regulatory capacity to modulate Q biosynthesis, similar to its role in regulating the activity of Ilv2p (Pang and Duggleby, 2001). Additional characterization of the potential interaction of Ilv6p with the Coq complex is required to further understand this possible function. There are functional homologs of Ilv6p in prokaryotes and plants but not other eukaryotes, making this possible regulatory scheme unique to prokaryotes, fungi, and plants (Cullin *et al.*, 1996; Hershey *et al.*, 1999; Lee and Duggleby, 2001). There were additional protein candidates from proteomic analysis of the tandem affinity purification eluates and further characterization of these proteins may yield a more complete understanding of the proteins associated with the Q biosynthetic complex and the regulation of this pathway.

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]
W303ΔCOQ8	Mat **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq8*::*HIS3*	(Do *et al.*, 2001)
CNAP3	Mat **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 COQ3*::*COQ3-CNAP-HIS3*	This study
CNAP6	Mat **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 COQ6*::*COQ6-CNAP-HIS3*	This study
CNAP9	Mat **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 COQ9*::*COQ9-CNAP-HIS3*	This study
CA-1	Mat **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 YLR290C*::*YLR290C-CNAP-HIS3*	This study
BY4741	MAT **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Brachmann *et al.*, 1998)
BY4741Δ*COQ3*	MAT **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 coq3*::*kanMX4*	(Winzeler *et al.*, 1999)
BY4741Δ*ILV6*	MAT **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ilv6*::*kanMX4*	(Giaever *et al.*, 2002)[b]
BY4741Δ*YLR290C*	MAT **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ylr290c*::*kanMX4*	(Giaever *et al.*, 2002)[b]

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

[b] GE Healthcare Yeast Knockout Collection, available on-line

Table 2

Primers used to integrate 3' CNAP tag coding sequence

Primer	Sequence[a]
COQ3-CNAP F	5'-ACGATTGTTCCGATGTCGGTAATTATTTTATGGCTATTCAGAGACTGAATCACCACCACCACCATCACC
COQ3-CNAP R	5'-TATTTATATAAGAAGATATTTACAGTCAGATACCTACTTTTCGTTTGATTGAATTCGAGCTCGTTTAAAC
COQ6-CNAP F	5'-TCGGTCCAGTTAAGAATATGATCATTGACACATTAGGAGGAAATGAGAAACACCACCACCACCATCACC
COQ6-CNAP R	5'-CATAAATAATTCTTAAAAGTGGAGCTAGTCTATTTCTATTTACATACCTCGAATTCGAGCTCGTTTAAAC
COQ9-CNAP F	5'-ATACGTTAATGTCTACGGTAAATTTAATCAAATCTCAATTAGTTAGGGGTCACCACCACCACCATCACC
COQ9-CNAP R	5'-ATACCTACGCATTTCACTGTCCTAAACTTCAAATTGACATGTAAGATTGCGAATTCGAGCTCGTTTAAAC
YLR290C-CNAP F	5'-AAAACCCAGATTTTAAAGGGGTAGTTACACTTGAGGAAATACTTAAAGCACACCACCACCACCATCACC
YLR290C-CNAP R	5'-TGTAAATAAAAGAGAAAAATAGATTAATAAATATAATAATGGCATAATCAGAATTCGAGCTCGTTTAAAC

[a] Underlined nucleotides denote 50 bp flanking the stop codon upstream (F) and downstream (R) of the target gene

Table 3

Description and source of antibodies

Antibody	Source
Atp2p	C.M. Koehler[a]
Coq1p	(Gin and Clarke, 2005)
Coq2p	(Hsieh *et al.*, 2007)
Coq3p	(Poon *et al.*, 1999)
Coq4p	(Belogrudov *et al.*, 2001)
Coq5p	(Baba *et al.*, 2004)
Coq6p	(Gin *et al.*, 2003)
Coq7p	(Tran *et al.*, 2006)
Coq8p	(Hsieh *et al.*, 2007)
Coq9p	(Hsieh *et al.*, 2004)
Coq10p	(Barros *et al.*, 2005)
Mdh1p	L. McAlister-Henn[b]
Protein C	Antibodies-online Inc.

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

b Dr. L. McAlister-Henn, Department of Molecular Biophysics and Biochemistry, University of Texas Health Sciences Center, San Antonio

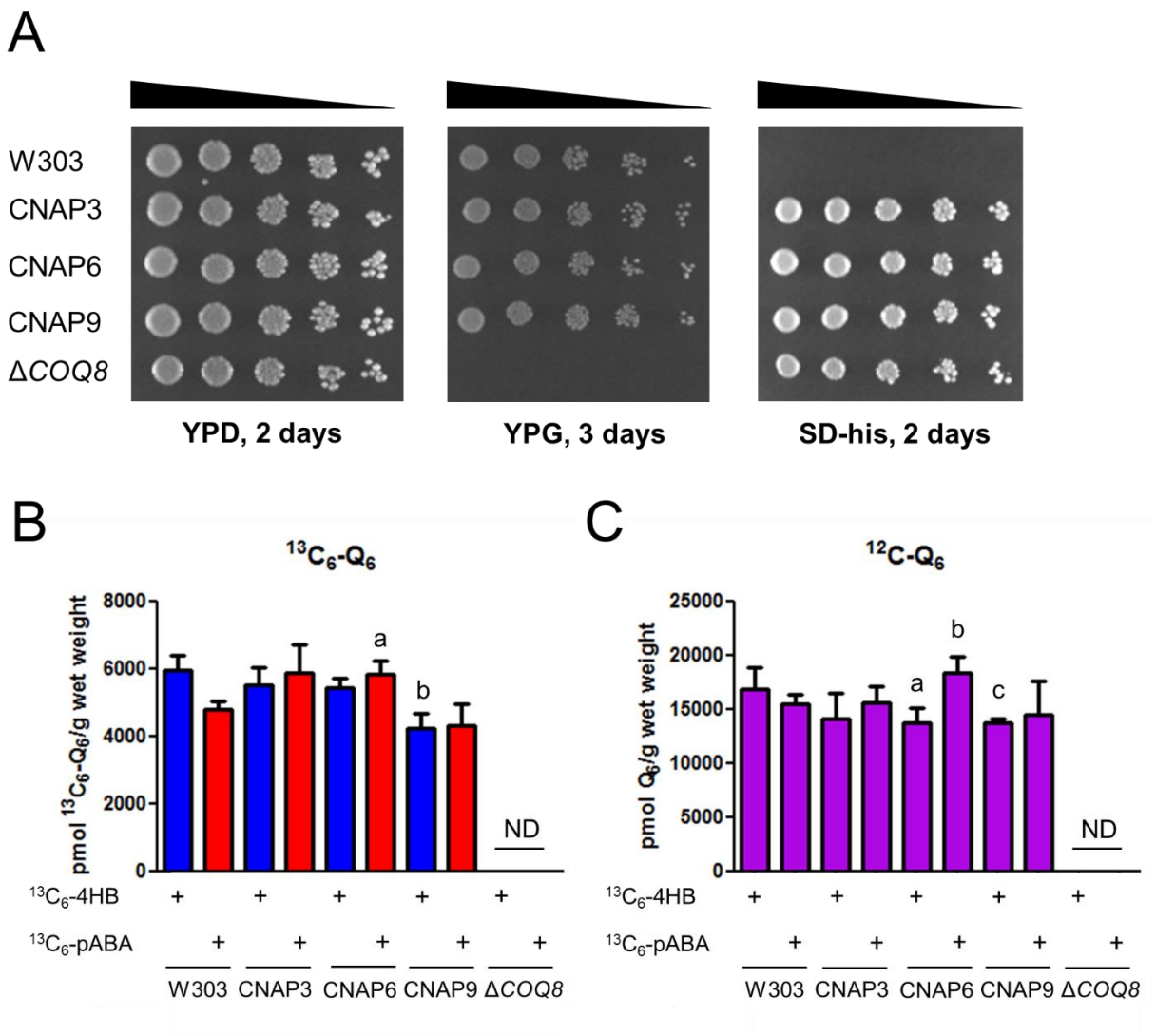


Figure 1. Expression of CNAP-tagged Coq3, Coq6, or Coq9 proteins preserves growth on a non-fermentable carbon source and *de novo* Q biosynthesis. (A) Designated yeast strains were grown overnight in 5 mL YPD, diluted to an OD_{600} of 0.2 with sterile PBS, and 2 µL of 5-fold serial dilutions were spotted onto each type of plate media, corresponding to a final OD_{600} of 0.2, 0.04, 0.008, 0.0016, and 0.00032. Plates were incubated at 30°C and growth is depicted after two days for both YPD and SD-his, and three days for YPG. Total $^{13}C_6$-Q_6 ($^{13}C_6$-Q_6 + $^{13}C_6$-Q_6H_2) **(B)** and total ^{12}C-Q_6 (^{12}C-Q_6 + ^{12}C-Q_6H_2) **(C)** were measured in the designated yeast strains by HPLC-MS/MS. Yeast strains were grown overnight in 5 mL SD-complete, diluted to

an OD_{600} of 0.05 in 50 mL DOD-complete, labeled with either $^{13}C_6$-4HB or $^{13}C_6$-pABA at an OD_{600} of 0.5, and harvested after three hours of labeling. $^{13}C_6$–Q_6 was detected using precursor-to-product ion transitions of 597.4/203.0 and 616.4/203.0, and ^{12}C-Q_6 with transitions of 591.4/197.0 and 610.4/197.0. Lipid measurements were normalized by the wet weight of extracted cells. Each bar represents the mean of four measurements from two biological samples with two injections each. Error bars represent standard deviations. Statistical significance was determined with the two-tailed Student's t-test and lower case letters above bars are indicative of statistical significance. In **(B)** the content of total $^{13}C_6$-Q_6 in CNAP3, CNAP6, CNAP9, and Δ*COQ8* was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0052; b, p=0.0018; ND, not detected). In **(C)** the content of total ^{12}C-Q_6 in CNAP3, CNAP6, CNAP9, and Δ*COQ8* was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0423; b, p=0.0179; c, p=0.0224; ND, not detected).

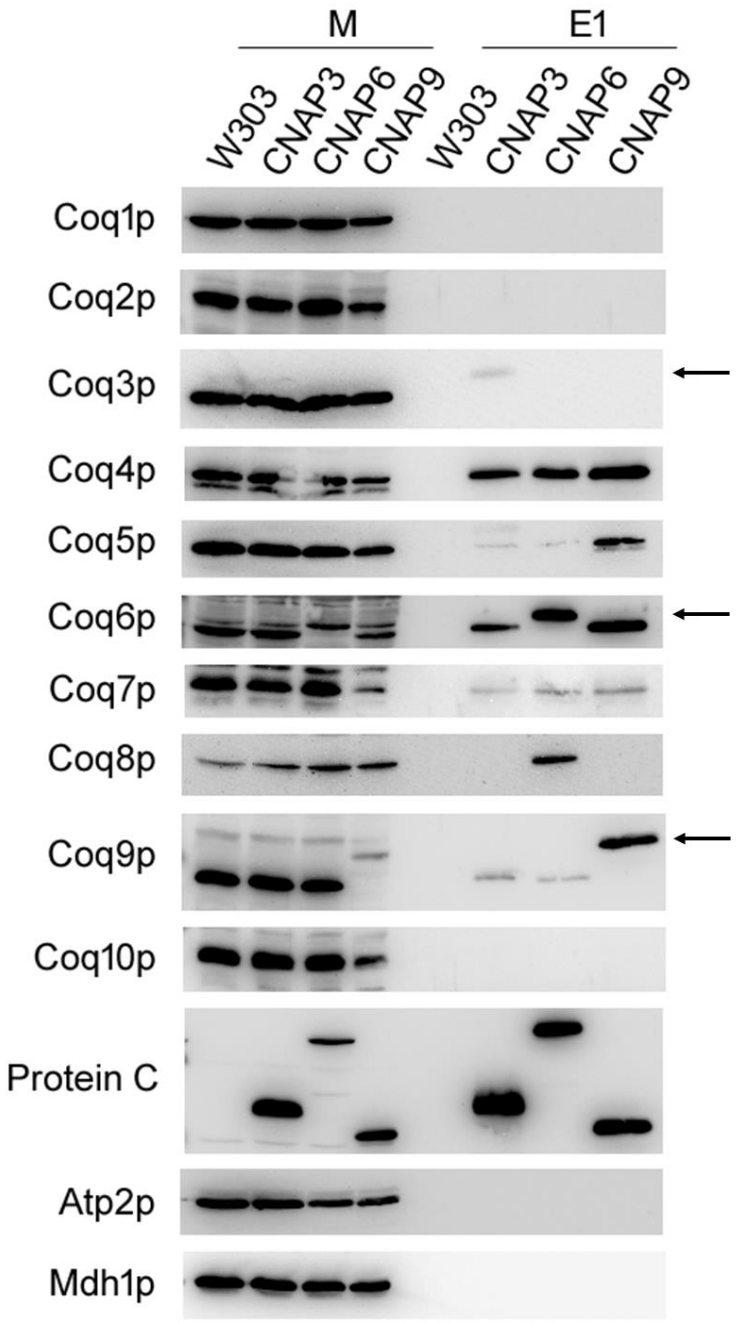


Figure 2. CNAP-tagged Coq proteins co-precipitate several other Coq proteins. W303, CNAP3, CNAP6, and CNAP9 purified mitochondria (15 mg protein) were solubilized with digitonin and subject to tandem affinity purification with Ni-NTA resin (Qiagen) followed by anti-PC agarose (Roche). Samples were separated on 12% SDS-PAGE gels followed by transfer

to PVDF membranes for Western blotting with antisera to the designated yeast polypeptides. 25 µg mitochondria protein were analyzed for each strain (M) and 2.5% of the first anti-PC elution (E1) volume was loaded per strain (25 µL). Arrows denote each tagged protein in their respective blots. The predominant band in the Coq3p blot represents a background protein and not Coq3p, accounting for its presence in CNAP3 M.

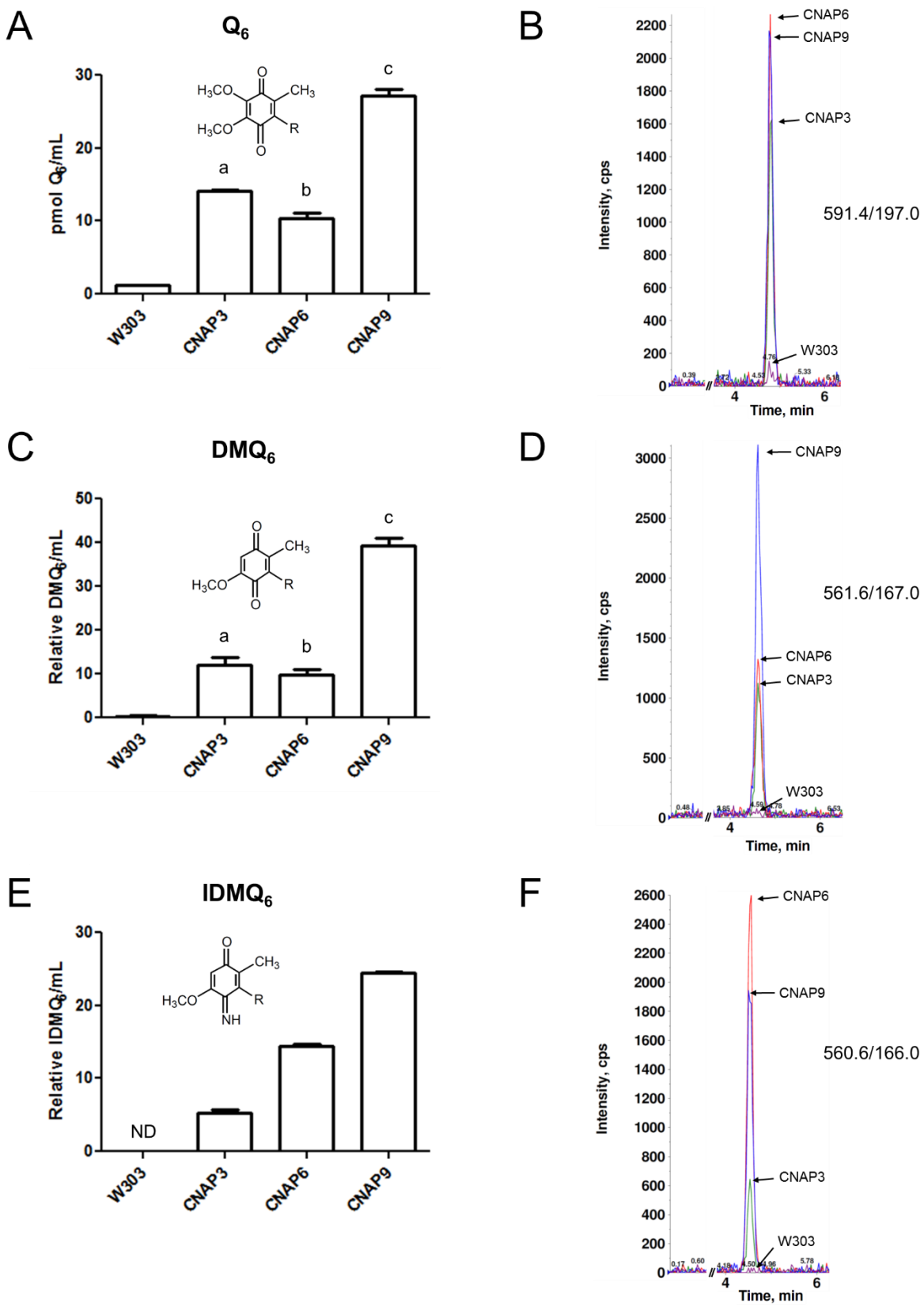

A
Q6
pmol Q6/mL
30
20
10
0
a
b
c
W303
CNAP3
CNAP6
CNAP9
B
CNAP6
CNAP9
CNAP3
591.4/197.0
Intensity, cps
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
W303
4
6
Time, min
C
DMQ6
Relative DMQ6/mL
50
40
30
20
10
0
a
b
c
W303
CNAP3
CNAP6
CNAP9
D
CNAP9
561.6/167.0
Intensity, cps
3000
2500
2000
1500
1000
500
0
CNAP6
CNAP3
W303
4
6
Time, min
E
IDMQ6
Relative IDMQ6/mL
30
20
10
0
ND
W303
CNAP3
CNAP6
CNAP9
F
CNAP6
CNAP9
560.6/166.0
Intensity, cps
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CNAP3
W303
4
6
Time, min

Figure 3. Tandem affinity-purified Coq complexes co-purify with Q_6 and late-stage Q_6 intermediates. For each strain, aliquots of the anti-PC eluates (29% of E1 and 29% of E2) were combined and subjected to lipid extraction with methanol and petroleum ether. Lipid extracts were analyzed by HPLC-MS/MS for Q_6 **(A)**, DMQ_6 **(C)**, and $IDMQ_6$ **(E)**. Total Q_6 ($Q_6 + Q_6H_2$) was detected using precursor-to-product ion transitions of 591.4/197.0 and 610.4/197.0, DMQ_6 with a transition of 561.6/167.0, and $IDMQ_6$ with a transition of 560.6/166.0. Measured lipids were normalized by the extracted eluate volume. Bars represent the mean of two measurements and error bars represent the standard deviation. Statistical significance was determined with the two-tailed Student's t-test and lower case letters above bars are indicative of statistical significance. In **(A)** the content of Q_6 in CNAP3, CNAP6, and CNAP9 was compared to wild type (a, p=0.0001; b, p=0.0040; c, p=0.0006). In **(C)** the content of DMQ_6 in CNAP3, CNAP6, and CNAP9 was compared to wild type (a, p=0.0110; b, p=0.0113; c, p=0.0010). In **(E)** there was no detectable $IDMQ_6$ in the wild type (ND, not detected). Representative overlaid traces of all four strains (W303, *purple*; CNAP3, *green*; CNAP6, *red*; CNAP9, *blue*) are shown for Q_6 **(B)**, DMQ_6 **(D)**, and $IDMQ_6$ **(F)**.

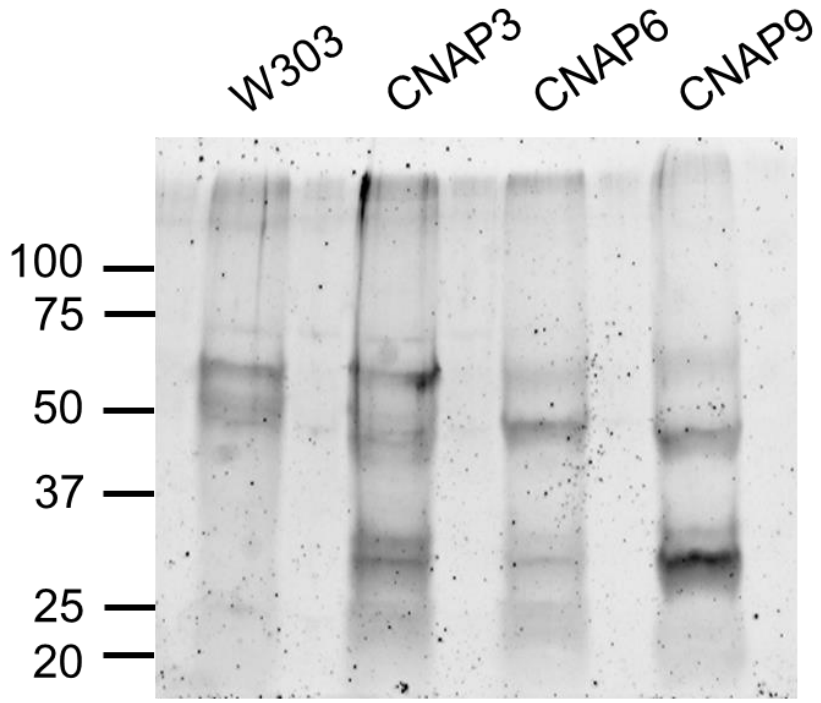


Figure 4. SYPRO Ruby staining for total protein reveals unique bands in CNAP tagged eluates. For each strain designated, aliquots of the anti-PC eluates (20% of E1 and 20% of anti-PC eluate two (E2)) were combined and dried with a speedvac at 60°C for two h. Samples were resuspended in 42 µL SDS sample buffer and separated with an 8-16% Criterion SDS-PAGE gel (Bio-Rad). Following separation proteins were fixed and the gel was stained overnight with a 1:1 mixture of fresh and used SYPRO Ruby (Life Technologies). The gel was subsequently washed and visualized with an FX Pro Plus Molecular Imager (Bio-Rad) at 532 nm excitation and emission was measured with a 555 nm long pass filter. The ladder denotes protein masses in kDa.

Table 3

Top protein hits from proteomic analysis of tandem affinity purification eluates

W303			CNAP3			CNAP6			CNAP9		
Protein	Function	Hits	Protein	Function	Hits	Protein	Function	Hits	Protein	Function	Hits
Rga2p	GTPase-activating protein	14	Coq3p	Q biosynthesis	12	Coq6p	Q biosynthesis	5	Coq6p	Q biosynthesis	9
Flo8p	Transcription factor	4	Coq6p	Q Biosynthesis	10	Coq9p	Q biosynthesis	3	Coq5p	Q biosynthesis	8
Act1p	Actin	2	Coq5p	Q biosynthesis	8	Hsp60p	Heat shock protein	2	Coq9p	Q biosynthesis	7
Pfd4p	Chaperone prefolding complex	2	Coq9p	Q biosynthesis	8	Coq5p	Q biosynthesis	1	Ilv6p	Regulatory subunit of acetolactate synthase	4
Spt16p	Associated with chromatin	2	YLR290C	Unknown	6	Dld3p	D-lactate dehydrogenase	1	Caj1p	Heat shock protein	3
Ilv6p	Regulatory subunit of acetolactae synthase	1	Coq4p	Q biosynthesis	5	His4p	Histidine biosynthesis	1	Coq4p	Q biosynthesis	3
			Coq7p	Q biosynthesis	5	Ilv6p	Regulatory subunit of acetolactate synthase	1	Coq7p	Q biosynthesis	3
			Htb1p	Histone B2	5	Mmt1p	Putative metal transporter	1	Mmt2p	Putative metal transporter	2
			Act1p	Actin	3	Mmt2p	Putative metal transporter	1	Msh1p	DNA binding protein	2
			Bmh1p	14-3-3 protein	3	Npl4p	Regulation of polyubiquitinated proteins	1	Rga2p	GTPase-activating protein	2
			Bmh2p	14-3-3 protein	3	Sld3p	Initiation of DNA replication	1	Mmt1p	Putative metal transporter	1
			Rga2p	GTPase-activating protein	3	Ura2p	Pyrimidine biosynthesis	1	Mrp21p	Mitochondrial ribosome	1
			Hpc2p	Nucleosome assembly complex	2	Yhm2p	Citrate/oxoglutarate carrier	1			
			Mas2p	Mitochondrial processing protease	2						
			Pef1p	Polar bud growth	2						
			Por1p	Porin	2						
			Sas4p	Histone acetylation	2						
			Ssa1p	Nuclear transport	2						
			Ssa2p	Nuclear transport	2						
			Ssa3p	Protein unfolding	2						
			Ssa4p	Heat shock protein	2						
			Tim11p	ATP synthase	2						
			Ilv6p	Regulatory subunit of acetolactate synthase	1						
			Mrp21p	Mitochondrial ribosome	1						

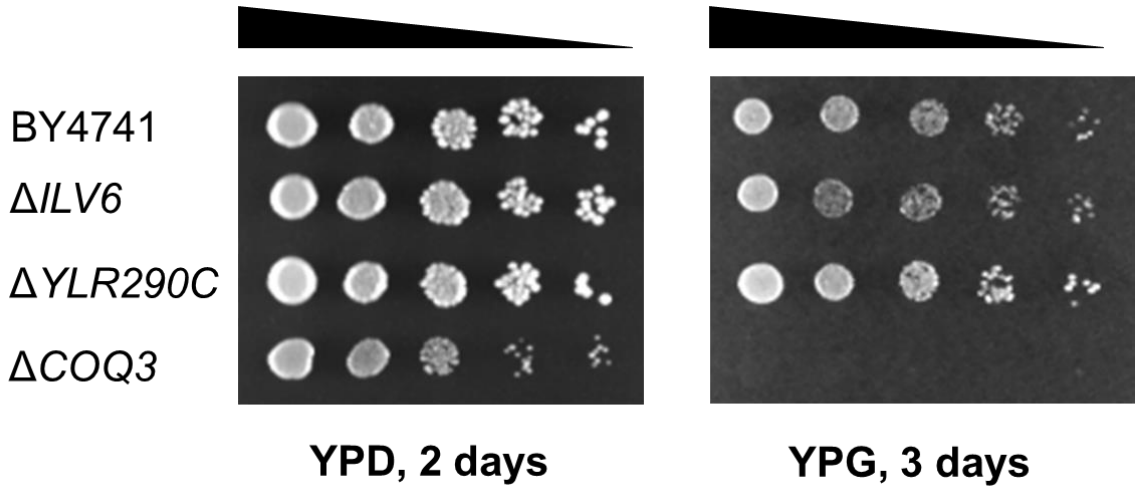
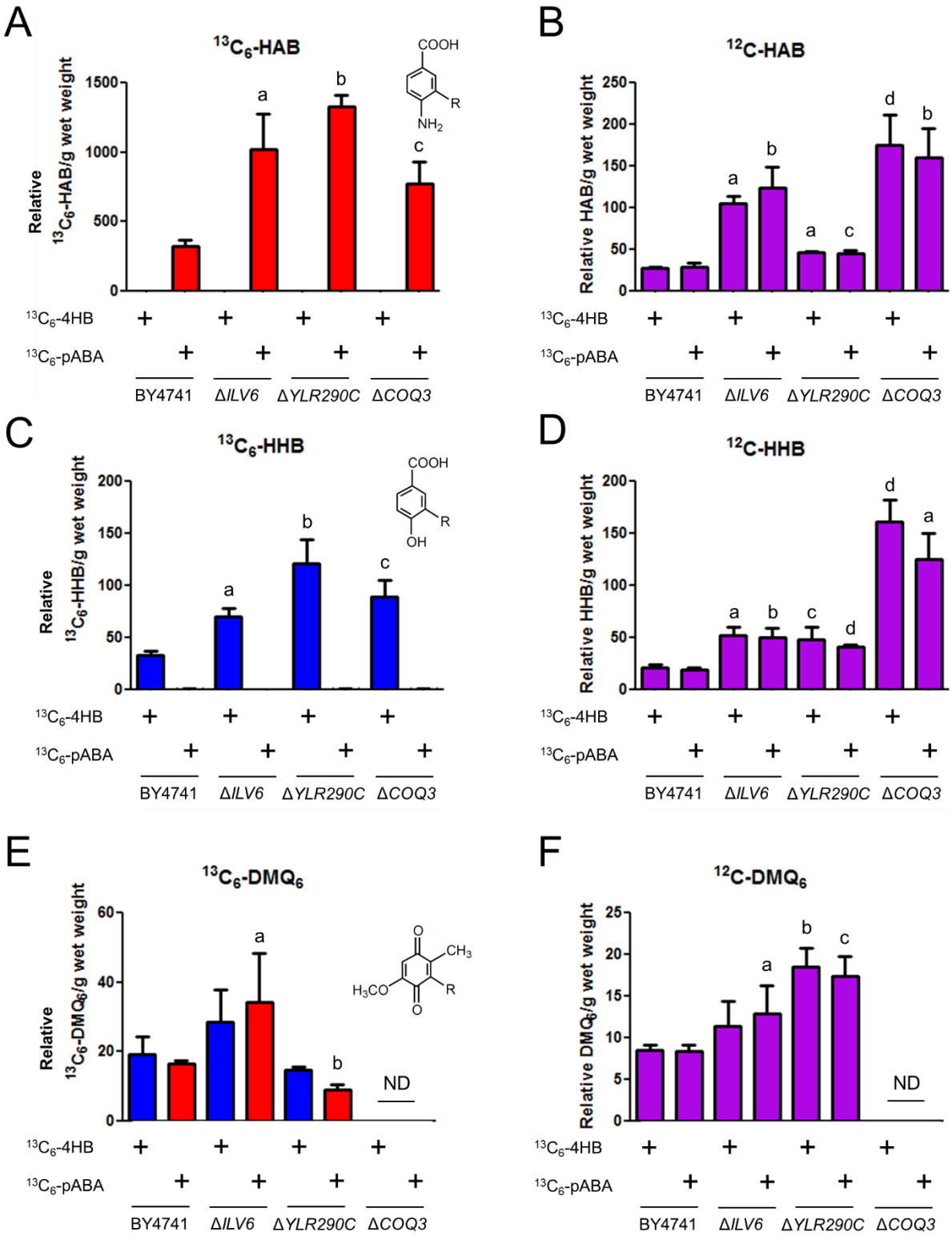


Figure 5. Yeast *ILV6* and *YLR290C* null mutants retain the ability to grow on a non-fermentable carbon source. Designated yeast strains were grown overnight in 5 mL YPD, diluted to an OD_{600} of 0.2 with sterile PBS, and 2 µL of 5-fold serial dilutions were spotted onto each type of plate media, corresponding to a final OD_{600} of 0.2, 0.04, 0.008, 0.0016, and 0.00032. Plates were incubated at 30°C and growth is depicted after two days for YPD and three days for YPG.


A
$^{13}C_6$-HAB
Relative $^{13}C_6$-HAB/g wet weight
1500
1000
500
0
a
b
c
COOH
R
NH_2
$^{13}C_6$-4HB + + + +
$^{13}C_6$-pABA + + + +
BY4741 ΔILV6 ΔYLR290C ΔCOQ3
B
^{12}C-HAB
Relative HAB/g wet weight
250
200
150
100
50
0
a
b
a
c
d
b
$^{13}C_6$-4HB + + + +
$^{13}C_6$-pABA + + + +
BY4741 ΔILV6 ΔYLR290C ΔCOQ3
C
$^{13}C_6$-HHB
Relative $^{13}C_6$-HHB/g wet weight
200
150
100
50
0
a
b
c
COOH
R
OH
$^{13}C_6$-4HB + + + +
$^{13}C_6$-pABA + + + +
BY4741 ΔILV6 ΔYLR290C ΔCOQ3
D
^{12}C-HHB
Relative HHB/g wet weight
200
150
100
50
0
a
b
c
d
d
a
$^{13}C_6$-4HB + + + +
$^{13}C_6$-pABA + + + +
BY4741 ΔILV6 ΔYLR290C ΔCOQ3
E
$^{13}C_6$-DMQ$_6$
Relative $^{13}C_6$-DMQ$_6$/g wet weight
60
40
20
0
a
b
ND
O
CH_3
H_3CO
R
O
$^{13}C_6$-4HB + + + +
$^{13}C_6$-pABA + + + +
BY4741 ΔILV6 ΔYLR290C ΔCOQ3
F
^{12}C-DMQ$_6$
Relative DMQ$_6$/g wet weight
25
20
15
10
5
0
a
b
c
ND
$^{13}C_6$-4HB + + + +
$^{13}C_6$-pABA + + + +
BY4741 ΔILV6 ΔYLR290C ΔCOQ3

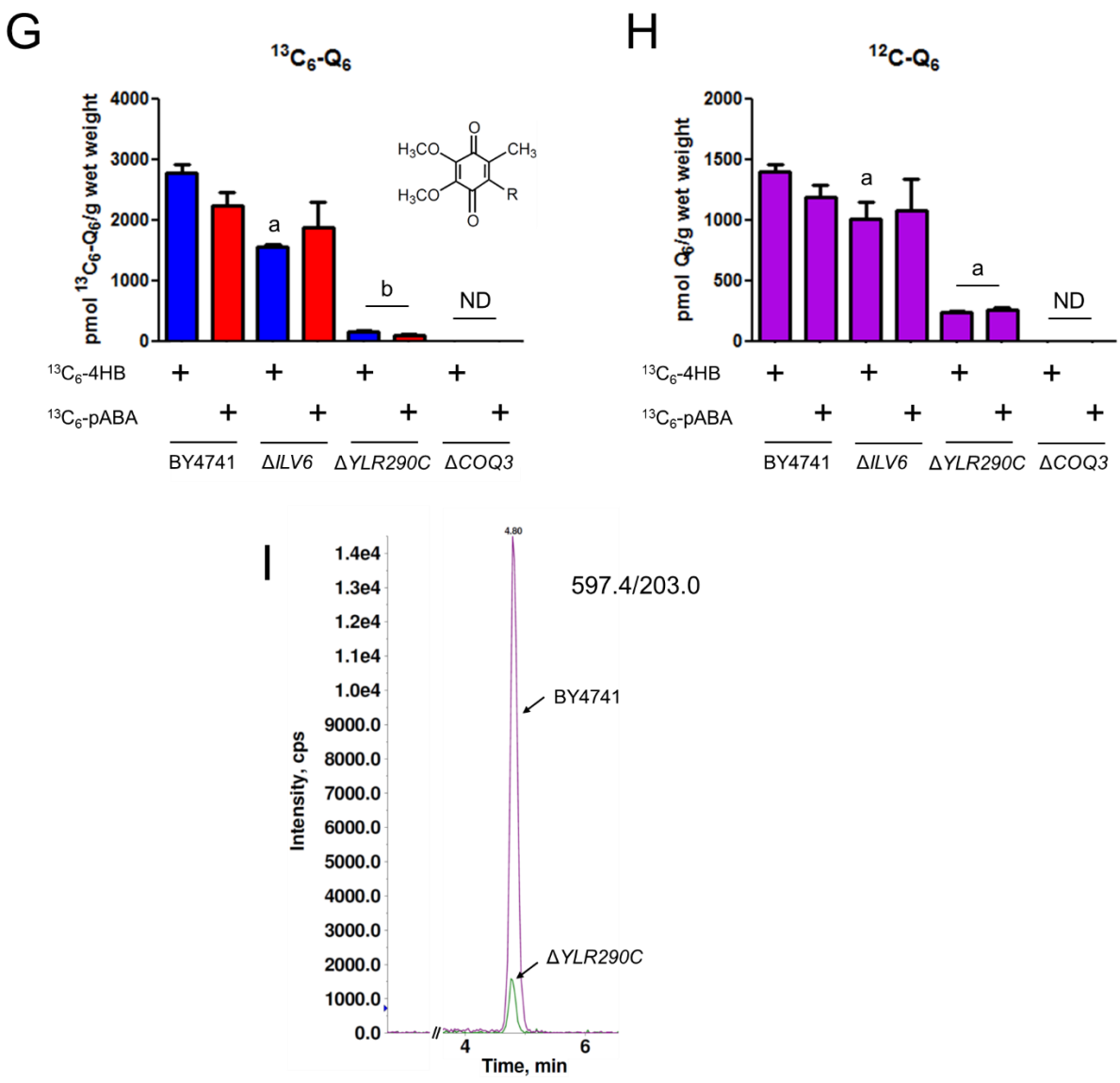


Figure 6. The yeast Δ*YLR290C* mutant, but not the Δ*ILV6* mutant, shows impaired *de novo* Q biosynthesis. $^{13}C_6$-HAB **(A)**, ^{12}C-HAB **(B)**, $^{13}C_6$-HHB **(C)**, ^{12}C-HHB **(D)**, $^{13}C_6$-DMQ_6 **(E)**, ^{12}C-DMQ_6 **(F)**, total $^{13}C_6$-Q_6 ($^{13}C_6$-Q_6 + $^{13}C_6$-Q_6H_2) **(G)**, and total ^{12}C-Q_6 (^{12}C-Q_6 + ^{12}C-Q_6H_2) **(H)** were measured in the designated yeast strains by HPLC-MS/MS. Yeast strains were grown overnight in 5 mL SD-complete, diluted to an OD_{600} of 0.05 in 50 mL DOD-complete, labeled with either $^{13}C_6$-4HB or $^{13}C_6$-pABA at an OD_{600} of 0.5, and harvested after three hours of labeling. Precursor-to-product ion transitions were detected as follows: $^{13}C_6$-HAB 552.4/156.0,

^{12}C-HAB 546.4/150.0, $^{13}C_6$-HHB 553.4/157.0, ^{12}C-HHB 547.4/151.0, $^{13}C_6$-DMQ_6 567.6/173.0, ^{12}C-DMQ_6 561.6/167.0, $^{13}C_6$-Q_6 597.4/203.0, $^{13}C_6$-Q_6H_2 616.4/203.0, ^{12}C-Q_6 591.4/197.0, and ^{12}C-Q_6H_2 610.4/197.0. Lipid measurements were normalized by the wet weight of extracted cells. Each bar represents the mean of four measurements from two biological samples with two injections each. Error bars represent standard deviations. Statistical significance was determined with the two-tailed Student's t-test and lower case letters above bars are indicative of statistical significance. In **(A)** the relative content of $^{13}C_6$-HAB in the three null mutants was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0017; b, p<0.0001; c, p=0.0014). In **(B)** the relative content of ^{12}C-HAB in the three null mutants was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p<0.0001; b, p=0.0003; c, p=0.0014; d=0.0002). In **(C)** the relative content of $^{13}C_6$-HHB in the three null mutants was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0001; b, p=0.0003; c, p=0.0005). In **(D)** the relative content of ^{12}C-HHB in the three null mutants was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0002; b, p=0.0004; c, p=0.0047; d, p<0.0001). In **(E)** the relative content of $^{13}C_6$-DMQ_6 in the three null mutants was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0441; b, p<0.0001; ND, not detected). In **(F)** the relative content of ^{12}C-DMQ_6 in the three null mutants was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0453; b, p=0.0002; c, p=0.0003; ND, not detected). In **(G)** the content of $^{13}C_6$-Q_6 in the three null mutants was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0017; b, p<0.0001; ND, not detected). In **(H)** the content of ^{12}C-Q_6 in the three null mutants was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p<0.0001; ND, not detected). **(I)** Representative overlaid traces for BY4741 and the *YLR290C* null mutant labeled with $^{13}C_6$-4HB are shown for $^{13}C_6$-Q_6.

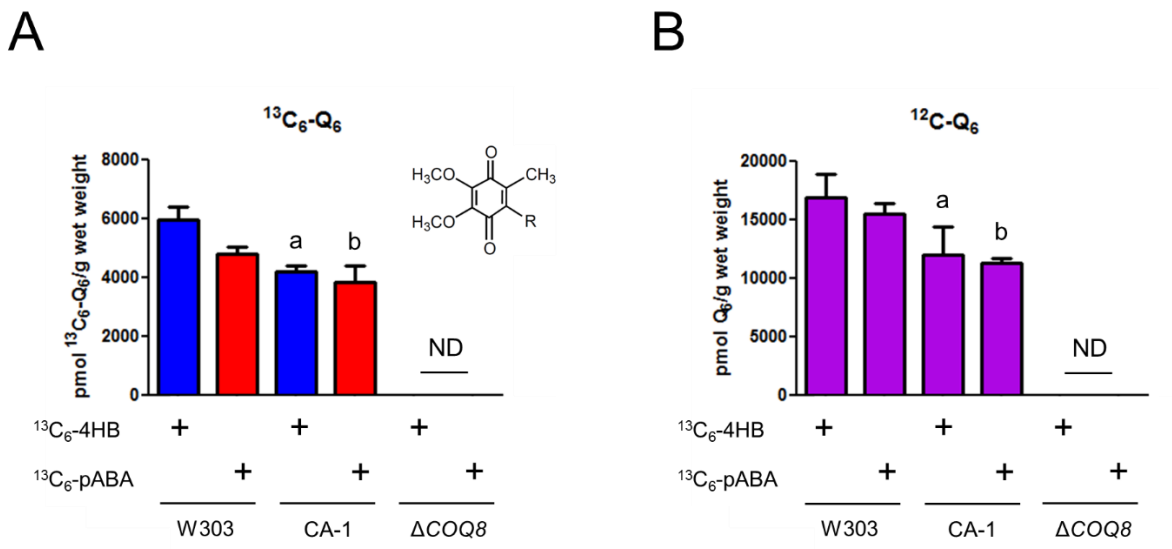


Figure 7. Expression of CNAP-tagged YLR290C preserves *de novo* Q biosynthesis. Levels of total $^{13}C_6$-Q_6 ($^{13}C_6$-Q_6 + $^{13}C_6$-Q_6H_2) **(A)** and total ^{12}C-Q_6 (^{12}C-Q_6 + ^{12}C-Q_6H_2) **(B)** were measured in the designated yeast strains by HPLC-MS/MS. Yeast strains were grown overnight in 5 mL SD-complete, diluted to an OD_{600} of 0.05 in 50 mL DOD-complete, labeled with either $^{13}C_6$-4HB or $^{13}C_6$-pABA at an OD_{600} of 0.5, and harvested after three hours of labeling. $^{13}C_6$–Q_6 was detected using precursor-to-product ion transitions of 597.4/203.0 and 616.4/197.0, and ^{12}C-Q_6 with transitions of 591.4/197.0 and 610.4/197.0. Lipid measurements were normalized by the wet weight of extracted cells. Each bar represents the mean of four measurements from two biological samples with two injections each. Error bars represent standard deviations. Statistical significance was determined with the two-tailed Student's t-test and lower case letters above bars are indicative of statistical significance. In **(A)** the content of $^{13}C_6$-Q_6 in CA-1 and Δ*COQ8* was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0005; b, p=0.0196; ND, not detected). In **(B)** the content of ^{12}C-Q_6 in CA-1 and Δ*COQ8* was compared to wild type with the corresponding $^{13}C_6$-labeled precursor (a, p=0.0202; b, p=0.0001; ND, not detected).

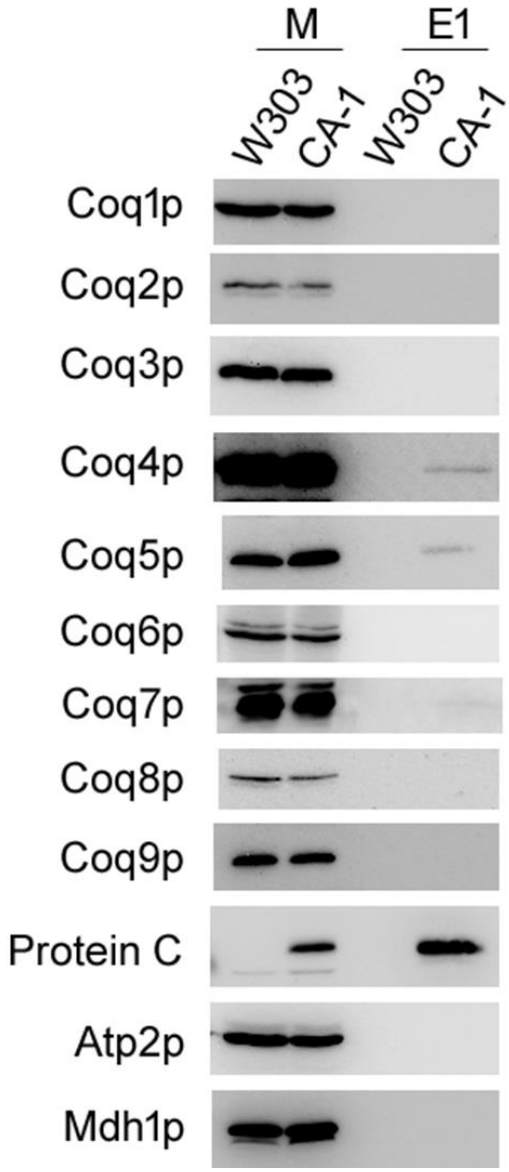


Figure 8. Coq4p, Coq5p, and Coq7p co-precipitate with YLR290C-CNAP. Purified mitochondria from W303 and CA-1 (15 mg protein) were solubilized with digitonin and subject to tandem affinity purification using Ni-NTA resin (Qiagen) followed by anti-PC agarose (Roche). Samples were separated on 12% SDS-PAGE gels followed by transfer to PVDF membranes for Western blotting. Mitochondria (25 µg protein) (M) and 2.5% of the first anti-PC elution (E1) were analyzed for each of the two strains.

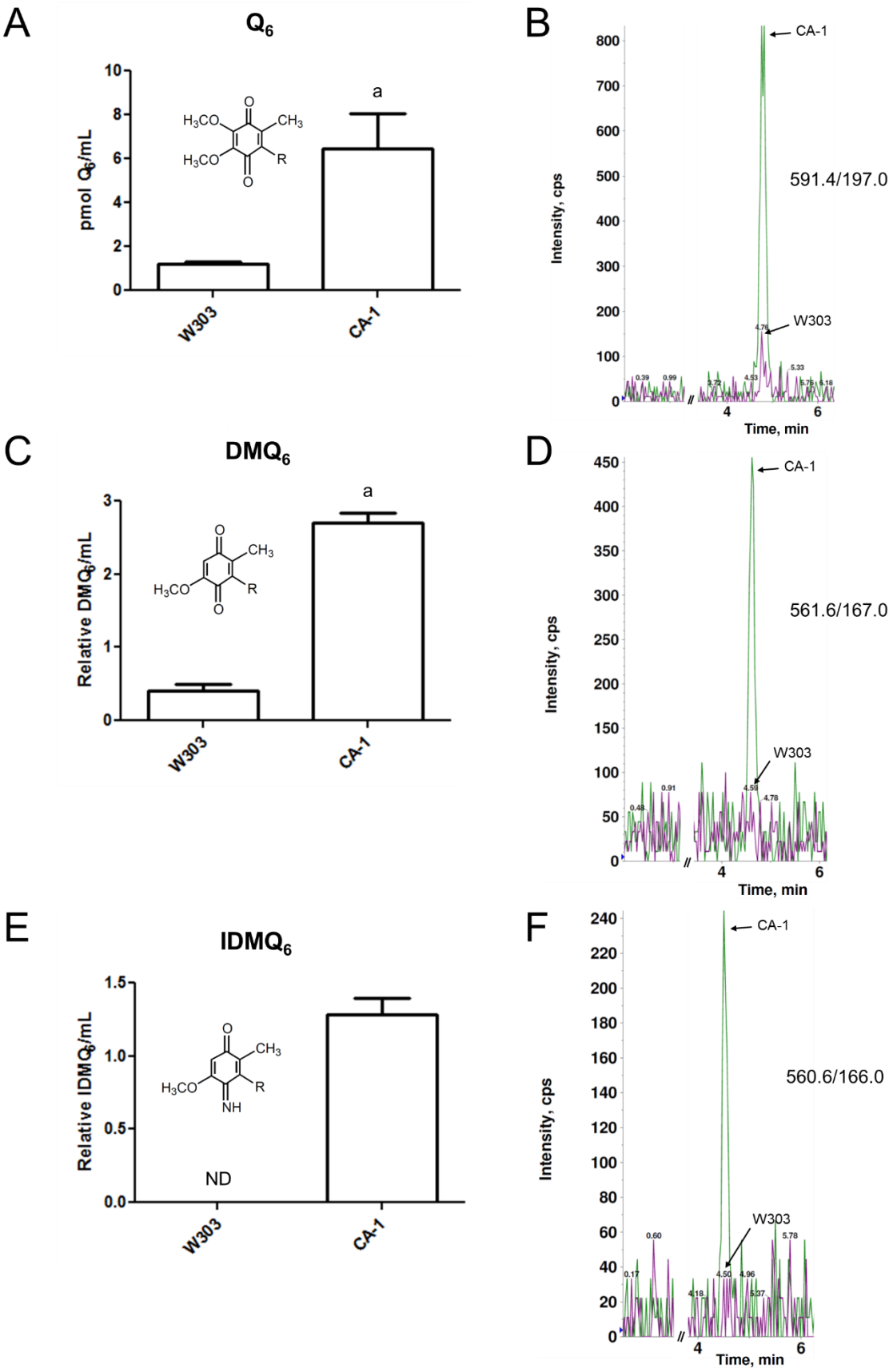

A
Q_6
pmol Q_6/mL
a
W303
CA-1
B
CA-1
W303
591.4/197.0
Intensity, cps
Time, min
C
DMQ_6
Relative DMQ_6/mL
a
W303
CA-1
D
CA-1
W303
561.6/167.0
Intensity, cps
Time, min
E
$IDMQ_6$
Relative $IDMQ_6$/mL
ND
W303
CA-1
F
CA-1
W303
560.6/166.0
Intensity, cps
Time, min

Figure 9. Tandem affinity-purified YLR290C-CNAP co-purifies with Q and late-stage Q intermediates. For each strain, aliquots of the anti-PC eluates (29% of E1 and 29% of E2) were combined and subjected to lipid extraction with methanol and petroleum ether. Lipid extracts were analyzed by HPLC-MS/MS for total Q_6 (Q_6 + Q_6H_2) **(A)**, DMQ_6 **(C)**, and $IDMQ_6$ **(E)**. Q_6 was detected using a precursor-to-product ion transitions of 591.4/197.0 and 610.4/197.0, DMQ_6 with a transition of 561.6/167.0, and $IDMQ_6$ with a transition of 560.6/166.0. Measured lipids were normalized by the extracted eluate volume. Bars represent the mean of two measurements and error bars represent the standard deviation. Statistical significance was determined with the two-tailed Student's t-test and lower case letters above bars are indicative of statistical significance. In **(A)** the content of Q_6 in CA-1 was compared to wild type (a, p=0.0418). In **(C)** the content of DMQ_6 in CA-1 was compared to wild type (a, p=0.0025). In **(E)** there was no detectable $IDMQ_6$ in the wild type (ND, not detected). Representative overlaid traces of both strains (W303, *magenta*; CA-1, *green*) are shown for Q_6 **(B)**, DMQ_6 **(D)**, and $IDMQ_6$ **(F)**.

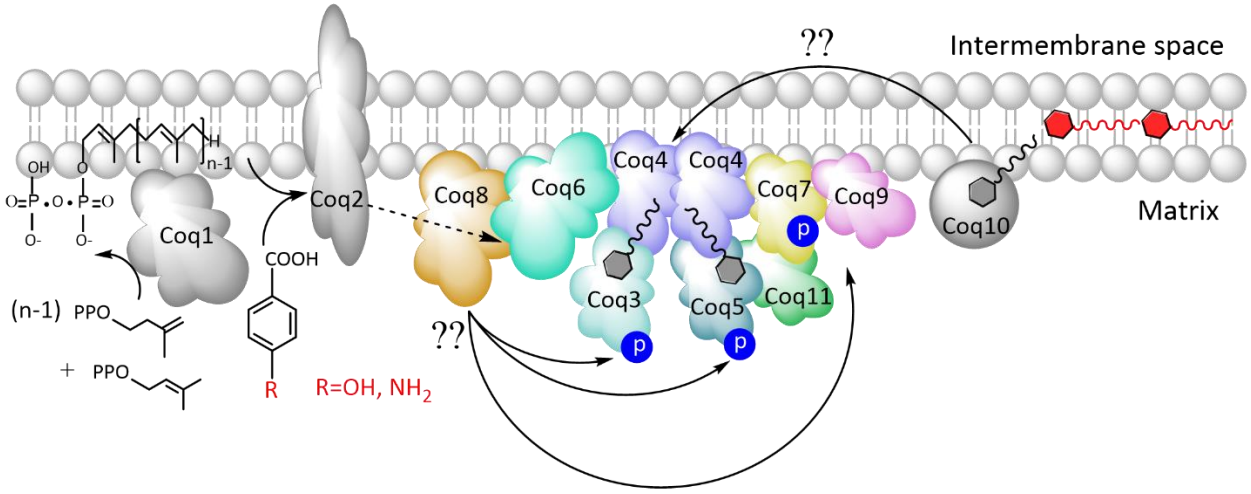


Figure 10. Model of the Q biosynthetic complex. The organization of the complex is based on co-precipitation experiments performed in previous work (Hsieh *et al.*, 2007) and this study, as well as two-dimensional blue native-PAGE analysis (He *et al.*, 2014). Coq1p, Coq2p, and Coq10p have not been shown to associate with the complex. Coq10p binds Q and particular Q intermediates and is postulated to function as a Q chaperone for efficient respiration and *de novo* Q biosynthesis (Allan *et al.*, 2012). Coq8p is required for the phosphorylation of Coq3p, Coq5p, and Coq7p (Xie *et al.*, 2011), and co-precipitation of CNAP-tagged Coq6p demonstrates their physical association. Co-precipitation of tagged YLR290C, here designated Coq11p, demonstrated its association with Coq4p, Coq5p, and Coq7p. Lipid analysis of co-precipitation eluates demonstrated the association of Q_6 and the late-stage intermediates DMQ_6 and $IDMQ_6$ with the complex, illustrated as small molecules in association with Coq4p.

References

Allan, C. M., Hill, S., Morvaridi, S., Saiki, R., Johnson, J. S., Liau, W. S., Hirano, K., Kawashima, T., Ji, Z., Loo, J. A., Shepherd, J. N. and Clarke, C. F. (2012). A conserved START domain coenzyme Q-binding polypeptide is required for efficient Q biosynthesis, respiratory electron transport, and antioxidant function in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1831**(4), 776-791.

Baba, S. W., Belogrudov, G. I., Lee, J. C., Lee, P. T., Strahan, J., Shepherd, J. N. and Clarke, C. F. (2004). Yeast Coq5 *C*-methyltransferase is required for stability of other polypeptides involved in coenzyme Q biosynthesis. *J Biol Chem* **279**(11), 10052-10059.

Barkovich, R. J., Shtanko, A., Shepherd, J. A., Lee, P. T., Myles, D. C., Tzagoloff, A. and Clarke, C. F. (1997). Characterization of the *COQ5* gene from *Saccharomyces cerevisiae*. Evidence for a *C*-methyltransferase in ubiquinone biosynthesis. *J Biol Chem* **272**, 9182-9188.

Barros, M. H., Johnson, A., Gin, P., Marbois, B. N., Clarke, C. F. and Tzagoloff, A. (2005). The *Saccharomyces cerevisiae COQ10* gene encodes a START domain protein required for function of coenzyme Q in respiration. *J Biol Chem* **280**(52), 42627-42635.

Barros, M. H. and Nobrega, F. G. (1999). *YAH1* of *Saccharomyces cerevisiae*: a new essential gene that codes for a protein homologous to human adrenodoxin. *Gene* **233**(1-2), 197-203.

Barros, M. H., Nobrega, F. G. and Tzagoloff, A. (2002). Mitochondrial ferredoxin is required for heme A synthesis in *Saccharomyces cerevisiae*. *J Biol Chem* **277**(12), 9997-10002.

Belogrudov, G. I., Lee, P. T., Jonassen, T., Hsu, A. Y., Gin, P. and Clarke, C. F. (2001). Yeast *COQ4* encodes a mitochondrial protein required for coenzyme Q synthesis. *Arch Biochem Biophys* **392**, 48-58.

Bentinger, M., Tekle, M. and Dallner, G. (2010). Coenzyme Q - Biosynthesis and functions. *Biochem Biophys Res Commun* **396**(1), 74-79.

Boratyn, G. M., Camacho, C., Cooper, P. S., Coulouris, G., Fong, A., Ma, N., Madden, T. L., Matten, W. T., McGinnis, S. D., Merezhuk, Y., Raytselis, Y., Sayers, E. W., Tao, T., Ye, J. and Zaretskaya, I. (2013). BLAST: a more efficient report with usability improvements. *Nucleic Acids Res* **41**(W1), W29-W33.

Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J. C., Hieter, P. and Boeke, J. D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**(2), 115-132.

Brandt, U. and Trumpower, B. (1994). The protonmotive Q cycle in mitochondria and bacteria. *Crit Rev Biochem Mol Biol* **29**(3), 165-197.

Burke, D., Dawson, D. and Stearns, T. (2000). Methods in Yeast Genetics. Plainview, NY, Cold Spring Harbor Laboratory Press.

Claypool, S. M., Oktay, Y., Boontheung, P., Loo, J. A. and Koehler, C. M. (2008). Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. *J Cell Biol* **182**(5), 937-950.

Crane, F. L. (1965). Distribution of ubiquinones. Biochemistry of quinones. R. A. Morton. London, Academic Press**:** 183-206.

Cullin, C., BaudinBaillieu, A., Guillemet, E. and OzierKalogeropoulos, O. (1996). Functional analysis of YCL09C: Evidence for a role as the regulatory subunit of acetolactate synthase. *Yeast* **12**(15), 1511-1518.

Do, T. Q., Hsu, A. Y., Jonassen, T., Lee, P. T. and Clarke, C. F. (2001). A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in *Saccharomyces cerevisiae abc1* mutants. *J Biol Chem* **276**(21), 18161-18168.

Frei, B., Kim, M. C. and Ames, B. N. (1990). Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc Natl Acad Sci U S A* **87**(12), 4879-4883.

Geromanos, S. J., Vissers, J. P. C., Silva, J. C., Dorschel, C. A., Li, G. Z., Gorenstein, M. V., Bateman, R. H. and Langridge, J. I. (2009). The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. *Proteomics* **9**(6), 1683-1695.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., Arkin, A. P., Astromoff, A., El Bakkoury, M., Bangham, R., Benito, R., Brachat, S., Campanaro, S., Curtiss, M., Davis, K., Deutschbauer, A., Entian, K. D., Flaherty, P., Foury, F., Garfinkel, D. J., Gerstein, M., Gotte, D., Guldener, U., Hegemann, J. H., Hempel, S., Herman, Z., Jaramillo, D. F., Kelly, D. E., Kelly, S. L., Kotter, P., LaBonte, D., Lamb, D. C., Lan, N., Liang, H., Liao, H., Liu, L., Luo, C. Y., Lussier, M., Mao, R., Menard, P.,

Ooi, S. L., Revuelta, J. L., Roberts, C. J., Rose, M., Ross-Macdonald, P., Scherens, B., Schimmack, G., Shafer, B., Shoemaker, D. D., Sookhai-Mahadeo, S., Storms, R. K., Strathern, J. N., Valle, G., Voet, M., Volckaert, G., Wang, C. Y., Ward, T. R., Wilhelmy, J., Winzeler, E. A., Yang, Y. H., Yen, G., Youngman, E., Yu, K. X., Bussey, H., Boeke, J. D., Snyder, M., Philippsen, P., Davis, R. W. and Johnston, M. (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**(6896), 387-391.

Gietz, R. D. and Woods, R. A. (2006). Yeast transformation by the LiAc/SS Carrier DNA/PEG method. *Methods Mol Biol* **313**, 107-120.

Gin, P. and Clarke, C. F. (2005). Genetic Evidence for a Multi-subunit Complex in Coenzyme Q Biosynthesis in Yeast and the Role of the Coq1 Hexaprenyl Diphosphate Synthase. *J Biol Chem* **280**(4), 2676-2681.

Gin, P., Hsu, A. Y., Rothman, S. C., Jonassen, T., Lee, P. T., Tzagoloff, A. and Clarke, C. F. (2003). The *Saccharomyces cerevisiae COQ6* gene encodes a mitochondrial flavin-dependent monooxygenase required for coenzyme Q biosynthesis. *J Biol Chem* **278**(28), 25308-25316.

Glerum, D. M., Muroff, I., Jin, C. and Tzagoloff, A. (1997). *COX15* codes for a mitochondrial protein essential for the assembly of yeast cytochrome oxidase. *J Biol Chem* **272**(30), 19088-19094.

Glick, B. S. and Pon, L. A. (1995). Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods Enzymol* **260**, 213-223.

Grandier-Vazeille, X., Bathany, K., Chaignepain, S., Camougrand, N., Manon, S. and Schmitter, J. M. (2001). Yeast mitochondrial dehydrogenases are associated in a supramolecular complex. *Biochemistry* **40**(33), 9758-9769.

Gulmezian, M. (2006). Characterization of *Escherichia coli ubiX*, *Saccharomyces cerevisiae PAD1* and *YDR539W*, and a complex of polypeptides involved in coenzyme Q biosynthesis, UCLA.

Gulmezian, M., Hyman, K. R., Marbois, B. N., Clarke, C. F. and Javor, G. T. (2007). The role of UbiX in *Escherichia coli* coenzyme Q biosynthesis. *Arch Biochem Biophys* **467**(2), 144-153.

He, C. H., Xie, L. X., Allan, C. M., Tran, U. C. and Clarke, C. F. (2014). Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast coq null mutants. *Biochim Biophys Acta* **1841**(4), 630-644.

Hershey, H. P., Schwartz, L. J., Gale, J. P. and Abell, L. M. (1999). Cloning and functional expression of the small subunit of acetolactate synthase from *Nicotiana plumbaginifolia*. *Plant Mol Biol* **40**(5), 795-806.

Hildebrandt, T. M. and Grieshaber, M. K. (2008). Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria. *Febs Journal* **275**(13), 3352-3361.

Hsieh, E. J., Dinoso, J. B. and Clarke, C. F. (2004). A tRNA(TRP) gene mediates the suppression of *cbs2-223* previously attributed to *ABC1/COQ8*. *Biochem Biophys Res Commun* **317**(2), 648-653.

Hsieh, E. J., Gin, P., Gulmezian, M., Tran, U. C., Saiki, R., Marbois, B. N. and Clarke, C. F. (2007). *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex. *Arch Biochem Biophys* **463**(1), 19-26.

Johnson, A., Gin, P., Marbois, B. N., Hsieh, E. J., Wu, M., Barros, M. H., Clarke, C. F. and Tzagoloff, A. (2005). *COQ9*, a new gene required for the biosynthesis of coenzyme Q in *Saccharomyces cerevisiae*. *J Biol Chem* **280**(36), 31397-31404.

Kopec, J., Schnell, R. and Schneider, G. (2011). Structure of PA4019, a putative aromatic acid decarboxylase from *Pseudomonas aeruginosa*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67**, 1184-1188.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259), 680-685.

Lange, H., Kaut, A., Kispal, G. and Lill, R. (2000). A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins. *Proc Natl Acad Sci U S A* **97**(3), 1050-1055.

Laredj, L. N., Licitra, F. and Puccio, H. M. (2014). The molecular genetics of coenzyme Q biosynthesis in health and disease. *Biochimie* **100C**, 78-87.

Lee, Y. T. and Duggleby, R. G. (2001). Identification of the regulatory subunit of *Arabidopsis thaliana* acetohydroxyacid synthase and reconstitution with its catalytic subunit. *Biochemistry* **40**(23), 6836-6844.

Lenaz, G. and De Santis, A. (1985). A survey of the function and specificity of ubiquione in the mitochondrial respiratory chain. Coenzyme Q. G. Lenaz. Chichester, U.K., John Wiley & Sons**:** 165-199.

Li, J., Saxena, S., Pain, D. and Dancis, A. (2001). Adrenodoxin reductase homolog (Arh1p) of yeast mitochondria required for iron homeostasis. *J Biol Chem* **276**(2), 1503-1509.

Lohman, D. C., Forouhar, F., Beebe, E. T., Stefely, M. S., Minogue, C. E., Ulbrich, A., Stefely, J. A., Sukumar, S., Luna-Sánchez, M., Jochem, A., Lew, S., Seetharaman, J., Xiao, R., Wang, H., Westphall, M. S., Wrobel, R. L., Everett, J. K., Mitchell, J. C., López, L. C., Coon, J. J., Tong, L. and Pagliarini, D. J. (2014). Mitochondrial COQ9 is a lipid-binding protein that associates with COQ7 to enable coenzyme Q biosynthesis. *Proc Natl Acad Sci U S A* **111**(44), E4697-E4705.

Manzella, L., Barros, M. H. and Nobrega, F. G. (1998). *ARH1* of *Saccharomyces cerevisiae*: A new essential gene that codes for a protein homologous to the human adrenodoxin reductase. *Yeast* **14**(9), 839-846.

Marbois, B., Gin, P., Faull, K. F., Poon, W. W., Lee, P. T., Strahan, J., Shepherd, J. N. and Clarke, C. F. (2005). Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q. *J Biol Chem* **280**, 20231-20238.

Marbois, B., Gin, P., Gulmezian, M. and Clarke, C. F. (2009). The yeast Coq4 polypeptide organizes a mitochondrial protein complex essential for coenzyme Q biosynthesis. *Biochim Biophys Acta* **1791**, 69-75.

Marbois, B., Xie, L. X., Choi, S., Hirano, K., Hyman, K. and Clarke, C. F. (2010). para-Aminobenzoic Acid Is a Precursor in Coenzyme Q(6) Biosynthesis in *Saccharomyces cerevisiae*. *J Biol Chem* **285**(36), 27827-27838.

Marchler-Bauer, A., Zheng, C. J., Chitsaz, F., Derbyshire, M. K., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., Lanczycki, C. J., Lu, F., Lu, S. N., Marchler, G. H., Song, J. S., Thanki, N., Yamashita, R. A., Zhang, D. C. and Bryant, S. H. (2013). CDD: conserved domains and protein three-dimensional structure. *Nucleic Acids Res* **41**(D1), D348-D352.

Mukai, N., Masaki, K., Fujii, T., Kawamukai, M. and Iefuji, H. (2010). *PAD1* and *FDC1* are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J Biosci Bioeng* **109**(6), 564-569.

Nguyen, T. P. T., Casarin, A., Desbats, M. A., Doimo, M., Trevisson, E., Santos-Ocana, C., Navas, P., Clarke, C. F. and Salviati, L. (2014). Molecular characterization of the human COQ5 *C*-methyltransferase in coenzyme Q10 biosynthesis. *Biochim Biophys Acta* **1841**(11), 1628-1638.

Padilla, S., Tran, U. C., Jimenez-Hidalgo, M., Lopez-Martin, J. M., Martin-Montalvo, A., Clarke, C. F., Navas, P. and Santos-Ocana, C. (2009). Hydroxylation of demethoxy-Q6 constitutes a control point in yeast coenzyme Q6 biosynthesis. *Cell Mol Life Sci* **66**(1), 173-186.

Pang, S. S. and Duggleby, R. G. (2001). Regulation of yeast acetohydroxyacid synthase by valine and ATP. *Biochem J* **357**, 749-757.

Perocchi, F., Jensen, L. J., Gagneur, J., Ahting, U., von Mering, C., Bork, P., Prokisch, H. and Steinmetz, L. M. (2006). Assessing systems properties of yeast mitochondria through an interaction map of the organelle. *Plos Genetics* **2**(10), 1612-1624.

Pierrel, F., Hamelin, O., Douki, T., Kieffer-Jaquinod, S., Muhlenhoff, U., Ozeir, M., Lill, R. and Fontecave, M. (2010). Involvement of Mitochondrial Ferredoxin and Para-Aminobenzoic Acid in Yeast Coenzyme Q Biosynthesis. *Chem Biol* **17**(5), 449-459.

Poon, W. W., Barkovich, R. J., Hsu, A. Y., Frankel, A., Lee, P. T., Shepherd, J. N., Myles, D. C. and Clarke, C. F. (1999). Yeast and rat Coq3 and *Escherichia coli* UbiG polypeptides catalyze both *O*-methyltransferase steps in coenzyme Q biosynthesis. *J Biol Chem* **274**(31), 21665-21672.

Rangarajan, E. S., Li, Y., Iannuzzi, P., Tocilj, A., Hung, L. W., Matte, A. and Cygler, M. (2004). Crystal structure of a dodecameric FMN-dependent UbiX-like decarboxylase (Pad1) from *Escherichia coli* O157: H7. *Protein Sci* **13**(11), 3006-3016.

Rea, S. L., Graham, B. H., Nakamaru-Ogiso, E., Kar, A. and Falk, M. J. (2010). Bacteria, yeast, worms, and flies: exploiting simple model organisms to investigate human mitochondrial diseases. *Dev Disabil Res Rev* **16**(2), 200-218.

Rossmann, M. G., Moras, D. and Olsen, K. W. (1974). Chemical and biological evolution of a nucleotide-binding protein. *Nature* **250**(5463), 194-199.

Shirasaki, D. I., Greiner, E. R., Al-Ramahi, I., Gray, M., Boontheung, P., Geschwind, D. H., Botas, J., Coppola, G., Horvath, S., Loo, J. A. and Yang, X. W. (2012). Network Organization of the Huntingtin Proteomic Interactome in Mammalian Brain. *Neuron* **75**(1), 41-57.

Silva, J. C., Denny, R., Dorschel, C., Gorenstein, M. V., Li, G. Z., Richardson, K., Wall, D. and Geromanos, S. J. (2006). Simultaneous qualitative and quantitative analysis of the *Escherichia coli* Proteome - A sweet tale. *Mol Cell Proteomics* **5**(4), 589-607.

Silva, J. C., Gorenstein, M. V., Li, G. Z., Vissers, J. P. C. and Geromanos, S. J. (2006). Absolute quantification of proteins by LCMSE - A virtue of parallel MS acquisition. *Mol Cell Proteomics* **5**(1), 144-156.

Tauche, A., Krause-Buchholz, U. and Rodel, G. (2008). Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of *O*-methylase Coq3p depends on Abc1p/Coq8p. *FEMS Yeast Res* **8**(8), 1263-1275.

Tran, U. C. and Clarke, C. F. (2007). Endogenous synthesis of coenzyme Q in eukaryotes. *Mitochondrion* **7S**, S62-S71.

Tran, U. C., Marbois, B., Gin, P., Gulmezian, M., Jonassen, T. and Clarke, C. F. (2006). Complementation of *Saccharomyces cerevisiae coq7* mutants by mitochondrial targeting of the *Escherichia coli* UbiF polypeptide: two functions of yeast Coq7 polypeptide in coenzyme Q biosynthesis. *J Biol Chem* **281**(24), 16401-16409.

Turunen, M., Olsson, J. and Dallner, G. (2004). Metabolism and function of coenzyme Q. *Biochim Biophys Acta* **1660**(1-2), 171-199.

Tzagoloff, A. and Dieckmann, C. L. (1990). *PET* genes of *Saccharomyces cerevisiae*. *Microbiol Rev* **54**, 211-225.

Tzagoloff, A., Yue, J., Jang, J. and Paul, M. F. (1994). A new member of a family of ATPases is essential for assembly of mitochondrial respiratory chain and ATP synthetase complexes in *Saccharomyces cerevisiae*. *J Biol Chem* **269**(42), 26144-26151.

UniProt, C. (2014). Activities at the Universal Protein Resource (UniProt). *Nucleic Acids Res* **42**(Database issue), D191-198.

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., El Bakkoury, M., Foury, F., Friend, S. H., Gentalen, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D. J., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J. L., Riles, L., Roberts, C. J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R. K., Veronneau, S., Voet, M., Volckaert, G., Ward, T. R., Wysocki, R., Yen, G. S., Yu, K. X., Zimmermann, K., Philippsen, P., Johnston, M. and Davis, R. W. (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**(5429), 901-906.

Xie, L. T. X., Hsieh, E. J., Watanabe, S., Allan, C. M., Chen, J. Y., Tran, U. C. and Clarke, C. F. (2011). Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants. *Biochim Biophys Acta* **1811**(5), 348-360.

Xie, L. X., Ozeir, M., Tang, J. Y., Chen, J. Y., Kieffer-Jaquinod, S., Fontecave, M., Clarke, C. F. and Pierrel, F. (2012). Over-expression of the Coq8 kinase in S*accharomyces cerevisiae coq* null

mutants allows for accumulation of diagnostic intermediates of the Coenzyme Q6 biosynthetic pathway. *J Biol Chem* **287**(28), 23571-23581.

Chapter 4

Perspectives and Future Directions

This work describes progress made towards characterizing the function of the coenzyme Q (Q)-binding protein Coq10p and the multi-subunit Q biosynthetic complex in *Saccharomyces cerevisiae* mitochondria. In Chapter 2 the function of Coq10p in Q biosynthesis and activity was studied using the prokaryotic homolog CC1736. The tertiary structure of CC1736 was previously determined by NMR and was shown to be a START domain protein (Shen *et al.*, 2005), a superfamily known for binding hydrophobic substrates such as phospholipids, cholesterol, and polyketides via a hydrophobic pocket (Ponting and Aravind, 1999; Miller, 2007). In our work we demonstrated that expression of CC1736 harboring an N-terminal mitochondrial leader sequence in a yeast *coq10* null mutant could restore growth on a non-fermentable carbon source and NADH-dependent cytochrome *c* reduction, indicating a conservation of function. Given this conservation of function, availability of structural information, and ease of purification with respect to Coq10p, we performed *in vitro* characterization of CC1736 as a surrogate for Coq10p to assess its potential function as a lipid-binding protein. Binding assays were performed with purified recombinant CC1736 and we showed that the protein is capable of binding isoforms of Q with varying isoprenoid tail lengths at a 1:1 ligand-protein stoichiometry, including Q_2, Q_3, and Q_{10}. In addition binding assays were also performed with DMQ_3, a late-stage Q intermediate, FHB, a farnesylated analog of an early Q intermediate, and ergosterol, a lipid structurally distinct to Q. CC1736 binds DMQ_3 with a 1:1 ligand-protein stoichiometry, but is unable to bind FHB or ergosterol. These binding studies showed that CC1736 is a lipid-binding protein with specificity for Q and late-stage Q intermediates, but does not discriminate between isoprenoid chains of different length (n = 2 to 10). A recent study demonstrated that supplementation with particular isoforms of Q_2 where the double bond in the first isoprenoid unit was shifted or removed failed to restore growth of Q-less

yeast mutants on a non-fermentable carbon source, but still supported respiration in isolated mitochondria (James *et al.*, 2010). It will be interesting to determine whether this *in vivo* discrimination occurs at the level of binding specificity with CC1736 and by extension Coq10p. A recent study with *Schizosaccharomyces pombe* Coq10, which has been shown to bind Q and is required for efficient mitochondrial respiration and sulfide metabolism in *S. pombe*, used an azido analog of Q to demonstrate that the quinone ring of Q interacts with the N-terminal region F39-K45 of Coq10, which corresponds to the ligand-binding pocket of several START domain proteins (Cui and Kawamukai, 2009; Murai *et al.*, 2014).

To further understand the role of Coq10p in Q biosynthesis we measured *de novo* Q synthesis using $^{13}C_6$-labeled precursors in both wild type and *coq10* null yeast strains. We determined that *de novo* Q biosynthesis decreases as a function of growth phase, with the greatest level of Q synthesis occurring at early-log phase. Yeast *coq10* null mutants synthesize significantly less *de novo* Q compared to wild type, most dramatically at early-log phase, indicating a role for Coq10p in efficient Q biosynthesis. We also measured *de novo* Q synthesis in complemented yeast *coq10* null mutants and observed that over-expression of *COQ10*, *COQ8*, or CC1736 significantly increased Q synthesis with respect to an empty vector control, however the rescue was modest and levels of *de novo* synthesized Q were still significantly lower than the wild type control, possibly due in part to the tendency of *coq10* null mutants to go rho minus (Barros *et al.*, 2005).

Our data demonstrated that Coq10p is required for efficient *de novo* biosynthesis of Q_6 in yeast and that Coq10p is likely a Q- and Q intermediate-binding protein through characterization of its functional homolog CC1736. Previous studies have suggested a function for Coq10p in

efficient electron transport, potentially as a chaperone to the N-site of the complex III (Busso *et al.*, 2010), while other studies have suggested a role in the stability of the Q biosynthetic complex due to the observation that certain Coq polypeptides are destabilized in the *coq10* null mutant (Hsieh *et al.*, 2007; He *et al.*, 2014). We propose that the Q-binding function of Coq10p allows it to play a role both in Q biosynthesis and the electron transport chain. Previous work has shown the association of bound DMQ_6 with the Q biosynthetic complex and supplementation of various *coq* null mutants with exogenous Q_6 stabilizes particular Coq polypeptides, suggesting a structural function for Q or a Q intermediate in the complex (Marbois *et al.*, 2005; Tran *et al.*, 2006; He *et al.*, 2014). Coq10p may serve as a chaperone for Q in which it delivers Q to the biosynthetic complex to maintain efficient *de novo* Q synthesis, as well as a chaperone for Q to the electron transport chain, potentially to one of the two Q-binding sites required for the Q cycle in complex III.

To better characterize the putative chaperone function of Coq10p it is necessary to characterize its potential protein-binding partners. Previous studies have suggested that Coq10p may exist in high molecular mass complex by sucrose gradient sedimentation and 2D blue native-PAGE (Barros *et al.*, 2005; Tauche *et al.*, 2008), however these experiments did not address whether Coq10p was associated with other proteins or with itself in an oligomeric complex. Initial attempts to generate a C-terminal tandem affinity tag on Coq10p for co-purification studies have proved unsuccessful, as the tag appeared to destabilize Coq10p (unpublished observations). However more recent work in which Coq10p has been tagged at its C-terminus with the cMyc epitope appears more promising. This tag does not disrupt steady-state levels of Coq10p and allows for co-immunoprecipitation of the tagged protein (Hui Tsui, unpublished observations). It will be informative to scale-up purification of Coq10p and subject

the eluates to Western blotting and proteomic analysis to determine what other proteins may be associated. It is anticipated that Coq10p may interact transiently with the Q biosynthetic complex and one or more components of the electron transport chain due to its hypothesized chaperone function, however it may also interact with a previously unanticipated protein, potentially giving insight to the regulation of Q biosynthesis.

In yeast several of the proteins required for Q biosynthesis are associated in a macromolecular complex on the matrix-face of the inner mitochondrial membrane (He *et al.*, 2014). The existence of this complex is postulated to be important for improved catalytic efficiency via substrate channeling as well as to sequester potentially reactive intermediates. In Chapter 3 work to characterize the Q biosynthetic complex through the use of tandem affinity purification tags was described. We took advantage of the CNAP (consecutive non-denaturing affinity purification) tag to purify the complex under gentle conditions and characterize the protein composition both by Western blotting and proteomic analysis. We generated yeast strains expressing C-terminal CNAP-tagged Coq3p, Coq6p, or Coq9p and demonstrated that the presence of this tag does not interfere with *de novo* Q biosynthesis or growth on a non-fermentable carbon source, making them suitable systems for characterizing the native Q biosynthetic complex. Eluates from tandem affinity purification of the tagged Coq proteins as well as a wild-type control from detergent-solubilized mitochondria were analyzed by Western blotting and it was shown that Coq3p, Coq4p, Coq5p, Coq6p, Coq7p, and Coq9 are all associated in one or more Q biosynthetic complexes. The eluates were also analyzed for the presence of Q_6 and Q_6 intermediates and we found that the Coq complex also co-purified with Q_6 and the two late-stage intermediates DMQ_6 and $IDMQ_6$, but not the early intermediates HHB or

HAB, consistent with previous reports that Q or a Q intermediate is important for stability of the Coq polypeptide complex.

In addition Western blotting demonstrated that tagged Coq6p co-purified Coq8p, giving the first direct evidence of Coq8p association with the biosynthetic complex. Coq8p is a putative atypical protein kinase and previous work has shown that Coq3p, Coq5p, and Coq7p are phosphorylated in a Coq8p-dependent manner, although direct kinase activity for Coq8p has not yet been demonstrated (Tauche *et al.*, 2008; Xie *et al.*, 2011). Furthermore it has been shown that overexpression of *COQ8* in certain *coq* null mutants stabilizes steady-state levels of sensitive Coq polypeptides and leads to accumulation of later-stage Q biosynthetic intermediates, suggesting a role for phosphorylation in regulation and stability of the complex (Padilla *et al.*, 2009; Xie *et al.*, 2012; He *et al.*, 2014). Interestingly this observation is in contrast to another study which showed that dephosphorylation of Coq7p by the phosphatase Ptc7p was required for upregulation of Q biosynthesis (Martin-Montalvo *et al.*, 2011; Martin-Montalvo *et al.*, 2013), however it is not clear whether the putative sites of dephosphorylation in that study were Coq8p-dependent or had a role in mitochondrial import given their proximity to the Coq7p N-terminus. Our work is the first to shown a direct interaction of Coq8p with the Coq polypeptide complex and may suggest that it is either directly responsible for phosphorylation of the particular Coq proteins or that it potentially plays a structural role in the complex. The observation that it only co-purified with tagged Coq6p and not tagged Coq3p or Coq9p is suggestive that this interaction is transient and that it may be more closely associated with Coq6p.

The association of potentially novel binding partners with the Q biosynthetic complex was explored through proteomic analysis using mass spectrometry. This approach identified

several potential proteins interacting with the Coq polypeptide complex, most notably YLR290C and Ilv6p. The potential role of these two proteins in Q biosynthesis was investigated by measuring *de novo* Q synthesis in the corresponding null mutants and while the *ilv6* null did not appear to have an effect on Q synthesis the *ylr290c* null had significantly decreased levels of *de novo* synthesized Q, suggesting a function for YLR290C in Q biosynthesis. To further assess this, YLR290C was also tagged at its C-terminus with the CNAP tag and subjected to tandem affinity purification. Western blotting showed that tagged YLR290C co-purified with Coq4p, Coq5p, and Coq7p, however detection of these proteins was much lower than the levels detected with co-purification of the tagged Coq proteins. Associated lipids were also measured in the tagged YLR290C eluate and we found that Q_6, DMQ_6, and $IDMQ_6$ co-purified with the YLR290C-containing complex, albeit in much lower levels compared to the complexes purified from the tagged Coq proteins. These results demonstrate that YLR290C is a novel interacting protein with the Q biosynthetic complex, however its association may be transient or more easily disrupted.

While the function of YLR290C is unknown sequence analysis indicates that it is part of the short-chain dehydrogenase/reductase (SDR) superfamily and contains a conserved Rossmann fold, a motif typically associated with binding nucleotide-containing cofactors such as NAD(P), FAD, and FMN (Rossmann *et al.*, 1974). Members of the SDR superfamily include a diverse family of oxidoreductases and catalyze reactions such as isomerization, decarboxylation, epimerization, imine reduction, and carbonyl-alcohol oxidoreduction (Marchler-Bauer *et al.*, 2013). The crystal structures of UbiX, a prokaryotic decarboxylase involved in Q biosynthesis, and a homolog to another decarboxylase involved in Q biosynthesis, Pad1, were solved and shown to contain a typical Rossmann fold with a bound FMN (Rangarajan *et al.*, 2004; Kopec *et*

al., 2011). While the structure of YLR290C is not known its identification as a member of the SDR superfamily with a conserved Rossmann fold, as well as the effects of its corresponding null mutant on Q biosynthesis make it tempting to speculate that YLR290C may function as the previously unknown decarboxylase in the yeast Q biosynthetic pathway. A confounding observation to this hypothesis is that the *ylr290c* null still synthesizes small levels of Q_6 and retains the ability to grow on a non-fermentable carbon source. One explanation is that YLR290C does function as the sole decarboxylase in Q biosynthesis due to the potential existence of redundant decarboxylases. *Escherichia coli* contains two decarboxylases that function in Q biosynthesis, UbiD and UbiX, and expression of yeast *PAD1* in an *E. coli ubiX* mutant restores Q_8 synthesis, while the yeast protein YDR539W, now designated Fdc1p, is a homolog to *E. coli* UbiD (Gulmezian *et al.*, 2007). In spite of this yeast mutants lacking either Pad1p or Fdc1p, or both Pad1p and Fdc1p, synthesize normal levels of Q_6 (Gulmezian, 2006), and it was later shown that these two proteins are required for decarboxylation of phenylacrylic acids (Mukai *et al.*, 2010). These observations are consistent with the hypothesis that Pad1p and Fdc1p may act as redundant decarboxylases with YLR290C, accounting for the small levels of synthesized Q_6 in the *ylr290c* null and the apparently normal levels of Q_6 in the *pad1* and *fdc1* null mutants. To assess this possibility a *pad1 fdc1 ylr290c* triple mutant should be constructed and its ability to synthesize Q_6 should be measured, in addition to any accumulated Q biosynthetic intermediates. This triple mutant and the *ylr290c* null single mutant should also be analyzed for their effects on the steady-state levels of the Coq polypeptides as well as the stability of the high molecular mass Coq polypeptide complex. Interestingly, the decreased *de novo* Q synthesis measured in the *ylr290c* null mutant was only observed in minimal media but not rich media (unpublished observations), suggesting an alternative ring precursor may be

present in rich media compared to minimal media, bypassing the requirement for YLR290C in Q biosynthesis. An experiment testing this hypothesis would be to determine *de novo* Q synthesis in the *ylr290c* null mutant grown in minimal media supplemented with either yeast extract or peptone, the two distinct components of rich media, to assess whether either component can restore *de novo* Q synthesis to wild-type levels.

Given the identification of YLR290C as a member of the SDR superfamily with a conserved Rossmann fold, it might act as an oxidoreductase to maintain the redox poise of particular Q biosynthetic intermediates to increase the efficiency of particular steps in the pathway. Generating and purifying recombinant YLR290C and performing the appropriate *in vitro* enzymes assays with synthetic farnesylated analogs of substrates should assess the potential function of YLR290C as either a decarboxylase or an oxidoreductase. The reactants and products of these reactions can be easily measured by LC-MS/MS. Purification of YLR290C will also serve to allow identification of potentially bound cofactors such as FMN, yielding further insight as to its possible function. Antibodies should also be generated against the YLR290C protein to allow elucidation of sub-mitochondrial localization, determine the effects of various *coq* null mutants on steady-state levels of YLR290C, assess its oligomeric state by 2D blue native-PAGE, and verify its presence in the eluates of the CNAP-tagged Coq proteins.

The CNAP-tagged Coq proteins generated for this study can also serve as a powerful tool to characterize phosphorylation of the Coq proteins. While it was shown that Coq3p, Coq5p, and Coq7p are phosphorylated by 2D-IEF the location of phosphorylation was not determined and the potential phosphorylation of other Coq proteins was not determined due to the limitations of both IEF and Western blotting (Xie *et al.*, 2011). Preliminary results from the proteomic

analyses in this study suggested phosphorylation on additional Coq proteins such as Coq6p and also narrowed down the likely sites of this modification to particular tryptic peptides (unpublished observations). Tryptic peptides from the tandem affinity purification eluates of the CNAP-tagged Coq proteins can be subjected to phospho-enrichment using a resin such as titanium dioxide to concentrate the phosphorylated peptides in order to improve detection by mass spectrometry as protein phosphorylation is often sub-stoichiometric, confounding detection of phosphorylated peptides in non-enriched samples. This analysis will show not only which proteins associated with the Coq polypeptide complex are phosphorylated but also approximately where they are phosphorylated. These putative sites can then be subjected to mutagenesis to determine the possible role of phosphorylation in both Q biosynthesis and assembly of the complex.

As the protein and lipid composition of the Q biosynthetic complex becomes clearer structural analysis should provide a more detailed schematic of the complex and its organization. Recently the crystal structure of yeast Coq5p was solved and shown to be a dimer (Dai *et al.*, 2014). The crystal structure of a prokaryotic homolog of Coq4p was also shown to exist as a dimer with a bound geranylgeranyl monophosphate, suggesting a role for polyisoprenoid binding for Coq4p (Rea *et al.*, 2010). The crystal structure of human COQ9 was recently solved and show to be a dimer, and in vitro analysis also demonstrated that it associated with COQ7 (Lohman *et al.*, 2014). The existence of these proteins as dimers is not surprising as blue native-PAGE results suggest that several copies of the Coq proteins may be present in the Q biosynthetic complex as its molecular mass exceed 669kDa, which is much greater than the additive mass of the individual Coq polypeptides known to be associated in the complex (He *et al.*, 2014). Taking into account the association of Coq8p and YLR290C with the complex, as

well as the known or putative dimeric state of particular Coq proteins, the expected mass of the Q biosynthetic complex is approximately 350kDa, suggesting that additional Coq proteins are in oligomeric states or that the core complex is in a higher-order multimeric state, similar to the respiratory supercomplexes. Purification and crystallization of the intact Q biosynthetic complex is technically challenging, so a more feasible approach to determine the structure of the holo-complex may be cryo-electron microscopy. Complexes purified with the CNAP tag may be subjected to negative staining and visualized by cryo-electron microscopy to yield an approximate three dimensional structure of the entire complex. Available structural information for the individual Coq proteins may then be used to model the individual proteins into the complex structure. A more accurate and thorough understanding of the organization and composition of the Q biosynthetic complex will give insight to both the synthesis of Q as well as its potential modes of regulation.

References

Barros, M. H., Johnson, A., Gin, P., Marbois, B. N., Clarke, C. F. and Tzagoloff, A. (2005). The *Saccharomyces cerevisiae COQ10* gene encodes a START domain protein required for function of coenzyme Q in respiration. *J Biol Chem* **280**(52), 42627-42635.

Busso, C., Tahara, E. B., Ogusucu, R., Augusto, O., Ferreira, J. R., Tzagoloff, A., Kowaltowski, A. J. and Barros, M. H. (2010). Saccharomyces cerevisiae coq10 null mutants are responsive to antimycin A. *Febs Journal* **277**(21), 4530-4538.

Cui, T. Z. and Kawamukai, M. (2009). Coq10, a mitochondrial coenzyme Q binding protein, is required for proper respiration in *Schizosaccharomyces pombe*. *Febs J* **276**(3), 748-759.

Dai, Y. N., Zhou, K., Cao, D. D., Jiang, Y. L., Meng, F., Chi, C. B., Ren, Y. M., Chen, Y. X. and Zhou, C. Z. (2014). Crystal structures and catalytic mechanism of the C-methyltransferase Coq5 provide insights into a key step of the yeast coenzyme Q synthesis pathway. *Acta Crystallographica Section D-Biological Crystallography* **70**, 2085-2092.

Gulmezian, M. (2006). Characterization of *Escherichia coli ubiX*, *Saccharomyces cerevisiae PAD1* and *YDR539W*, and a complex of polypeptides involved in coenzyme Q biosynthesis, UCLA.

Gulmezian, M., Hyman, K. R., Marbois, B. N., Clarke, C. F. and Javor, G. T. (2007). The role of UbiX in *Escherichia coli* coenzyme Q biosynthesis. *Arch Biochem Biophys* **467**(2), 144-153.

He, C. H., Xie, L. X., Allan, C. M., Tran, U. C. and Clarke, C. F. (2014). Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast coq null mutants. *Biochim Biophys Acta* **1841**(4), 630-644.

Hsieh, E. J., Gin, P., Gulmezian, M., Tran, U. C., Saiki, R., Marbois, B. N. and Clarke, C. F. (2007). *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex. *Arch Biochem Biophys* **463**(1), 19-26.

James, A. M., Cocheme, H. M., Murai, M., Miyoshi, H. and Murphy, M. P. (2010). Complementation of coenzyme Q-deficient yeast by coenzyme Q analogues requires the isoprenoid side chain. *Febs Journal* **277**(9), 2067-2082.

Kopec, J., Schnell, R. and Schneider, G. (2011). Structure of PA4019, a putative aromatic acid decarboxylase from *Pseudomonas aeruginosa*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67**, 1184-1188.

Lohman, D. C., Forouhar, F., Beebe, E. T., Stefely, M. S., Minogue, C. E., Ulbrich, A., Stefely, J. A., Sukumar, S., Luna-Sánchez, M., Jochem, A., Lew, S., Seetharaman, J., Xiao, R., Wang, H., Westphall, M. S., Wrobel, R. L., Everett, J. K., Mitchell, J. C., López, L. C., Coon, J. J., Tong, L. and Pagliarini, D. J. (2014). Mitochondrial COQ9 is a lipid-binding protein that associates with COQ7 to enable coenzyme Q biosynthesis. *Proc Natl Acad Sci U S A* **111**(44), E4697-E4705.

Marbois, B., Gin, P., Faull, K. F., Poon, W. W., Lee, P. T., Strahan, J., Shepherd, J. N. and Clarke, C. F. (2005). Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q. *J Biol Chem* **280**, 20231-20238.

Marchler-Bauer, A., Zheng, C. J., Chitsaz, F., Derbyshire, M. K., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., Lanczycki, C. J., Lu, F., Lu, S. N., Marchler, G. H., Song, J. S., Thanki, N., Yamashita, R. A., Zhang, D. C. and Bryant, S. H. (2013). CDD: conserved domains and protein three-dimensional structure. *Nucleic Acids Res* **41**(D1), D348-D352.

Martin-Montalvo, A., Gonzalez-Mariscal, I., Padilla, S., Ballesteros, M., Brautigan, D. L., Navas, P. and Santos-Ocana, C. (2011). Respiratory-induced coenzyme Q biosynthesis is regulated by a phosphorylation cycle of Cat5p/Coq7p. *Biochemical Journal* **440**, 107-114.

Martin-Montalvo, A., Gonzalez-Mariscal, I., Pomares-Viciana, T., Padilla-Lopez, S., Ballesteros, M., Vazquez-Fonseca, L., Gandolfo, P., Brautigan, D. L., Navas, P. and Santos-Ocana, C. (2013). The Phosphatase Ptc7 Induces Coenzyme Q Biosynthesis by Activating the Hydroxylase Coq7 in Yeast. *Journal of Biological Chemistry* **288**(39), 28126-28137.

Miller, W. L. (2007). Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* **1771**(6), 663-676.

Mukai, N., Masaki, K., Fujii, T., Kawamukai, M. and Iefuji, H. (2010). *PAD1* and *FDC1* are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J Biosci Bioeng* **109**(6), 564-569.

Murai, M., Matsunobu, K., Kudo, S., Ifuku, K., Kawamukai, M. and Miyoshi, H. (2014). Identification of the Binding Site of the Quinone-Head Group in Mitochondrial Coq10 by Photoaffinity Labeling. *Biochemistry* **53**(24), 3995-4003.

Padilla, S., Tran, U. C., Jimenez-Hidalgo, M., Lopez-Martin, J. M., Martin-Montalvo, A., Clarke, C. F., Navas, P. and Santos-Ocana, C. (2009). Hydroxylation of demethoxy-Q6 constitutes a control point in yeast coenzyme Q6 biosynthesis. *Cell Mol Life Sci* **66**(1), 173-186.

Ponting, C. P. and Aravind, L. (1999). START: a lipid binding domain in StAR, HD-ZIP and signalling proteins. *Trends in Biochemical Sciences* **24**(4), 130-132.

Rangarajan, E. S., Li, Y., Iannuzzi, P., Tocilj, A., Hung, L. W., Matte, A. and Cygler, M. (2004). Crystal structure of a dodecameric FMN-dependent UbiX-like decarboxylase (Pad1) from *Escherichia coli* O157: H7. *Protein Sci* **13**(11), 3006-3016.

Rea, S. L., Graham, B. H., Nakamaru-Ogiso, E., Kar, A. and Falk, M. J. (2010). Bacteria, yeast, worms, and flies: exploiting simple model organisms to investigate human mitochondrial diseases. *Dev Disabil Res Rev* **16**(2), 200-218.

Rossmann, M. G., Moras, D. and Olsen, K. W. (1974). Chemical and biological evolution of a nucleotide-binding protein. *Nature* **250**(5463), 194-199.

Shen, Y., Goldsmith-Fischman, S., Atreya, H. S., Acton, T., Ma, L., Xiao, R., Honig, B., Montelione, G. T. and Szyperski, T. (2005). NMR structure of the 18 kDa protein CC1736 from *Caulobacter crescentus* identifies a member of the START domain superfamily and suggests residues mediating substrate specificity. *Proteins* **58**(3), 747-750.

Tauche, A., Krause-Buchholz, U. and Rodel, G. (2008). Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of *O*-methylase Coq3p depends on Abc1p/Coq8p. *FEMS Yeast Res* **8**(8), 1263-1275.

Tran, U. C., Marbois, B., Gin, P., Gulmezian, M., Jonassen, T. and Clarke, C. F. (2006). Complementation of *Saccharomyces cerevisiae coq7* mutants by mitochondrial targeting of the *Escherichia coli* UbiF polypeptide: two functions of yeast Coq7 polypeptide in coenzyme Q biosynthesis. *J Biol Chem* **281**(24), 16401-16409.

Xie, L. T. X., Hsieh, E. J., Watanabe, S., Allan, C. M., Chen, J. Y., Tran, U. C. and Clarke, C. F. (2011). Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants. *Biochim Biophys Acta* **1811**(5), 348-360.

Xie, L. X., Ozeir, M., Tang, J. Y., Chen, J. Y., Kieffer-Jaquinod, S., Fontecave, M., Clarke, C. F. and Pierrel, F. (2012). Over-expression of the Coq8 kinase in S*accharomyces cerevisiae coq* null mutants allows for accumulation of diagnostic intermediates of the Coenzyme Q6 biosynthetic pathway. *J Biol Chem* **287**(28), 23571-23581.

Appendix I

Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants

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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 Q_6. 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.

1. Introduction

Coenzyme Q (ubiquinone or Q) 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 reduced hydroquinone (QH_2) 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]. QH_2 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; Q_{10}, Q_9, Q_8 and Q_6 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 Q_6 [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

Abbreviations: 2D-IEF/SDS-PAGE, two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Q, coenzyme Q; ORF, open reading frame

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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 *cbs2* mutant allele (*cbs2-223*) 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 Q_6 and the growth defect in media containing a nonfermentable carbon source can be rescued by the addition of exogenous Q_6 to the growth medium. The suppression of the *cbs2* mutation was shown to be due to a neighboring tRNA and not to *COQ8* [23]. *E. coli* UbiB and *Providencia 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 Q_{10} 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 Q_{10} deficiencies harboring mutations in *ADCK3* indicated it is required for Q_{10} 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 Q_6 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 DMQ_6 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 Q_6, 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 Q_6 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% $(NH_4)_2SO_4$ and amino acids were added at final concentrations as described in Ref. [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 $OD_{600} = 2.5$ in YPD. Yeast cells from 1 ml of culture were pelleted by centrifugation 12,000 × *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

Table 1
Genotypes and sources of *S. cerevisiae* strains.

Strain	Genotype	Source
W303-1A	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303ΔABC1	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq8::HIS3*	[22]
FY251	MAT a *ura3-52 his3Δ200 leu2Δ1 trp1Δ63*	F. Winston[b]
FYΔABC1	MAT a *ura3-52 his3Δ200 leu2Δ1 trp1Δ63 coq8::TRP1*	[22]
CH130-A1	MAT a *his3-1,15 leu2-3,112 trp1-1 ura3-1 coq8-1*	[22]
CF130-2B	MAT α *his3Δ200 ura3-52 trp1Δ63 coq8-1*	C130[c] × FY251
C177	MAT α *met6 coq8-2*	A. Tzagoloff[c]
W177-3B	MAT a *his3-1,15 leu2-3,112 met6 ura3-1 coq8-2*	C177 × W303-1A
C183	MAT α *met6 coq8-3*	A. Tzagoloff[c]
W183-2A	MAT a *his3-1,15 trp1-1 ura3-1 coq8-3*	C183 × W303-1A
C194	MAT α *met6 coq8-4*	A. Tzagoloff[c]
W194-3A	MAT α *ade2-1 his3-1,15 met6 ura3-1 coq8-4*	C194 × W303-1A
C222	MAT α *met6 coq8-5*	A. Tzagoloff[c]
W222-7D	MAT a *ade2-1 his3-1,15 leu2-3,112 ura3-1 coq8-5*	C222 × W303-1A
C240	MAT α *met6 coq8-6*	A. Tzagoloff[c]
W240-1A	MAT a *leu2-3,112 ura3-1 coq8-6*	C240 × W303-1A
C275	MAT α *met6 coq8-9*	A. Tzagoloff[c]
C308	MAT α *met6 coq8-7*	A. Tzagoloff[c]
W308-4C	MAT α *ade2-1 his3-1,15 met6 trp1-1 ura3-1 coq8-7*	C308 × W303-1A
C315	MAT α *met6 coq8-8*	A. Tzagoloff[c]
W315-4A	MAT α *trp1-1 ura3-1 coq8-8*	C315 × W303-1A
JM6	MAT a, *his4* ρ^0	[78]
JM8	MAT α *ade1* ρ^0	[78]

[a] Dr. Rodney Rothstein, Department of Human Genetics, Columbia University.
[b] Dr. Fred Winston, C. Dollard, and S. Ricapero-Hovasse, Department of Genetics, Harvard University.
[c] Dr. Alex Tzagoloff, Department of Biological Sciences, Columbia University.

(1 M sorbitol, 50 mM Tris–Cl pH 7.5, 6 mg/ml zymolyase-20 T (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′ CGCGGAATTCAACTTTATAGGCAAAAATCT 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-Cla1-(+1 to +31)-3′ of *ADCK3*) and a reverse complement primer, ADCK3Rev (5′-clamp-Kpn1-(+1944 to +1927)-3′ of *ADCK3*) were used with MHS1011 as template DNA to generate a PCR-amplifed 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 (multicopy), respectively.

Table 2
Plasmid constructs used in this study.

Plasmid	Genes	Copy number	Source
pRS316	Yeast shuttle vector only	Low copy	Sikorski and Hieter [79]
pRS426	Yeast shuttle vector only	Multicopy	Christianson et al. [80]
pAH01	Yeast vector with *CYC1* promoter	Low copy	Hsu et al. [81]
pCH1	Yeast vector with *CYC1* promoter	Multicopy	Hsu et al. [81]
pQM	pAH01 with *COQ3* mito leader	Low copy	Hsu et al. [41]
pRCM	pCH1 with *COQ3* mito leader	Multicopy	Saiki and Morvaridi
p3HN4	Yeast *ABC1/COQ8*	Low copy	Do et al. [82]
pK216A	Yeast *abc1/coq8-K216A*	Low copy	This work
p4HN4	Yeast *ABC1/COQ8*	Multicopy	Hsieh et al. [23]
p4K216A	Yeast *abc1/coq8-K216A*	Multicopy	This work
pBT8-1	Yeast *ABC1/COQ8*-biotin	Multicopy	This work
pBT8KA	Yeast *abc1/coq8-K216A*-biotin	Multicopy	This work
psCABC1	pAH01/Human *CABC1/ADCK3* with *CYC1* promoter	Low copy	This work
pmCABC1	pCH1/Human *CABC1/ADCK3* with *CYC1* promoter	Multicopy	This work
plcADCK3	pQM/Human CABC1/*ADCK3* with *CYC1* promoter and *COQ3* mito leader	Low copy	This work
pmcADCK3	pRCM/Human CABC1/*ADCK3* with *CYC1* promoter and *COQ3* mito leader	Multicopy	This work

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′ CATTTCGTACGCCCCTTTCCTATCTCTCAATGTGG 3′ +1194 to 1160 bp). Nucleotides that are underlined are the mutated nucleotides. The PCR fragments were digested with AflII and BsiWI. The plasmids p3HN4 [22], p4HN4 [23] and pBT8-1 were treated with AflII and BsiWI to remove a fragment of *COQ8*. The AflII/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′ ACTCGAGAAAAGCAATCTGGTAGATTTATGGG 3′ −300 to −276 (underlined) relative to the *COQ8* ATG) and Abc1Seq-R (5′ CCTCGAGTTAAACTTTATAGGCAAAAATCTCTT 3′ +1506 to +1481 (underlined) relative to the *COQ8* ATG). The second segment was amplified with the primers KAbc1A-F1 (5′ CGTCTCTTAAGAACTATACAGAACG 3′ +55 to +74 relative to the *COQ8* ATG) and Abc1Seq-R2 (5′ TCTGTTTTATCTTTTTTTTTTGTTCTTCGAGATT 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 l of YPGal+0.1% Dextrose and harvested at an OD_{600} of approximately 1. Crude mitochondria were isolated and purified on a Histodenz (Sigma) gradient as described in Ref. [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 Na_2CO_3 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, 13 cm, 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 dithiothreiotol), 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 μL λ-phosphatase (100 U/μL) (New England Bio Labs).

2.9. Immunoblot analyses

Proteins were transferred from SDS-polyacrylamide gels to polyvinylidene difuloride (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 Q_6 and Q_4 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 homologs 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 nonfermentable carbon source. No growth was detected after 3 to 10 days of incubation of transformants expressing Coq8-K216A (Fig. 2). Identical results were obtained with another *coq8Δ* null mutant FYΔABC1, 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-

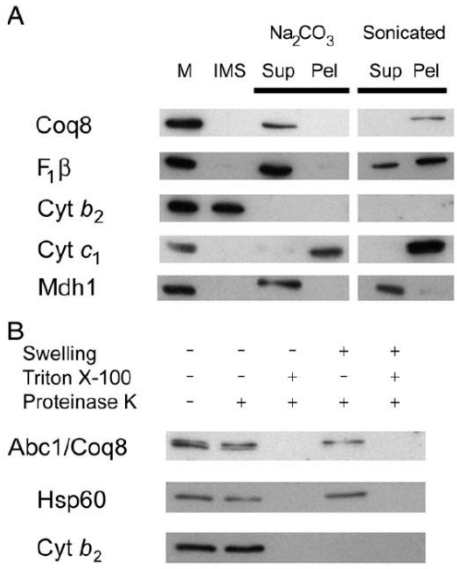

Fig. 1. Coq8p is a peripheral membrane protein, associated with the mitochondrial inner membrane on the matrix side. *A.* Coq8p is peripherally associated with mitochondria membranes. Nycodenz purified mitochondria (M) were hypotonically swelled to generate the intermembrane space (IMS) fraction and mitoplasts. Mitoplasts were then treated with 0.1 M Na_2CO_3 pH 11.5, or sonicated. The treated mitoplasts were centrifuged and the supernatant (Sup) and pellet (Pel) fractions were 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 F_1-ATPase ($F_1\beta$) 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.

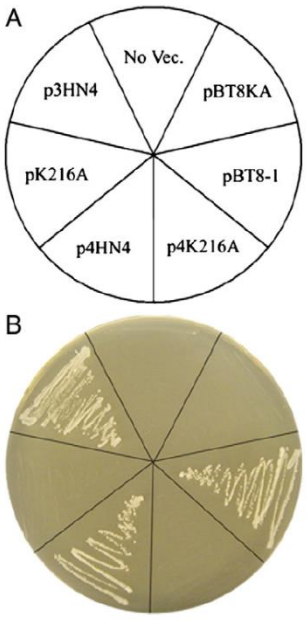

Fig. 2. The Lysine216 in kinase subdomain II of Coq8 is required for rescue of glycerol growth in a yeast *coq8* null mutant. The yeast *coq8* null mutant (W303ΔABC1) was transformed with plasmids containing either wild-type *COQ8* or *coq8-K216A*. p3HN4 (*COQ8*) and pK216A (*coq8-K216A*) are low-copy vectors; p4HN4 (*COQ8*), p4K216A (*coq8-K216A*), pBT8-1 (*COQ8*) and pBT8KA (*coq8-K216A*) are multicopy vectors. Yeast transformants were incubated 3 days on YPG plate media at 30 °C.

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 Q_6 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 Q_6 to yeast *coq7* null mutant cultures restores assembly of a biosynthetic complex enabling synthesis of DMQ_6, the penultimate intermediate in Q biosynthesis [33]. Addition of Q_6 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 Q_6 to cultures of W303ΔABC1:pK216A yeast did not rescue steady-state Coq8 polypeptide levels. Similarly, addition of Q_6 did not restore the Q-biosynthetic complex in *coq8* null mutants [34]. Antibodies against the β subunit of the F_1-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 produce 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

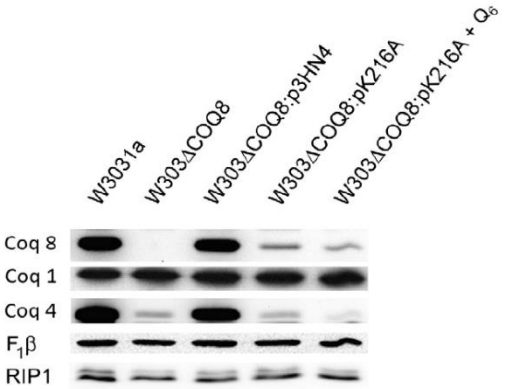

Fig. 3. The yeast Coq8-K216A polypeptide is not present at normal steady-state levels. Yeast were grown in 1 l of YPGal medium with 0.1% dextrose and harvested at an OD_{600nm} of approximately 4. W303ΔABC1:pK216A + Q_6 was grown in YPGal + 0.1% Dextrose media supplemented with 1.2 μM Q_6. Purified mitochondria from W303-1A, W303ΔABC1 and W303ΔABC1 harboring the designated plasmids were subjected to Western blot analysis.

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 was rescued for growth on glycerol-medium following transformation with p3HN4 (low-copy yeast *COQ8*), while transformation with pRS426 (multi-copy 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 Q_{10} 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 Q_6 content, lipids were extracted from wild-type and mutant yeast, and Q_6 levels were measured with a QT4000 mass spectrometer coupled in-line with reversed phase HPLC. Q_6 was not detected in W303ΔCOQ8, W183, or W222 harboring empty vector, pRS426. In contrast, Q_6 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 nonfermentable carbon source, glycerol. Each of the *coq8* mutants tested was rescued by pmcADCK3, and rescue was also observed after 10 days with plcADCK3 (Fig. 6). The pmcADCK3 plasmid partially restored Q_6 content in yeast lipid extracts from transformed yeast (Fig. 7). Yeast *abc1/coq8* mutants transformed with pmcADCK3 contained a small amount of Q_6, while the same mutants transformed with the empty vector (pRS426) contained no detectable Q_6.

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 Q_6. Yeast growth is observed in the spot containing only 20,000 cells (2 μl of yeast cells at $A_{600nm} = 1.0$ or 1×10^7 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 Q_6 content shown in Fig. 7, it seems likely that the content of Q_6 as measured in YPGal liquid medium (Fig. 7) may not be indicative of the Q_6 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 Q_6 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 Q_6 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,

Table 3
Amino acid and nucleotide substitutions of *coq8* alleles.

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

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

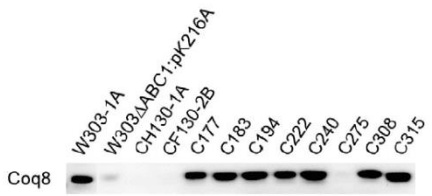

Fig. 4. Seven yeast *coq8* mutant strains have normal steady-state levels of the Coq8 polypeptide. The designated yeast strains were grown in YPD, lysed with 2% SDS and 425–600 μm glass beads (Sigma). Aliquots of whole cell lysates (corresponding to 0.1 OD_{600} of yeast cells) were separated by SDS-PAGE and then transferred to a PVDF membrane for Western blot analyses with antibodies to Coq8p.

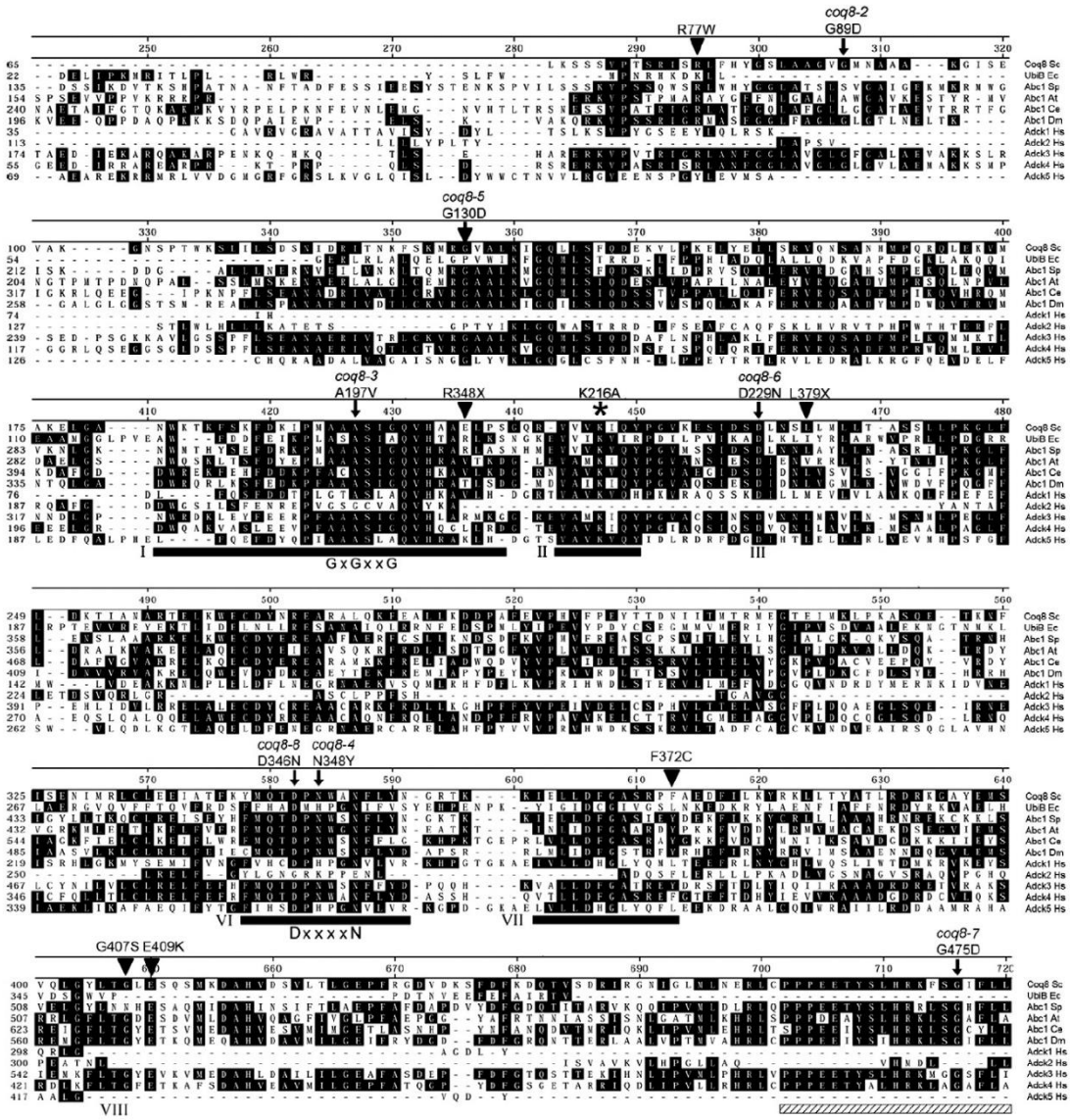

Fig. 5. Amino acid alignment of Abc1/Coq8p homologs. An alignment of Abc1/Coq8p and its homologs was created using DNASTAR™ Megalign. Clustal method was used with PAM250 residue weight table. Homologs are *E. coli* (GenBank™ accession number P27854), *S. pombe* (GenBank™ accession number CAA62818), *A. thaliana* (GenBank™ accession number AAC72875), *C. elegans* (GenBank™ accession number AAA62560), *D. melanogaster* (GenBank™ accession number AAA62560NP_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.

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.

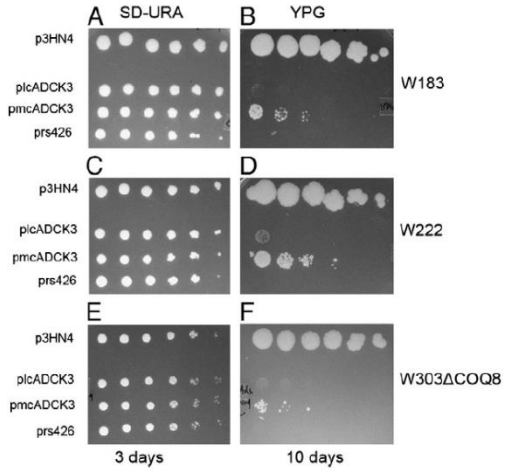

Fig. 6. Expression of the human ADCK3 polypeptide rescues the growth of yeast *coq8* mutants on nonfermentable carbon source media. Yeast *coq8* point mutants W183 and W222, and *coq8* null mutant (W303ΔABC1) were transformed with the designated plasmids: p3HN4, yeast low-copy *COQ8*; plcADCK3, low-copy ADCK3; pmcADCK3, multicopy 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 μl 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*, and *E*, Growth is depicted on SD-Ura plates after 3 days of incubation at 30 °C; *B*, *D*, and *F*, YPG plates after 10 days of incubation at 30 °C.

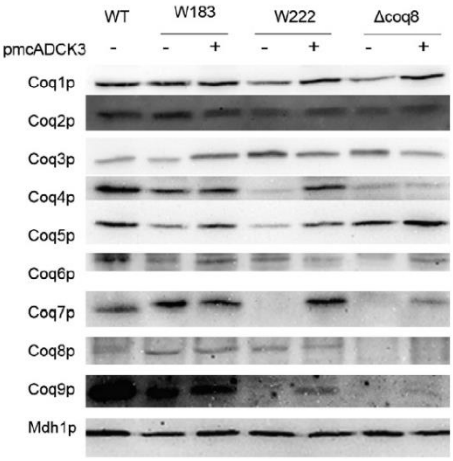

Fig. 8. Expression of the human ADCK3 polypeptide in yeast *coq8* mutants partially restores steady-state levels of some of the yeast Coq polypeptides. Mitochondria were isolated from wild-type yeast (W303-1A) or from the indicated *coq8* mutant yeast strains with or without pmcADCK3 (a multicopy plasmid 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.

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 W303ΔCOQ8) 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.

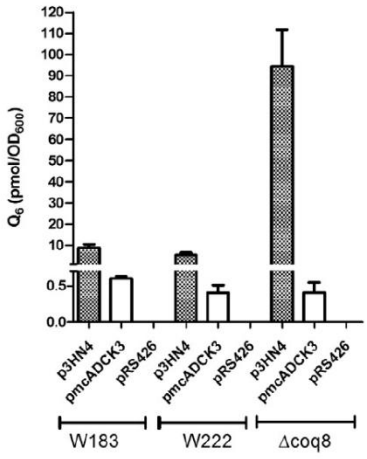

Fig. 7. Expression of the human ADCK3 polypeptide partially restores Q_6 content in yeast *coq8* mutants. Yeast cells were cultured in YPGal + 0.1% dextrose and collected at an optical density (A_{600nm}) of about 1.0. The content of Q_6 in yeast cell lipid extracts was determined by HPLC/MS-MS as described in Materials and methods. Yeast *coq8* mutants were transformed with plasmids expressing yeast *ABC1/COQ8* (low-copy, p3HN4), human CABC1/ADCK3 (multicopy, pmcADCK3), or empty vector control (multicopy, pRS426). Each bar represents a total of four measurements from two independent samples each with two injections. Standard deviations are represented with error bars. Q_4 was used as internal standard.

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 that 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

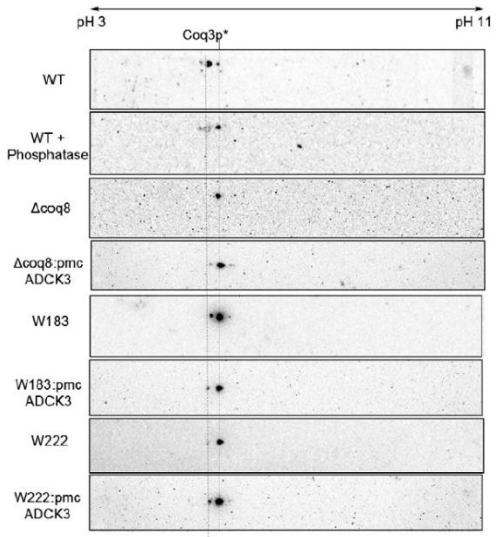

Fig. 9. Expression of the human ADCK3 polypeptide in yeast *coq8* mutants partially restores the phosphorylation state of the yeast Coq3 polypeptide. Isolated mitochondria (200 μg protein) from wild-type yeast (W303-1A), or from the designated *coq8* mutants, W183, W222, or W303ΔCOQ8, either without 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.

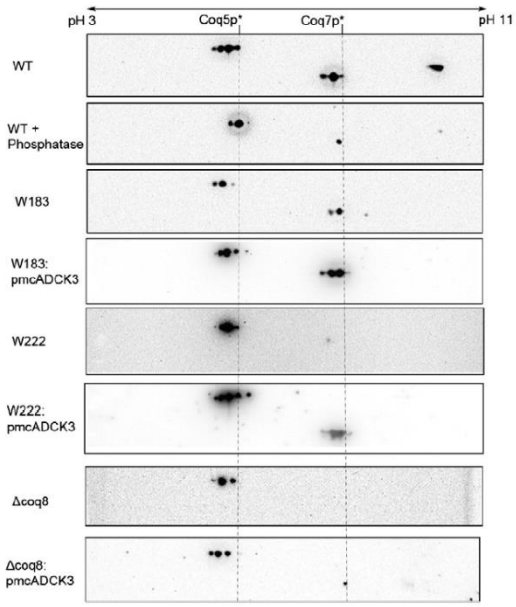

Fig. 10. Expression of the human ADCK3 polypeptide in yeast *coq8* mutants partially restores the phosphorylation state of the yeast Coq5 and Coq7 polypeptides. Isolated mitochondria (200 μg protein) from wild-type yeast (W303-1A), or from the designated *coq8* mutants, W183, W222, or W303ΔCOQ8, either 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 Coq7 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.

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 homolog 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 Q_6 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 Q_6 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 homologs (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 Q_{10} 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 Q_6 content, respectively [26]. We confirm that the corresponding yeast *coq8* mutant harboring G130D-Coq8p lacked Q_6 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 homologs (Fig. 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 homologs (Fig. 11). 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. subtillis*, 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.

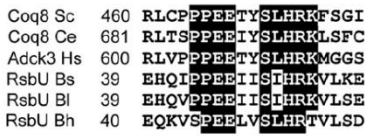

Fig. 11. The carboxyl terminal segment of Coq8 homologs and the amino terminal segment of RsbU homologs share a region of high identity. In RsbU the sequence designated by the black bar forms an alpha-helix, and side chains interact with a neighboring alpha-helix in a RsbU homodimer. Numbers indicate the amino acid position of the segment. The RsbU homologs shown are from *Bacillus subtilis* (GenBank accession number NP_388351), *Bacillus lichenformis* (GenBank accession number YP_077796), and *Bacillus halodurans* (GenBank accession number NP_241392). Shading indicates identical residues present in the majority of the sequences.

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 (Q_{151}STOP) *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 Q_6 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 nonfermentable carbon source and enabled synthesis of Q_6. 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 Q_6 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 Q_{10} can sometimes elicit dramatic improvement [74]. Partial rescue of kidney disease with oral Q_{10} 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 homologs 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 Q_{10} 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 homologs 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 homologs 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) homologs 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 homolog) 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.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbalip.2011.01.009.

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References

[1] S. Ghisla, C. Thorpe, Acyl-CoA dehydrogenases. A mechanistic overview, Eur. J. Biochem. 271 (2004) 494–508.
[2] G. Lenaz, A. De Santis, A survey of the function and specificity of ubiquione in the mitochondrial respiratory chain, in: G. Lenaz (Ed.), Coenzyme Q, John Wiley & Sons, Chichester, U.K., 1985, pp. 165–199.
[3] T.M. Hildebrandt, M.K. Grieshaber, Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria, FEBS J. 275 (2008) 3352–3361.
[4] S. Hill, K. Hirano, V.V. Shmanai, B.N. Marbois, D. Vidovic, A.V. Bekish, B. Kay, V. Tse, J. Fine, C.F. Clarke, M.S. Shchepinov, Isotope-reinforced polyunsaturated fatty acids protect yeast cells from oxidative stress, Free Radic. Biol. Med. 50 (2010) 130–138.
[5] M. Bentinger, K. Brismar, G. Dallner, The antioxidant role of coenzyme Q, Mitochondrion 7S (2007) S41–S50.
[6] L. Papucci, N. Schiavone, E. Witort, M. Donnini, A. Lapucci, A. Tempestini, L. Formigli, S. Zecchi-Orlandini, G. Orlandini, G. Carella, R. Brancato, S. Capaccioli, Coenzyme Q10 prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property, J. Biol. Chem. 278 (2003) 28220–28228.
[7] L. Walter, H. Miyoshi, X. Leverve, P. Bernard, E. Fontaine, Regulation of the mitochondrial permeability transition pore by ubiquinone analogs. A progress report, Free Radic. Res. 36 (2002) 405–412.
[8] F. Devun, L. Walter, J. Belliere, C. Cottet-Rousselle, X. Leverve, E. Fontaine, Ubiquinone analogs: a mitochondrial permeability transition pore-dependent pathway to selective cell death, PLoS ONE 5 (2010) e11792.
[9] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 281 (1998) 1309–1312.
[10] L. Azzolin, S. von Stockum, E. Basso, V. Petronilli, M.A. Forte, P. Bernardi, The mitochondrial permeability transition from yeast to mammals, FEBS Lett. 584 (2010) 2504–2509.
[11] M. Kawamukai, Biosynthesis and bioproduction of coenzyme Q10 by yeasts and other organisms, Biotechnol. Appl. Biochem. 53 (2009) 217–226.
[12] R.E. Olson, H. Rudney, Biosynthesis of ubiquinone, Vitam. Horm. 40 (1983) 1–43.
[13] B. Marbois, L.X. Xie, S. Choi, K. Hirano, K. Hyman, C.F. Clarke, *para*-Aminobenzoic acid is a precursor in coenzyme Q6 biosynthesis in *Saccharomyces cerevisiae*, J. Biol. Chem. 285 (2010) 27827–27838.
[14] F. Pierrel, O. Hamelin, T. Douki, S. Kieffer-Jaquinod, U. Muhlenhoff, M. Ozeir, R. Lill, M. Fontecave, Involvement of mitochondrial ferredoxin and para-aminobenzoic acid in yeast coenzyme Q biosynthesis, Chem. Biol. 17 (2010) 449–459.
[15] U.C. Tran, C.F. Clarke, Endogenous synthesis of coenzyme Q in eukaryotes, Mitochondrion 7S (2007) S62–S71.
[16] B. Marbois, P. Gin, K.F. Faull, W.W. Poon, P.T. Lee, J. Strahan, J.N. Shepherd, C.F. Clarke, Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q, J. Biol. Chem. 280 (2005) 20231–20238.
[17] B. Marbois, P. Gin, M. Gulmezian, C.F. Clarke, The yeast Coq4 polypeptide organizes a mitochondrial protein complex essential for coenzyme Q biosynthesis, Biochim. Biophys. Acta 1791 (2009) 69–75.
[18] E.J. Hsieh, P. Gin, M. Gulmezian, U.C. Tran, R. Saiki, B.N. Marbois, C.F. Clarke, *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex, Arch. Biochem. Biophys. 463 (2007) 19–26.
[19] S.L. Rea, B.H. Graham, E. Nakamaru-Ogiso, A. Kar, M.J. Falk, Bacteria, yeast, worms, and flies: exploiting simple model organisms to investigate human mitochondrial diseases, Dev. Disabil. Res. Rev. 16 (2010) 200–218.
[20] I. Bousquet, G. Dujardin, P.P. Slonimski, *ABC1*, a novel yeast nuclear gene has a dual function in mitochondria: it suppresses a cytochrome *b* mRNA translation defect and is essential for the electron transfer in the bc_1 complex, EMBO J. 10 (1991) 2023–2031.
[21] G. Brasseur, G. Tron, G. Dujardin, P.P. Slonimski, P. Brivet-Chevillotte, The nuclear *ABC1* gene is essential for the correct conformation and functioning of the

cytochrome *bc1* complex and the neighbouring complexes II and IV in the mitochondrial respiratory chain, Eur. J. Biochem. 246 (1997) 103–111.
[22] T.Q. Do, A.Y. Hsu, T. Jonassen, P.T. Lee, C.F. Clarke, A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in *Saccharomyces cerevisiae abc1* mutants, J. Biol. Chem. 276 (2001) 18161–18168.
[23] E.J. Hsieh, J.B. Dinoso, C.F. Clarke, A tRNA(TRP) gene mediates the suppression of *cbs2-223* previously attributed to *ABC1/COQ8*, Biochem. Biophys. Res. Commun. 317 (2004) 648–653.
[24] D.R. Macinga, G.M. Cook, R.K. Poole, P.N. Rather, Identification and characterization of *aarF*, a locus required for production of ubiquinone in *Providencia stuartii* and *Escherichia coli* and for expression of 2′-*N*-acetyltransferase in *P. stuartii*, J. Bacteriol. 180 (1998) 128–135.
[25] W.W. Poon, D.E. Davis, H.T. Ha, T. Jonassen, P.N. Rather, C.F. Clarke, Identification of *Escherichia coli ubiB*, a gene required for the first monooxygenase step in ubiquinone biosynthesis, J. Bacteriol. 182 (2000) 5139–5146.
[26] J. Mollet, A. Delahodde, V. Serre, D. Chretien, D. Schlemmer, A. Lombes, N. Boddaert, I. Desguerre, P. de Lonlay, H.O. de Baulny, A. Munnich, A. Rotig, CABC1 gene mutations cause ubiquinone deficiency with cerebellar ataxia and seizures, Am. J. Hum. Genet. 82 (2008) 623–630.
[27] C. Lagier-Tourenne, M. Tazir, L.C. Lopez, C.M. Quinzii, M. Assoum, N. Drouot, C. Busso, S. Makri, L. Ali-Pacha, T. Benhassine, M. Anheim, D.R. Lynch, C. Thibault, F. Plewniak, L. Bianchetti, C. Tranchant, O. Poch, S. DiMauro, J.L. Mandel, M.H. Barros, M. Hirano, M. Koenig, ADCK3, an ancestral kinase, is mutated in a form of recessive ataxia associated with coenzyme Q10 deficiency, Am. J. Hum. Genet. 82 (2008) 661–672.
[28] C.J. Leonard, L. Aravind, E.V. Koonin, Novel families of putative protein kinases in bacteria and archaea: evolution of the "eukaryotic" protein kinase superfamily, Genome Res. 8 (1998) 1038–1047.
[29] G. Manning, D.B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, The protein kinase complement of the human genome, Science 298 (2002) 1912–1934.
[30] A. Johnson, P. Gin, B.N. Marbois, E.J. Hsieh, M. Wu, M.H. Barros, C.F. Clarke, A. Tzagoloff, *COQ9*, a new gene required for the biosynthesis of coenzyme Q in *Saccharomyces cerevisiae*, J. Biol. Chem. 280 (2005) 31397–31404.
[31] M.H. Barros, A. Johnson, P. Gin, B.N. Marbois, C.F. Clarke, A. Tzagoloff, The *Saccharomyces cerevisiae COQ10* gene encodes a START domain protein required for function of coenzyme Q in respiration, J. Biol. Chem. 280 (2005) 42627–42635.
[32] C. Busso, E.B. Tahara, R. Ogusucu, O. Augusto, J.R. Ferreira-Junior, A. Tzagoloff, A.J. Kowaltowski, M.H. Barros, *Saccharomyces cerevisiae coq10* null mutants are responsive to antimycin A, FEBS J. (2010).
[33] S. Padilla, U.C. Tran, M. Jimenez-Hidalgo, J.M. Lopez-Martin, A. Martin-Montalvo, C.F. Clarke, P. Navas, C. Santos-Ocana, Hydroxylation of demethoxy-Q6 constitutes a control point in yeast coenzyme Q6 biosynthesis, Cell. Mol. Life Sci. 66 (2009) 173–186.
[34] A. Tauche, U. Krause-Buchholz, G. Rodel, Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of *O*-methylase Coq3p depends on Abc1p/Coq8p, FEMS Yeast Res. 8 (2008) 1263–1275.
[35] C.J. Sippel, R.R. Goewert, F.N. Slachman, R.E. Olson, The regulation of ubiquinone-6 biosynthesis by *Saccharomyces cerevisiae*, J. Biol. Chem. 258 (1983) 1057–1061.
[36] D. Burke, D. Dawson, T. Stearns, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Plainview, NY, 2000.
[37] R.J. Barkovich, A. Shtanko, J.A. Shepherd, P.T. Lee, D.C. Myles, A. Tzagoloff, C.F. Clarke, Characterization of the *COQ5* gene from *Saccharomyces cerevisiae*. Evidence for a *C*-methyltransferase in ubiquinone biosynthesis, J. Biol. Chem. 272 (1997) 9182–9188.
[38] R. Elble, A simple and efficient procedure for transformation of yeasts, Biotechniques 13 (1992) 18–20.
[39] S.H. Ackerman, J. Martin, A. Tzagoloff, Characterization of *ATP11* and detection of the encoded protein in mitochondria of *Saccharomyces cerevisiae*, J. Biol. Chem. 267 (1992) 7386–7394.
[40] D.M. Glerum, T.J. Koerner, A. Tzagoloff, Cloning and characterization of *COX14*, whose product is required for assembly of yeast cytochrome oxidase, J. Biol. Chem. 270 (1995) 15585–15590.
[41] A.Y. Hsu, W.W. Poon, J.A. Shepherd, D.C. Myles, C.F. Clarke, Complementation of *coq3* mutant yeast by mitochondrial targeting of the *Escherichia coli* UbiG polypeptide: evidence that UbiG catalyzes both *O*-methylation steps in ubiquinone biosynthesis, Biochemistry 35 (1996) 9797–9806.
[42] B. Cormack, Directed mutagenesis using the polymerase chain reaction, in: V.B. Chanda (Ed.), Current Protocols in Molecular Biology, vol. 1, John Wiley & Sons, 1997, pp. 8.5.1–8.5.10.
[43] B.S. Glick, L.A. Pon, Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*, Meth. Enzymol. 260 (1995) 213–223.
[44] B.S. Glick, A. Brandt, K. Cunningham, S. Muller, R.L. Hallberg, G. Schatz, Cytochromes c_1 and b_2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism, Cell 69 (1992) 809–822.
[45] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[46] T. Jonassen, C.F. Clarke, Isolation and functional expression of human *COQ3*, a gene encoding a methyltransferase required for ubiquinone biosynthesis, J. Biol. Chem. 275 (2000) 12381–12387.
[47] M. Jasinski, D. Sudre, G. Schansker, M. Schellenberg, S. Constant, E. Martinoia, L. Bovet, AtOSA1, a member of the Abc1-like family, as a new factor in cadmium and oxidative stress response, Plant Physiol. 147 (2008) 719–731.
[48] G.I. Belogrudov, P.T. Lee, T. Jonassen, A.Y. Hsu, P. Gin, C.F. Clarke, Yeast *COQ4* encodes a mitochondrial protein required for coenzyme Q synthesis, Arch. Biochem. Biophys. 392 (2001) 48–58.
[49] Y. Fujiki, A.L. Hubbard, S. Fowler, P.B. Lazarow, Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum, J. Cell Biol. 93 (1982) 97–102.
[50] M.P. Kamps, B.M. Sefton, Neither arginine nor histidine can carry out the function of lysine-295 in the ATP-binding site of p60src, Mol. Cell. Biol. 6 (1986) 751–757.
[51] S.K. Hanks, T. Hunter, Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification, FASEB J. 9 (1995) 576–596.
[52] P. Gin, C.F. Clarke, Genetic evidence for a multi-subunit complex in coenzyme Q biosynthesis in yeast and the role of the Coq1 hexaprenyl diphosphate synthase, J. Biol. Chem. 280 (2005) 2676–2681.
[53] U.C. Tran, B. Marbois, P. Gin, M. Gulmezian, T. Jonassen, C.F. Clarke, Complementation of *Saccharomyces cerevisiae coq7* mutants by mitochondrial targeting of the *Escherichia coli* UbiF polypeptide: two functions of yeast Coq7 polypeptide in coenzyme Q biosynthesis, J. Biol. Chem. 281 (2006) 16401–16409.
[54] A. Tzagoloff, C.L. Dieckmann, *PET* genes of *Saccharomyces cerevisiae*, Microbiol. Rev. 54 (1990) 211–225.
[55] A. Tzagoloff, A. Akai, R.B. Needleman, Assembly of the mitochondrial membrane system. Characterization of nuclear mutants of *Saccharomyces cerevisiae* with defects in mitochondrial ATPase and respiratory enzymes, J. Biol. Chem. 250 (1975) 8228–8235.
[56] A. Tzagoloff, A. Akai, R.B. Needleman, G. Zulch, Assembly of the mitochondrial membrane system. Cytoplasmic mutants of *Saccharomyces cerevisiae* with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase, J. Biol. Chem. 250 (1975) 8236–8242.
[57] Z. Vajo, L.M. King, T. Jonassen, D.J. Wilkin, N. Ho, A. Munnich, C.F. Clarke, C.A. Francomano, Conservation of the *Caenorhabditis elegans* timing gene *clk-1* from yeast to human: a gene required for ubiquinone biosynthesis with potential implications for aging, Mamm. Genome 10 (1999) 1000–1004.
[58] S.W. Baba, G.I. Belogrudov, J.C. Lee, P.T. Lee, J. Strahan, J.N. Shepherd, C.F. Clarke, Yeast *coq5 C*-methyltransferase is required for stability of other polypeptides involved in coenzyme Q biosynthesis, J. Biol. Chem. 279 (2004) 10052–10059.
[59] E.J. Hsieh, Characterization of the Coenzyme Q Biosynthetic Genes *ABC1/COQ8* and *COQ9* in Yeast *Saccharomyces cerevisiae*, Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, 2006, p. 126.
[60] S. Facchin, R. Lopreiato, S. Stocchetto, G. Arrigoni, L. Cesaro, O. Marin, G. Carignani, L.A. Pinna, Structure–function analysis of yeast piD261/Bud32, an atypical protein kinase essential for normal cell life, Biochem. J. 364 (2002) 457–463.
[61] M. Spitaler, A. Villunger, H. Grunicke, F. Uberall, Unique structural and functional properties of the ATP-binding domain of atypical protein kinase C-iota, J. Biol. Chem. 275 (2000) 33289–33296.
[62] S.S. Taylor, E. Radzio-Andzelm, T. Hunter, How do protein kinases discriminate between serine/threonine and tyrosine? Structural insights from the insulin receptor protein-tyrosine kinase, FASEB J. 9 (1995) 1255–1266.
[63] L.N. Johnson, E.D. Lowe, M.E. Noble, D.J. Owen, The Eleventh Datta Lecture. The structural basis for substrate recognition and control by protein kinases, FEBS Lett. 430 (1998) 1–11.
[64] S.K. Hanks, Genomic analysis of the eukaryotic protein kinase superfamily: a perspective, Genome Biol. 4 (2003) 111.
[65] S.K. Hanks, A.M. Quinn, T. Hunter, The protein kinase family: conserved features and deduced phylogeny of the catalytic domains, Science 241 (1988) 42–52.
[66] O. Delumeau, S. Dutta, M. Brigulla, G. Kuhnke, S.W. Hardwick, U. Volker, M.D. Yudkin, R.J. Lewis, Functional and structural characterization of RsbU, a stress signaling protein phosphatase 2C, J. Biol. Chem. 279 (2004) 40927–40937.
[67] A.C. Olivier, S. Lemaire, F. Van Bambeke, P.M. Tulkens, E. Oldfield, Role of rsbU and staphyloxanthin in phagocytosis and intracellular growth of *Staphylococcus aureus* in human macrophages and endothelial cells, J. Infect. Dis. 200 (2009) 1367–1370.
[68] J.H. Shin, M.S. Brody, C.W. Price, Physical and antibiotic stresses require activation of the RsbU phosphatase to induce the general stress response in *Listeria monocytogenes*, Microbiology 156 (2010) 2660–2669.
[69] A. Chi, C. Huttenhower, L.Y. Geer, J.J. Coon, J.E. Syka, D.L. Bai, J. Shabanowitz, D.J. Burke, O.G. Troyanskaya, D.F. Hunt, Analysis of phosphorylation sites on proteins from *Saccharomyces cerevisiae* by electron transfer dissociation (ETD) mass spectrometry, Proc. Natl Acad. Sci. USA 104 (2007) 2193–2198.
[70] C. Stark, T.C. Su, A. Breitkreutz, P. Lourenco, M. Dahabieh, B.J. Breitkreutz, M. Tyers, I. Sadowski, PhosphoGRID: a database of experimentally verified in vivo protein phosphorylation sites from the budding yeast *Saccharomyces cerevisiae*, Database (Oxford) 2010 (2010) bap026.
[71] M.A. Zampol, C. Busso, F. Gomes, J.R. Ferreira-Junior, A. Tzagoloff, M.H. Barros, Over-expression of *COQ10* in *Saccharomyces cerevisiae* inhibits mitochondrial respiration, Biochem. Biophys. Res. Commun. 402 (2010) 82–87.
[72] M. Iiizumi, H. Arakawa, T. Mori, A. Ando, Y. Nakamura, Isolation of a novel gene, *CABC1*, encoding a mitochondrial protein that is highly homologous to yeast activity of bc_1 complex, Cancer Res. 62 (2002) 1246–1250.
[73] M. Gerards, B. van den Bosch, C. Calis, K. Schoonderwoerd, K. van Engelen, M. Tijssen, R. de Coo, A. van der Kooi, H. Smeets, Nonsense mutations in CABC1/ADCK3 cause progressive cerebellar ataxia and atrophy, Mitochondrion 10 (2010) 510–515.
[74] C.M. Quinzii, M. Hirano, Coenzyme Q and mitochondrial disease, Dev. Disabil. Res. Rev. 16 (2010) 183–188.
[75] R. Saiki, A.L. Lunceford, Y. Shi, B. Marbois, R. King, J. Pachuski, M. Kawamukai, D.L. Gasser, C.F. Clarke, Coenzyme Q10 supplementation rescues renal disease in Pdss2kd/kd mice with mutations in prenyl diphosphate synthase subunit 2, Am. J. Physiol. Ren. Physiol. 295 (2008) F1535–F1544.
[76] B. Cardazzo, P. Hamel, W. Sakamoto, H. Wintz, G. Dujardin, Isolation of an *Arabidopsis thaliana* cDNA by complementation of a yeast abc1 deletion mutant deficient in complex III respiratory activity, Gene 221 (1998) 117–125.
[77] A.J. Ytterberg, J.B. Peltier, K.J. van Wijk, Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes, Plant Physiol. 140 (2006) 984–997.

[78] J.E. McEwen, C. Ko, B. Kloeckner-Gruissem, R.O. Poyton, Nuclear functions required for cytochrome *c* oxidase biogenesis in *Saccharomyces cerevisiae*. Characterization of mutants in 34 complementation groups, J. Biol. Chem. 261 (1986) 11872–11879.
[79] R.S. Sikorski, P. Hieter, A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*, Genetics 122 (1989) 19–27.
[80] T.W. Christianson, R.S. Sikorski, M. Dante, J.H. Shero, P. Hieter, Multifunctional yeast high-copy-number shuttle vectors, Gene 110 (1992) 119–122.
[81] A.Y. Hsu, W.W. Poon, J.A. Shepherd, D.C. Myles, C.F. Clarke, Complementation of coq3 mutant yeast by mitochondrial targeting of the *Escherichia coli* UbiG polypeptide: evidence that UbiG catalyzes both O-methylation steps in ubiquinone biosynthesis, Biochemistry 35 (1996) 9797–9806.
[82] T.Q. Do, A.Y. Hsu, T. Jonassen, P.T. Lee, C.F. Clarke, A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in *Saccharomyces cerevisiae abc1* mutants, J. Biol. Chem. 276 (2001) 18161–18168.
[83] W.J. Chen, M.G. Douglas, Phosphodiester bond cleavage outside mitochondria is required for the completion of protein import into the mitochondrial matrix, Cell 49 (1987) 651–658.
[84] G. Daum, P.C. Bohni, G. Schatz, Import of proteins into mitochondria. Cytochrome *b*2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria, J. Biol. Chem. 257 (1982) 13028–13033.
[85] A. Ohashi, J. Gibson, I. Gregor, G. Schatz, Import of proteins into mitochondria. The precursor of cytochrome c1 is processed in two steps, one of them heme-dependent, J. Biol. Chem. 257 (1982) 13042–13047.
[86] L.M. Thompson, L. McAlister-Henn, Dispensable presequence for cellular localization and function of mitochondrial malate dehydrogenase from *Saccharomyces cerevisiae*, J. Biol. Chem. 264 (1989) 12091–12096.
[87] D.S. Reading, R.L. Hallberg, A.M. Myers, Characterization of the yeast HSP60 gene coding for a mitochondrial assembly factor, Nature 337 (1989) 655–659.

Appendix II

Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast *coq* null mutants

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Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast *coq* null mutants ☆

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ABSTRACT

Coenzyme Q biosynthesis in yeast requires a multi-subunit Coq polypeptide complex. Deletion of any one of the *COQ* genes leads to respiratory deficiency and decreased levels of the Coq4, Coq6, Coq7, and Coq9 polypeptides, suggesting that their association in a high molecular mass complex is required for stability. Over-expression of the putative Coq8 kinase in certain *coq* null mutants restores steady-state levels of the sensitive Coq polypeptides and promotes the synthesis of late-stage Q-intermediates. Here we show that over-expression of Coq8 in yeast *coq* null mutants profoundly affects the association of several of the Coq polypeptides in high molecular mass complexes, as assayed by separation of digitonin extracts of mitochondria by two-dimensional blue-native/SDS PAGE. The Coq4 polypeptide persists at high molecular mass with over-expression of Coq8 in *coq3*, *coq5*, *coq6*, *coq7*, *coq9*, and *coq10* mutants, indicating that Coq4 is a central organizer of the Coq complex. Supplementation with exogenous Q_6 increased the steady-state levels of Coq4, Coq7, and Coq9, and several other mitochondrial polypeptides in select *coq* null mutants, and also promoted the formation of late-stage Q-intermediates. Q supplementation may stabilize this complex by interacting with one or more of the Coq polypeptides. The stabilizing effects of exogenously added Q_6 or over-expression of Coq8 depend on Coq1 and Coq2 production of a polyisoprenyl intermediate. Based on the observed interdependence of the Coq polypeptides, the effect of exogenous Q_6, and the requirement for an endogenously produced polyisoprenyl intermediate, we propose a new model for the Q-biosynthetic complex, termed the CoQ-synthome.

1. Introduction

Coenzyme Q (ubiquinone, CoQ or Q) is a lipid composed of a fully substituted benzoquinone ring and a polyisoprenyl chain, which contains six isoprene units in *Saccharomyces cerevisiae* (Q_6), eight in *Escherichia coli* (Q_8), and ten in humans (Q_{10}) [1]. Q is an electron carrier in the mitochondrial respiratory chain, and is essential in cellular energy metabolism [2]. The oxidized quinone (Q) accepts electrons from NADH via complex I, or succinate via complex II, and the reduced hydroquinone (QH_2) donates electrons to cytochrome *c* via complex III. Instead of complex I, *S. cerevisiae* rely on the much simpler NADH:Q oxidoreductases that oxidize NADH external to the mitochondria (Nde1 and Nde2), or inside the matrix (Ndi1) [3]. In mammalian mitochondria Q functions to integrate the respiratory chain with many aspects of metabolism by serving as an electron acceptor for glycerol-3-phosphate, dihydroorotate, choline, sarcosine, sulfide, and several amino acid and fatty acylCoA dehydrogenases [4,5]. QH_2 also functions as a crucial lipid-soluble antioxidant [6] and decreased levels of Q are associated with mitochondrial, cardiovascular, kidney and neurodegenerative diseases [7–11]. A better understanding of the enzymatic steps and organization of the polypeptides and cofactors

Abbreviations: 4-AP, 3-hexaprenyl-4-aminophenol; Coq1, the Coq1 polypeptide; *COQ1*, designates the wild-type gene encoding the Coq1 polypeptide; *coq1*, designates a mutated gene; $DDMQ_6$, the oxidized form of demethyl-demethoxy-Q_6H_2; DMQ_6, demethoxy-Q_6; DMQ_6H_2, demethoxy-Q_6H_2; 4-HB, 4-hydroxybenzoic acid; HAB, 3-hexaprenyl-4-aminobenzoic acid; HHAB, 3-hexaprenyl-4-amino-5-hydroxybenzoic acid; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; HMAB, 3-hexaprenyl-4-amino-5-methoxybenzoic acid; 4-HP, 3-hexaprenyl-4-hydroxyphenol; $IDMQ_6$, 4-imino-demethoxy-Q_6; MRM, multiple reaction monitoring; pABA, *para*-aminobenzoic acid; Q, ubiquinone or coenzyme Q; Q_6H_2, ubiquinol or coenzyme Q_6H_2; RP-HPLC-MS/MS, Reverse phase-high performance liquid chromatography–tandem mass spectrometry; START, steroidogenic acute regulatory protein-related lipid transfer

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required for Q biosynthesis will aid efforts to determine how the content of this important lipid can be regulated for optimal metabolism and health.

Q biosynthesis in *S. cerevisiae* requires at least eleven proteins, Coq1–Coq9, Arh1, and Yah1 (Fig. 1) [12–14]. Yeast mutants lacking any of the Coq1–Coq9 polypeptides are respiratory deficient due to the lack of Q. The Coq1 polypeptide synthesizes the hexaprenyl diphosphate tail, and Coq2 attaches the tail to either 4-hydroxybenzoic acid (4HB) or para-aminobenzoic acid (pABA); both are used as aromatic ring precursors in the biosynthesis of Q in yeast [13,15]. The other Coq polypeptides catalyze ring modification steps including *O*-methylation (Coq3), *C*-methylation (Coq5), or hydroxylation (Coq6 and Coq7). Coq6 requires ferredoxin (Yah1) and ferredoxin reductase (Arh1), which presumably serve as electron donors for the ring hydroxylation step [13,16]. Coq4, Coq8, and Coq9 polypeptides are essential for Q biosynthesis but their functional roles are not yet completely understood. In the Q-biosynthetic pathway proceeding from pABA, Coq9 is required for the replacement of the ring amino substituent with a hydroxyl group, although it remains uncertain exactly how this step is carried out [17].

Both genetic and physical evidence indicate that a multi-subunit Coq polypeptide complex is essential for Q biosynthesis [12,18–20]. Deletion of any one of the *COQ* genes in *S. cerevisiae* leads to destabilization of several other Coq polypeptides; the levels of Coq4, Coq6, Coq7, and Coq9 polypeptides are significantly decreased in each of the *coq1–coq9* null mutant yeast strains [20]. Although steady-state levels of the Coq3 polypeptide were also found to be decreased [20], Coq3 levels in mitochondria isolated from the *coq4–coq9* null mutants were shown to be preserved in subsequent studies performed in the presence of phosphatase and protease inhibitors [17,21]. As a result of the interdependence of the Coq polypeptides, *coq3–coq9* null mutant yeast accumulate only the early intermediates 3-hexaprenyl-4-hydroxybenzoic acid (HHB) and 3-hexaprenyl-4-aminobenzoic acid (HAB), produced by the prenylation of 4HB and pABA, respectively (Fig. 1) [17]. Whereas each of the *coq* null mutants lacks the designated Coq polypeptide [20], several *coq* mutants harboring certain amino acid substitution mutations show a less drastic block in Q biosynthesis as compared to *coq* null mutants. For example, certain *coq7* point mutants retain steady-state levels of the Coq7 polypeptide and accumulate demethoxy-Q_6 (DMQ_6), a late-stage Q-intermediate missing just one methoxy group [22,23]. Some of the Coq polypeptides physically interact — biotinylated Coq3 co-purifies with Coq4 [18], and Coq9 tagged with the hemagglutinin epitope co-purifies with Coq4, Coq5, Coq6, and Coq7 polypeptides [20]. These studies were performed with

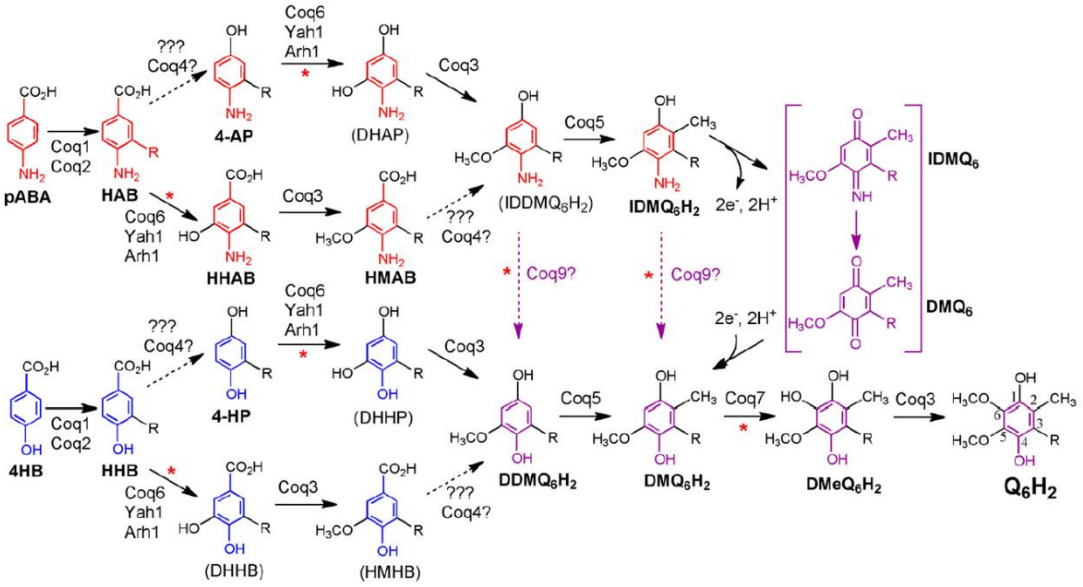

Fig. 1. Q_6 biosynthesis in *S. cerevisiae* proceeds from either 4HB or pABA. The classic Q biosynthetic pathway is shown in *blue* emanating from 4HB (4-hydroxybenzoic acid). *R* represents the hexaprenyl tail present in Q_6 and all intermediates. The numbering of the aromatic carbon atoms used throughout this study is shown on the reduced form of Q_6, Q_6H_2. Coq1 synthesizes the hexaprenyl-diphosphate tail, which is transferred by Coq2 to 4HB to form HHB (3-hexaprenyl-4-hydroxybenzoic acid). Alternatively, the *red* pathway indicates that pABA (*para*-aminobenzoic acid) is prenylated by Coq2 to form HAB (3-hexaprenyl-4-aminobenzoic acid). Both HHB and HAB are early Q_6-intermediates, readily detected in each of the *coq* null strains (*Δcoq3–Δcoq9*). Subsequent ring modification steps are thought to occur in the sequences shown, including hydroxylation by Coq6 in concert with ferredoxin (Yah1) and ferredoxin reductase (Arh1). Coq3 performs the two O-methylation steps, Coq5 the C-methylation step, and Coq7 performs the penultimate hydroxylation step. The functional roles of the Coq4, Coq8, and Coq9 polypeptides are elaborated in this study. Coq8p over-expression (*hcCOQ8*) in certain *Δcoq* strains leads to the accumulation of novel intermediates [17], suggesting these branched pathways. For example, in the presence of hcCOQ8, *coq6* or *coq9* mutants accumulate 4-AP (derived from pABA), and 4-HP (derived from 4HB) [17], indicating that in some cases decarboxylation and hydroxylation at position 1 of the ring may precede the Coq6 hydroxylation step. *Purple dotted arrows* designate the replacement of the C4-amine with a C4-hydroxyl and correspond to a proposed C4-deamination/deimination reaction, resulting in a convergence of the 4HB and pABA pathways. A putative mechanism to replace the C4-imino group with the C4-hydroxy group is shown in *purple brackets* for $IDMQ_6$ but could also occur on $IDDMQ_6$ (*not shown*). Several steps defective in the *Δcoq9* strain are designated with *red asterisks*. Intermediates previously detected are shown in bold: 4-AP (3-hexaprenyl-4-aminophenol); $DDMQ_6H_2$, the reduced form of demethyl-demethoxy-Q_6; DMQ_6, demethoxy-Q_6; DMQ_6H_2, demethoxy-Q_6H_2 (the reduced form of DMQ_6); HHAB, 3-hexaprenyl-5-hydroxy-4-aminobenzoic acid; HMAB, 3-hexaprenyl-5-methoxy-4-aminobenzoic acid; 4-HP (3-hexaprenyl-4-hydroxyphenol); $IDMQ_6$, (4-imino-demethoxy-Q_6); $IDMQ_6H_2$, 4-amino-demethoxy-Q_6H_2 (the reduced form of $IDMQ_6$). Intermediates shown in parentheses indicate that they have not yet been detected: DHAP, 5-hydroxy-4-aminohexaprenylphenol; DHHB, 3-hexaprenyl-4,5-dihydroxybenzoic acid; DHHP, 4,5-dihydroxy-hexaprenylphenol; HMHB, 3-hexaprenyl-4-hydroxy-5-methoxybenzoic acid; $IDDMQ_6H_2$, 4-amino-demethyl-demethoxyQ_6H_2.

digitonin-extracts of purified mitochondria. In such extracts the *O*-methyltransferase activity of Coq3 co-eluted with several of the other Coq polypeptides as high molecular mass complexes as determined by gel-filtration and by blue-native polyacrylamide gel electrophoresis (BN-PAGE) [18–21]. Indeed, the ability of Coq4 to organize high molecular mass complexes including Coq3 were shown to be essential for Q biosynthesis [19].

Several lines of evidence suggest that the Coq polypeptides and the multi-subunit Q-biosynthetic complex appear to be influenced by phosphorylation, either directly or indirectly due to Coq8. Coq8 (originally identified as Abc1) is a member of an ancient atypical kinase family [24]. Coq8/Abc1 homologs are required for Q biosynthesis in *E. coli* [25], yeast [26,27], and humans [28,29]. There is conservation of function as plant and human homologs of Coq8 are able to restore Q biosynthesis in yeast *coq8* mutants [30,31]. Conserved kinase motifs present in Coq8 are essential for maintenance of Q content [28,29,31], for the phosphorylation of Coq3, Coq5, and Coq7 polypeptides [21,31], and the association of Coq3 with a high molecular mass Coq polypeptide complex [21]. Collectively these studies suggest that maintenance or assembly of the Q-biosynthetic complex and phosphorylated forms of Coq3, Coq5, and Coq7 polypeptides depends on the presence of intact kinase motifs present in Coq8. However, it is important to note that kinase activity has not been demonstrated directly for yeast Coq8, or for the Coq8 homologs in prokaryotes, plants, or animals. Thus substrates of Coq8 have yet to be identified. In fact, there is evidence that phosphorylation may negatively regulate yeast Coq7 [32]. Moreover, recent work identified yeast Ptc7 as a mitochondrial phosphatase recognizing Coq7 and indicated that Ptc7 is required for optimal Q_6 content and function [33]. Thus although it appears that kinases and phosphatase activities modulate Q_6 biosynthesis and function in yeast, the role(s) played by Coq8 remain to be determined.

The content of Coq8 profoundly influences Q biosynthesis in *S. cerevisiae*. Over-expression of Coq8 was shown to restore synthesis of DMQ_6 in *coq7* null mutant yeast [17,34], suggesting the functional restoration of the Coq polypeptides up to this penultimate step of Q biosynthesis. In fact over-expression of Coq8 in the *coq3* and *coq5* null mutants restored steady-state levels of the Coq4, Coq6, Coq7, and Coq9 polypeptides [17,35]. Similarly, over-expression of Coq8 in the *coq3–coq9* null mutants restored steady-state levels of the unstable Coq polypeptides and resulted in the accumulation of late-stage Q-intermediates [17]. For example, over-expression of Coq8 in the *coq5* null mutant led to the synthesis of a late-stage Q intermediate diagnostic of the blocked *C*-methylation step (demethyl-demethoxy-Q_6, $DDMQ_6$) (Fig. 1) [17]. These results suggest a model whereby the over-expression of Coq8 stabilizes the remaining component Coq polypeptides, and allows the formation of high molecular mass Coq complexes.

A growing body of evidence indicates that Q or certain polyisoprenylated Q-intermediates also associate with the Q-biosynthetic complex. It was shown that DMQ_6 co-elutes with Coq3 *O*-methyltransferase activity and high molecular mass Coq polypeptide complexes during size exclusion chromatography of digitonin extracts of mitochondria [18]. Yeast *coq7* null mutants cultured in the presence of exogenous Q_6 were able to synthesize DMQ_6, and steady-state levels of Coq4 polypeptides were restored, indicating that the presence of Q_6 itself may stabilize the Coq polypeptide complexes [23,34]. Over-expression of Coq8 has no effect on either the *coq1* or *coq2* null mutants [17], which lack the ability to synthesize polyisoprenylated ring intermediates. This indicates that a polyisoprenylated component is essential for complex formation. Indeed, expression of diverse polyprenyl-diphosphate synthases, derived from prokaryotic species that do not synthesize Q, rescues Q synthesis in yeast *coq1* null mutants, and restores steady-state levels of the sensitive Coq polypeptides, including Coq4 and Coq6 [36]. Thus, exogenously supplied Q, or a polyisoprenylated Q-intermediate is postulated to interact with the complex and/or may stabilize certain of the Coq polypeptides.

Recent evidence suggests that the interaction between the Coq10 polypeptide and Q is essential for the function of Q in respiration and for efficient de novo synthesis of Q [37–39]. Respiration in mitochondria isolated from yeast *coq10* mutants can be rescued by the addition of Q_2, a soluble analog of Q_6. This is considered to be a hallmark phenotype of the yeast *coq* mutants unable to synthesize Q_6. However, unlike the *coq1–coq9* mutants, yeast *coq10* mutants retain the ability to synthesize Q_6, although its synthesis as measured with stable isotope-labeled ring precursors is less efficient [38]. The defects in Q respiratory function and de novo synthesis in the *coq10* mutant are rescued by human [37] or *Caulobacter crescentus* orthologs of Coq10 [38]. Structural determination of the *C. crescentus* Coq10 ortholog CC1736 identified a steroidogenic acute regulatory protein-related lipid transfer (START) domain [40]. The START domain forms a hydrophobic binding pocket and family members have been shown to bind sterols, phospholipids and other hydrophobic ligands. START domain proteins function as transporters and/or act as sensors of lipid ligands that regulate lipid metabolism and signaling [41,42]. The CC1736 START domain protein binds Q_{10}, Q_6, Q_3, Q_2 and DMQ_3, but not ergosterol or a farnesylated analog of HHB [38]. Thus, the Coq10 START polypeptide binds Q_n isoforms and facilitates both de novo Q biosynthesis and respiratory electron transport.

In this study we examine the sub-mitochondrial localization of the yeast Coq polypeptides, and determine the effects of over-expression of *COQ8* on the high molecular mass Coq polypeptide complexes in the *coq1–coq10* null mutants. The effects of Q supplementation on Coq polypeptide steady-state levels and the accumulation of Q-intermediates are also determined in each of the *coq* null mutants. The findings suggest that over-expression of Coq8 or Q_6 supplementation enhances the formation or maintenance of the Coq polypeptide complexes and are integrated into a new model of Q-biosynthesis.

2. Materials and methods

2.1. Yeast strains and plasmids

S. cerevisiae strains used in this study are listed in Table 1. Growth media for yeast were prepared as described [43], and included YPD (1% yeast extract, 2% peptone, 2% dextrose), YPGal (1% yeast extract, 2% peptone, 2% galactose, 0.1% dextrose) and YPEG (1% yeast extract,

Table 1
Genotype and source of yeast strains.

Strain	Genotype	Source
JM43	MAT α *leu2-3,112 ura3-52 trp1-289 his4-580*	[96]
W3031A	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
BY4741	MAT a *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
W303Δcoq1	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq1::LEU2*	[36]
W303Δcoq2	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq2::HIS3*	[97]
CC303	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq3::LEU2*	[98]
W303Δ*coq4*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq4::TRP1*	[99]
W303Δ*coq5*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq5::HIS3*	[45]
W303Δ*coq6*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq6::LEU2*	[49]
W303Δ*coq7*	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq7::LEU2*	[22]
W303Δcoq8	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq8::HIS3*	[99]
BY4741Δ*coq9*	MAT a *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 coq9::KanMX4*	Open Biosystems
W303Δ*coq10*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq10::HIS3*	[37]

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

2% peptone, 2% ethanol and 3% glycerol). Yeast were transformed with lithium acetate as described [44]. Transformed yeast strains were selected and maintained in SD-Ura (selective synthetic medium with 2% dextrose lacking uracil) [43], modified as described [45]. The p4HN4 plasmid used in this study (hcCOQ8) contains the *COQ8* gene in pRS426, a multi-copy yeast shuttle vector [46].

2.2. Mitochondrial isolation and immunoblot analyses with JM43 yeast

Yeast were cultured in YPGal medium (30 °C, 250 rpm) to an absorbance ($A_{600\ nm}$) of 2–4. Preparation of spheroplasts and fractionation of cell lysates were performed as described [47]. Crude mitochondria were isolated and further purified over a linear Nycodenz gradient as described previously [48]. Protein concentrations were determined with the bicinchoninic acid assay (Thermo). Indicated amounts of protein from the Nycodenz-purified mitochondrial fractions were analyzed by electrophoresis (SDS-PAGE) on 12% acrylamide, 2.5 M urea, Tris/glycine gels, then transferred to Hybond ECL Nitrocellulose (Amersham Biosciences). Subsequent immunoblot analyses and treatment of membranes for detection of antibodies were as described [49]. Primary antibodies to yeast mitochondrial polypeptides (Table 2) were used at the following concentrations: Coq1, 1:10,000; Coq2, 1:1000; Coq3, 1:1000; Coq4, 1:2000; Coq5, 1:5000; Coq6, 1:500; Coq7, 1:1000; affinity purified Coq8, 1:100; the beta subunit of F1-ATPase complex (Atp2), 1:4000; cytochrome b_2 ($Cytb_2$) 1:5000; cytochrome *c* (Cytc), 1:10,000; cytochrome c_1 ($Cytc_1$ 1:2000; and Heat shock protein 60 (Hsp60), 1:10,000. Goat anti-rabbit and goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (Calbiochem) were each used at a 1:10,000 dilution.

2.3. Sub-mitochondrial localization of Coq2 and Coq7 polypeptides

Mitochondria from JM43 yeast (3 mg protein, 150 μl) were suspended in five volumes of hypo-osmotic buffer (20 mM HEPES-KOH, pH 7.4), and incubated on ice for 30 min. The mixture was then centrifuged at 18,000 ×*g* for 20 min at 4 °C to separate the intermembrane space components (supernatant) from the mitoplasts (pellet), as described [50]. Mitoplasts were then sonicated (four 20-s-pulses on ice slurry, 20% duty cycle, 2.5 output setting; Sonifier W350, Branson Sonic Power Co.), then centrifuged at 100,000 ×*g* for 1 h at 4 °C to generate matrix (supernatant) and membrane (pellet) fractions. Alternatively, mitoplasts were subjected to alkaline carbonate extraction [51], and the mixture was then centrifuged at 100,000 ×*g* for 1 h at 4 °C to separate the integral membrane components (pellet) from the peripheral membrane and matrix components (supernatant). Equal aliquots of Nycodenz-purified mitochondria, untreated mitoplasts, pellet and supernatant fractions from either sonication or alkaline carbonate extraction, and intermembrane space components were subjected to SDS-PAGE analysis followed by immunoblot analyses.

Proteinase K treatment of mitochondria was performed as described [52] with some modifications. Proteinase K was added from a freshly made concentrated stock solution (10 mg/ml) to mitochondria suspended in buffer C (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4) (0.3 mg protein/ml) to a final concentration of 100 μg/ml. For treatment of mitoplasts, proteinase K was prepared in the hypo-osmotic buffer (20 mM HEPES-KOH, pH 7.4). When required, Triton-X100 or SDS were added to final concentration (w/v) of 1% or 0.5%, respectively, and incubated for 30 min at 4 °C. PMSF was added to inactivate the proteinase, followed by the addition of trichloroacetic acid (TCA; 60 °C) to a final concentration of 20%. The TCA pellets were subsequently collected by centrifugation and resuspended in Thorner buffer [53]; equal aliquots were processed for electrophoresis as described above.

Table 2
Description and source of antibodies.

Antibody	Source
Atp2	Carla. M. Koehler[a]
Coq1	[36]
Coq2	[20]
Coq3	[68]
Coq4	[67]
Coq5	[66]
Coq6	[49]
Coq7	[23]
Coq8	[20]
Coq9	[20]
Cytc	Carla M. Koehler[a]
$Cytb_2$	Carla M. Koehler[a]
$Cytc_1$	A. Tzagoloff[b]
Hsp60	Carla M. Koehler[a]
Mdh1	Lee McAlister-Henn[c]
Rip1	B. Trumpower[d]

[a] Dr. Carla. M. Koehler, Department of Chemistry and Biochemistry, UCLA.
[b] Dr. A. Tzagoloff, Department of Biological Sciences, Columbia University.
[c] Dr. Lee McAlister-Henn, Department of Molecular Biophysics and Biochemistry, University of Texas Health Sciences Center, San Antonio.
[d] Dr. B. Trumpower, Department Biochemistry, Dartmouth Medical School.

2.4. Salt-wash treatments of sonicated mitoplasts

Salt-wash treatments were performed as described previously [54] with some modifications. Equal volumes of sonication buffer (as a no salt control) or sonication buffer containing either KCl or NaCl were added to sonicated mitoplasts to final concentrations of 0.5 M or 1.0 M for KCl, and 0.5 M for NaCl. The samples were incubated on ice for 15 min, followed by centrifugation at 100,000 ×*g* for 1 h at 4 °C to separate the membrane associated components (pellet) from the soluble components (supernatant). Equal aliquots of starting mitochondria, unsonicated mitoplasts, intermembrane space components, membrane pellet, and supernatant fractions from salt-wash treatments of the sonicated mitoplasts were subjected to SDS-PAGE separation followed by immunoblot analyses.

2.5. Mitochondrial isolation and digitonin solubilization of W303 and BY4741 yeast strains

Yeast cultures were grown to an $A_{600\ nm}$ of 3–4 in YPGal media, and crude mitochondria were isolated from a total volume of 1.8 l of culture as described above. Crude mitochondria were further purified with an Optiprep discontinuous iodixanol gradient, and were collected from the interface of the gradient after ultracentrifugation. Briefly, the crude mitochondrial pellet was resuspended in 3 ml of Solution C. Solution C was prepared by adding 2 volumes of OptiPrep (60% w/v iodixanol; Sigma-Aldrich) to 1 volume of 0.8 M sorbitol, 60 mM HEPES-KOH, pH 7.4. Solutions of ρ (density) = 1.10 and 1.16 g/ml were prepared by mixing Solution C with Solution D (3 + 7 and 6.25 + 3.75, v/v respectively). Solution D contains 0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4. In centrifuge tubes, 3 ml of crude mitochondria suspended in Solution C was layered at the bottom of a 14 × 89 mm Ultra-Clear centrifuge tube (Beckman), followed by 4.5 ml of the ρ = 1.16 g/ml iodixanol solution, and finally 4.5 ml of the ρ = 1.10 g/ml iodixanol solution was layered on top. Tubes were subjected to centrifugation (80,000 ×*g* for 3 h, 4 °C). The band of mitochondria was collected and washed with 10 volumes of solution D. Purified mitochondria were harvested by centrifugation at 12,000 ×*g* for 10 min, 4 °C, and were resuspended in 1 ml of solution D, and stored at −80 °C. Aliquots of purified mitochondria (200 μg) were solubilized in 50 μl of 1.6% digitonin, 1× protease inhibitor EDTA-free (Roche), 1:100 phosphatase inhibitor cocktail sets I and II (Calbiochem), 1× NativePAGE sample buffer (Invitrogen), and mitochondria suspension buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4). Samples were incubated on ice for

1 h with mixing by pipetting up and down every 20 min. The soluble supernatant fraction was separated from the insoluble pellet by centrifugation in a Beckman Airfuge (100,000 ×g, 10 min, chilled rotor).

2.6. Rescue of coq mutants with exogenous Q_6

Medium containing a final concentration of 10 μM Q_6 was prepared with a 6.54 mM Q_6 stock in ethanol; vehicle control medium contained an equivalent volume of added ethanol (1.5 μl/ml). Both Q_6-supplemented ($+Q_6$) and unsupplemented ($-Q_6$) YPD were sterile filtered. Designated wild-type W3031B or *coq* null mutants were grown in 20 ml YPD overnight and diluted to 0.1 $A_{600\ nm}$ in 18 ml of ($+Q_6$) or ($-Q_6$) YPD. Yeast cells were grown at 30 °C for 42 h. Cells (30 $A_{600\ nm}$) were centrifuged for lipid extraction and 145 pmol Q_4 was added to each cell pellet as an internal standard prior to lipid extraction. Yeast pellets were washed twice with distilled water before lipid extraction. Lipid extracts were analyzed by RP-HPLC-MS/MS [17]. Data were processed with Analyst version 1.4.2 software (Applied Biosystems). Cells (10 $A_{600\ nm}$) were collected by centrifugation for protein extraction as described [55]. Aliquots (corresponding to 1.3 $A_{600\ nm}$) of yeast whole cell lysates were separated by SDS-PAGE on 10% acrylamide gels followed by immunoblot analyses as described below.

To determine de novo synthesis of $^{13}C_6$-DMQ$_6$ in the *coq7* null mutant strain in the presence or absence of exogenous Q_6, cells were diluted to 0.1 $A_{600\ nm}$ in 18 ml of Q_6-supplemented ($+Q_6$) or unsupplemented ($-Q_6$) YPD. Media also contained 10 μg/ml $^{13}C_6$-4HB. Incubations proceeded for 42 h, and cell pellets were processed by lipid extraction and RP-HPLC-MS/MS as described above.

2.7. Two-dimensional Blue Native/SDS-PAGE and immunoblot analyses

Protein concentrations of purified mitochondria were determined by the bicinchoninic acid assay (Thermo). 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 0.1%. BN-PAGE was performed as described in Native PAGE user manual with NativePAGE 4–16% Bis–Tris gel 1.0 mm × 10 wells (Invitrogen). First dimension gel slices were soaked in 65 °C 2× SDS sample buffer for 10 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, 0.1% Tween-20 (phosphate buffered saline is composed of 0.14 M NaCl, 1.2 mM NaH_2PO_4, and 8.1 mM Na_2HPO_4). Membranes were treated with the following primary antibodies (Table 2) at the dilution indicated: Coq4, 1:250; Coq7, 1:1000; Coq9, 1:1000; porin, 1:1000. Secondary antibodies were goat anti-rabbit IgG H&L chain specific peroxidase conjugate (Calbiochem).

3. Results

3.1. Sub-mitochondrial localization of Coq2 and Coq7 polypeptides

According to earlier studies [50,56], the yeast Coq2 and Coq7 proteins both reside in mitochondria. However, the sub-mitochondrial localization of these proteins (in their untagged forms) was not determined. To determine the sub-mitochondrial localizations of Coq2 and Coq7, yeast mitochondria were further fractionated as described in Materials and methods. Purified mitochondria were treated with hypotonic buffer, resulting in the disruption of the outer membrane and subsequent release of soluble components of the intermembrane space while keeping the inner membrane intact. Immunoblot analyses of the sub-mitochondrial fractions indicated that Coq2 and Coq7 polypeptides associated with the pellet (mitoplast fraction) and did not co-localize with cytochrome b_2 (Cytb2), the intermembrane space marker (Fig. 2A).

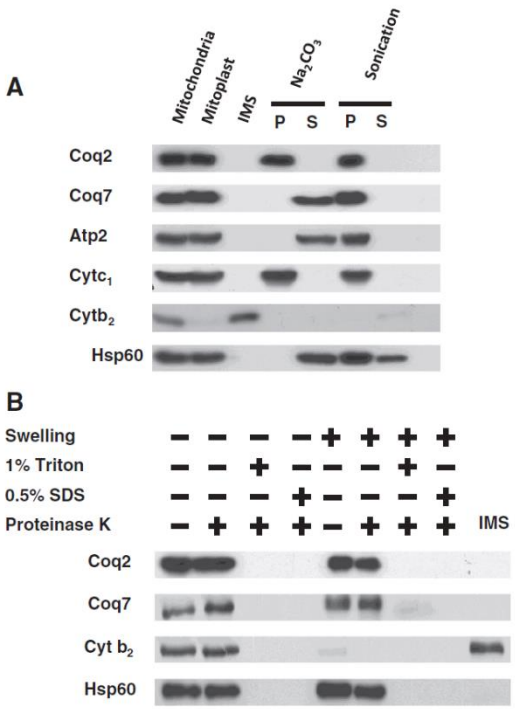

Fig. 2. Coq2 is an integral membrane protein while Coq7 is peripherally associated to the inner mitochondrial membrane, facing toward the matrix side. (*A*), Mitochondria were subjected to a hypotonic swelling and centrifugation to separate intermembrane space protein (*IMS*) and mitoplasts. The mitoplasts were treated with 0.1 M Na_2CO_3, pH 11.5, or sonicated, then separated by centrifugation (100,000 ×*g* for 1 h) into supernatant (*S*) or pellet (*P*) fractions. (*B*), Intact mitochondria or mitoplasts were treated with 100 μg/ml Proteinase K for 30 min on ice, with or without detergent. Equal aliquots of pellet and TCA-precipitated soluble fractions were analyzed. Mitochondrial control markers are: Atp2, peripheral inner membrane protein; $Cytb_2$, inter-membrane space protein; $Cytc_1$, integral inner membrane protein; and Hsp60, soluble matrix protein.

Mitoplasts were further fractionated either by sonication, releasing soluble matrix components into the supernatant following high speed centrifugation, or by extraction with alkaline carbonate, which releases peripherally bound membrane proteins into the supernatant [57]. Sonication treatment partially dissociated Hsp60, the matrix marker [58], however, neither Coq2 nor Coq7 was released from the membrane/pellet fraction (Fig. 2A). Coq7 was released into the supernatant by alkaline carbonate extraction in a manner similar to Atp2, a peripheral inner membrane protein [59], while Coq2 remained in the pellet, along with $Cytc_1$ an integral membrane marker [60]. These results indicated that Coq2 is an integral membrane protein while Coq7 behaves as a peripheral membrane protein.

To further characterize the membrane association of Coq2 and Coq7 proteins, purified mitochondria or mitoplasts were treated with Proteinase K in the absence and presence of detergent (1% Triton X-100 or 0.5% SDS). The results (Fig. 2B) showed that Coq2, Coq7, and Hsp60 polypeptides were protected from the protease both in intact mitochondria and in mitoplasts. As expected, detergent treatment of either mitochondria or mitoplasts rendered all proteins protease-sensitive. The results indicate that both Coq2 and Coq7 polypeptides are inner membrane proteins facing the matrix side in yeast mitochondria.

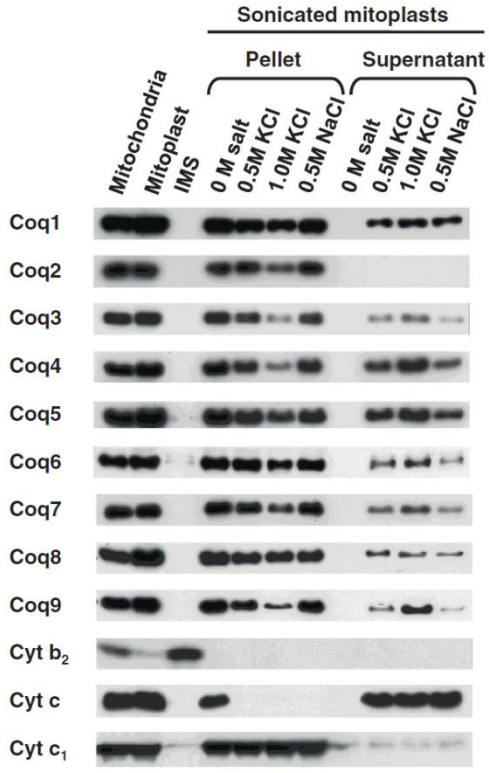

Fig. 3. Coq2 is resistant to salt extraction of sonicated mitoplasts while other Coq proteins are partially disassociated from the mitochondrial inner membrane. Purified mitochondria were subjected to hypotonic swelling to generate mitoplasts. Equal volumes of sonication buffer with or without salt were added to sonicated mitoplasts. Samples were incubated for 15 min on ice then separated by centrifugation (100,000 ×*g* for 1 h) into supernatant or pellet fractions. Equal aliquots of pellet and TCA-precipitated supernatant fractions were analyzed.

3.2. Coq2 is resistant to salt extraction while other Coq polypeptides are sensitive

The above sub-mitochondrial localization results indicated that Coq7 is a peripheral membrane protein. However, modeling studies have predicted Coq7 to be an interfacial inner mitochondrial membrane protein [61,62]. Interfacial membrane proteins, such as prostaglandin synthase [63] and squalene cyclase [64], are embedded in the membrane via interaction with only one leaflet of the bilayer. To distinguish between a peripheral and an interfacial membrane association for Coq7, salt extraction analyses (with 0.5 M KCl, 1.0 M KCl, or 0.5 M NaCl) were performed on sonicated mitoplasts prepared as described above. The resulting mixtures were subsequently separated into supernatant and pellet (membrane associated) fractions via high-speed centrifugation. Western blot analysis of the fractions (Fig. 3) showed that Coq7 and each of the Coq polypeptides, except for Coq2, were partially released from the membrane following the addition of salt. Interestingly, the degree of dissociation of these proteins depended on salt concentration and not on its identity per se, KCl versus NaCl. In contrast, Coq2 and the integral membrane marker, $Cytc_1$, were resistant to salt extraction and thus remained in the membrane fraction. Cytochrome *c*, which peripherally attaches to the inner mitochondrial membrane through electrostatic interactions with fatty acids and acidic phospholipids [65], was released from the sonicated mitoplasts following salt addition, as expected. These results provide further support for the sub-mitochondrial localization data indicating that yeast Coq7 is a peripheral membrane protein on the matrix side as are Coq1, Coq3, Coq4, Coq5, Coq6, Coq8, and Coq9 polypeptides [20,31,36,49,66–68].

3.3. The Coq4 and Coq9 polypeptides are sensitive indicators of the Coq polypeptide Q-biosynthetic complexes — and over-expression of COQ8 stabilizes these complexes in certain coq null mutants

The co-localization of the Coq polypeptides with the mitochondrial inner membrane is consistent with their interaction in Q-biosynthetic complexes. The yeast Coq4 and Coq9 polypeptides co-purify with other Coq polypeptides and both migrate at high molecular mass in separation of digitonin extracts of mitochondria [18–21]. Thus, we used the Coq4 and Coq9 polypeptides as sensitive indicators of the state of the high molecular mass Coq complexes. Mitochondria from wild type and *coq* null mutant yeast were purified, solubilized with digitonin, separated by two-dimensional blue native/SDS PAGE, and antibodies against Coq4 and Coq9 were used to detect their presence. In digitonin extracts of wild-type mitochondria, Coq4 and Coq9 polypeptides are detected in several high molecular mass complexes (from 669 to >880 kDa) (Fig. 4). The Coq4 and Coq9 polypeptides are also detected at lower molecular mass (66–440 kDa), perhaps indicating their presence in partial- or distinct sub-complexes. In contrast, Coq4 and Coq9 were not detected in digitonin extracts of mitochondria isolated from *coq3*, *coq4*, *coq5*, or *coq7* null mutant strains (Figs. 4 and 5). In each of the *coq3*–*coq9* null mutant strains, the lack of one of the Coq polypeptides is thought to destabilize the Coq polypeptide complex, and the mutants accumulate only the early Q-intermediates HHB and HAB, generated from the aromatic ring precursors 4HB and pABA, respectively (Figs. 1, 4 and 5). In contrast, the Coq4 and Coq9 polypeptides are detected at high molecular mass in the *coq6* null mutant, and the Coq4 polypeptide is detected in the *coq9* null mutant (Fig. 5). These observations are consistent with the presence of steady state levels of these polypeptides noted previously in these two null mutants [17]. Schematics showing possible interactions between the Coq polypeptides are depicted in Figs. 4 and 5.

The over-expression of Coq8, a putative kinase, has dramatic effects on the phenotypes of the *coq* null mutants. Over-expression of Coq8 restores steady state levels of several of the Coq polypeptides, and enables the synthesis of late-stage Q-intermediates in several of the *coq* null mutants [17]. To investigate whether over-expression of Coq8 stabilizes high molecular mass Coq polypeptide complexes, mitochondria were prepared from *coq* null mutant yeast over-expressing Coq8, and digitonin extracts were separated by two-dimensional blue native/SDS PAGE. We were particularly interested in examining the high molecular mass complexes in the *coq3* and *coq4* mutants, because over-expression of Coq8 stabilizes the Coq6, Coq7, and Coq9 polypeptides, yet the *coq3* and *coq4* mutants persist in accumulating early Q-intermediates. Over-expression of Coq8 in the *coq3* mutant restored Coq4 and Coq9 polypeptides to both high and low molecular mass complexes (440–880 kDa and 66 kDa) (Fig. 4), yet only early-stage intermediates HHB (with 4-HB as precursor) and HAB (with pABA as precursor) were detected in this strain [17]. Over-expression of Coq8 in the *coq4* null mutant restored the presence of the Coq9 polypeptide, although it was detected in only a low molecular mass complex (66 kDa) (Fig. 4); under these conditions, HHAB is detected (Fig. 1) [17], indicating the presence of functional Coq6. These results indicate that although over-expression of Coq8 stabilizes Coq6, Coq7 and Coq9 polypeptides in the *coq4* mutant, in the absence of the Coq4 polypeptide, a high molecular mass complex is not observed, and HHAB is the only novel Q-intermediate detected. Conversely, although the over-expression of Coq8 in the *coq3* mutant restores high molecular mass complexes of Coq4 and Coq9, this does not appear to result in production of new Q-intermediates.

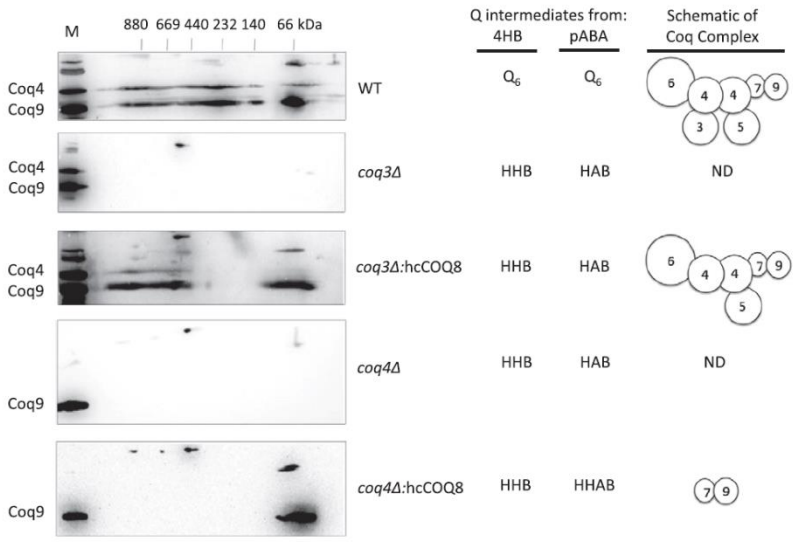

Fig. 4. Over-expression of Coq8 in the *coq3* null mutant, but not in the *coq4* null mutant, stabilizes the multi-subunit Coq polypeptide complex. Mitochondria were isolated from WT (W303-1A), *coq3* null or *coq4* null with and without the over-expression of Coq8 (hcCOQ8). Purified mitochondria (200 μg protein), were separated by two-dimensional blue native/SDS PAGE, and the immunoblots were probed with antibodies against Coq4 and Coq9. A sample of wild-type mitochondria separated just in the SDS-second dimension served as a positive control and is designated by *M*. Q or Q-intermediates derived from either 4HB or pABA that accumulated in the yeast strains were determined in the study by Xie et al. [17]. The *coq* mutants over-expressing Coq8 continue to produce HHB and HAB, but in addition the *coq4* mutant also accumulates HHAB. Schematics show interactions of the Coq polypeptides and illustrate interactions potentially favored by over-expression of Coq8; ND, Coq polypeptides not detected.

The effects of Coq8 over-expression on the native molecular mass of the Coq4 and Coq9 polypeptides were also studied in the *coq5*, *coq6*, *coq7*, and *coq9* null mutants. Over-expression of Coq8 restored the Coq4 and Coq9 polypeptides to several high and low molecular mass complexes in the *coq5* and *coq6* null mutants (Fig. 5). Over-expression of *COQ8* restored the Coq4 polypeptide to a high molecular mass complex and the Coq9 polypeptide to a low molecular mass complex in the *coq7* null yeast mutant (Fig. 5). In the *coq5*, *coq6* and *coq7* null mutants, over-expression of Coq8 enables synthesis of late-stage Q-intermediates: *coq5* null mutant accumulates $DDMQ_6$, *coq6* null accumulates 4-HP (with 4-HB as precursor) and 4-AP (with pABA as precursor), and *coq7* null accumulates DMQ_6 [17]. Coq4 steady-state levels decrease dramatically in the *coq9* null mutant, but a small amount of Coq4 is detected near 669 kDa. Over-expression of *COQ8* in the *coq9* null mutant has only mild effects on Coq4 steady-state levels [17], but Coq4 is present at a higher molecular weight (around 800 kDa) (Fig. 5). The *coq9* null mutant harboring multi-copy Coq8 accumulates 4-HP and DMQ_6 (with 4-HB as precursor) and 4-AP and $IDMQ_6$ (with pABA as precursor) [17]. These results indicate that over-expression of *COQ8* stabilizes Coq polypeptide complexes in several of the *coq* null mutants.

3.4. Over-expression of COQ8 enhances Coq4 and Coq9 levels in a coq10 null mutant

Previous work showed that steady-state levels of Coq4, Coq6, Coq7, and Coq9 polypeptides were decreased in the *coq10* null mutant [20]. Although the *coq10* null mutant produces Q_6, the rate of de novo Q_6 biosynthesis is decreased relative to that of wild-type yeast [38]. Moreover, the respiratory defect and Q_6 de novo biosynthesis in the *coq10* mutant is rescued by over-expression of *COQ8* [37,38]. Over-expression of *COQ8* enhances steady-state levels of Coq4 and Coq9 in the *coq10* null mutant (Fig. 6A). While both Coq4 and Coq9 are detected in high molecular mass complexes in the *coq10* null mutant, over-expression of *COQ8* appears to increase the association of Coq4 with the complex (Fig. 6B).

3.5. Q_6 supplementation changes steady-state levels of certain Coq polypeptides and promotes accumulation of late-stage Q-intermediates in certain coq null mutants

Exogenous Q_6 has been shown to rescue the growth of the *S. cerevisiae coq2*, *coq3*, *coq5*, *coq7*, *coq8*, *coq9*, and *coq10* null mutants on media containing non-fermentable carbon sources [12,26,37,50,69]. We were able to rescue the growth of each of the *coq1–coq9* null mutants on YPEG medium containing ethanol and glycerol as non-fermentable carbon sources, with the addition of 2 μM Q_6 to the medium (data not shown). To determine the effect of exogenous Q_6 on the Coq polypeptide levels, each of the *coq1–coq9* null mutants was cultured in YPD in the presence or absence of exogenous Q_6. For these experiments YPD medium was chosen because growth of the *coq* null mutants in the absence of Q_6 is supported by dextrose. Previous studies indicate that both plasma membrane and mitochondrial Q_6 content in *coq7* null mutant (W303 genetic background) were increased when cultured in YPD supplemented with 2 μM Q_6 [70]. Succinate-cytochrome *c* reductase activity also increased under these conditions, indicating exogenous Q_6 restored activity in the mitochondrial respiratory chain [70]. The YPD medium was supplemented with 10 μM Q_6, because this concentration is near optimal for restoration of growth [69]. Wild-type yeast and each of the *coq* null mutants in YPD were cultured in either the presence or absence of 10 μM Q_6. The addition of Q_6 does not appear to affect mitochondrial protein levels in wild-type yeast (Fig. 7). However, Q_6 supplementation increases Coq9 polypeptide steady-state levels in *coq3*, *coq4*, *coq6* and *coq7* null mutants and increases Coq4 in *coq3*, *coq6* and *coq7* null mutants (Fig. 7). The most significant increases in Coq4, Coq7 and Coq9 polypeptide levels were observed in the *coq6* null mutant supplemented with Q_6 (Fig. 7). In contrast, Q_6 supplementation decreases steady-state levels of Coq1 in *coq2–coq9* null mutants. To determine whether

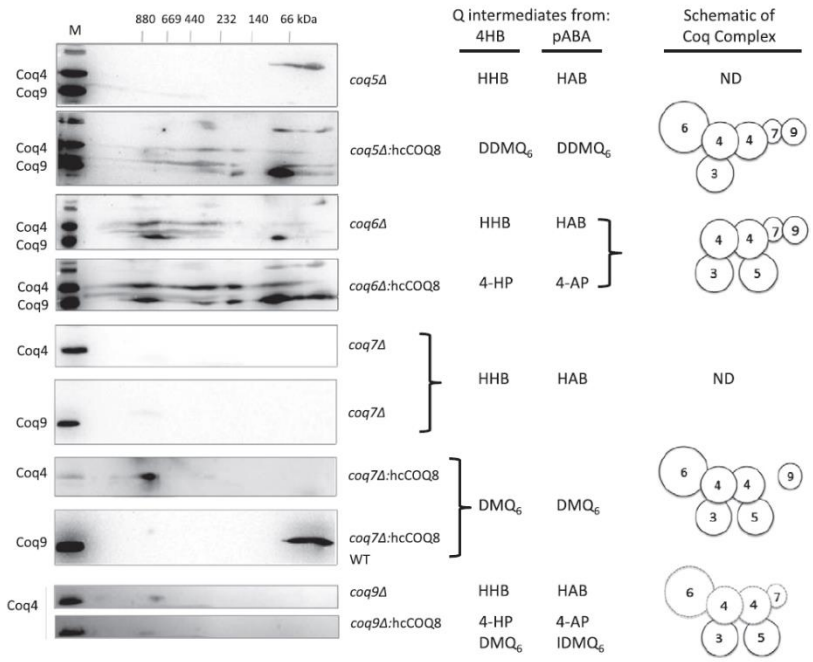

Fig. 5. Over-expression of Coq8 in *coq5*, *coq6*, *coq7* or *coq9* null mutant strains stabilizes the multi-subunit Coq polypeptide complex. Mitochondria were isolated from yeast strains harboring a deletion in one of the *coq5*, *coq6*, *coq7*, or *coq9* genes with and without the over-expression of Coq8 (hcCOQ8). Purified mitochondria (200 μg protein) were separated by two-dimensional blue native/SDS PAGE, and the immunoblots were probed with antibodies against Coq4 and Coq9. A sample of wild-type mitochondria separated only in the SDS-second dimension served as a positive control and is designated by *M*. Q-intermediates derived from either 4HB or pABA that accumulated in the yeast mutants were determined in the study by Xie et al. [17]. The *coq* mutants over-expressing Coq8 continue to produce HHB and HAB, but in addition the designated late-stage Q-intermediates are also observed. Schematics show interactions of the Coq polypeptides and illustrate interactions potentially favored by over-expression of Coq8; *dotted lines* indicate that steady state-Coq polypeptides are present but are decreased relative to wild type; ND, Coq polypeptides not detected.

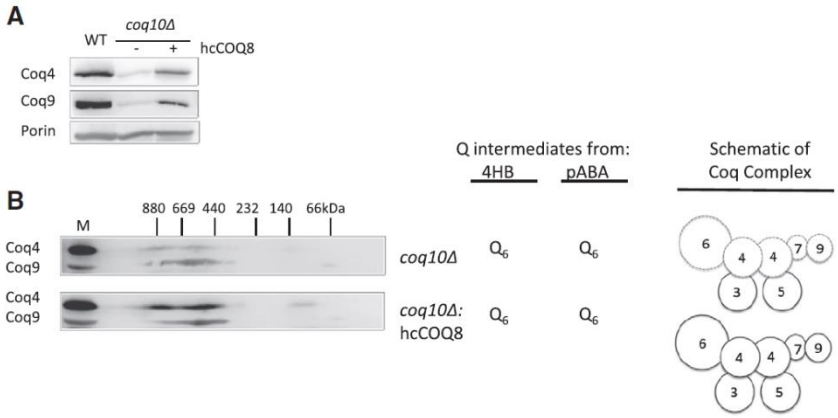

Fig. 6. Over-expression of Coq8 in *coq10* null mutant strain stabilizes the Coq4 and Coq9 polypeptide levels. Mitochondria were purified from *coq10* null mutant yeast strain with and without the over-expression of Coq8 (hcCOQ8). (*A*), Purified mitochondria (20 μg protein) were subject to SDS-PAGE and Western blot probing with antibodies against Coq4, Coq9, and Porin. (*B*), Purified mitochondria (200 μg protein), were subjected to two-dimensional Blue Native/SDS PAGE, and immunoblots were probed with antibodies against Coq4 and Coq9. A sample of wild-type mitochondria separated only in the SDS-second dimension served as a positive control and is designated by *M*. The *coq10* mutant produces Q_6 from 4HB and pABA and retains high molecular mass complexes of the Coq polypeptides as indicated by the schematic of the Coq complex; *dotted lines* indicate that steady state-Coq polypeptides are present but are decreased relative to wild type.

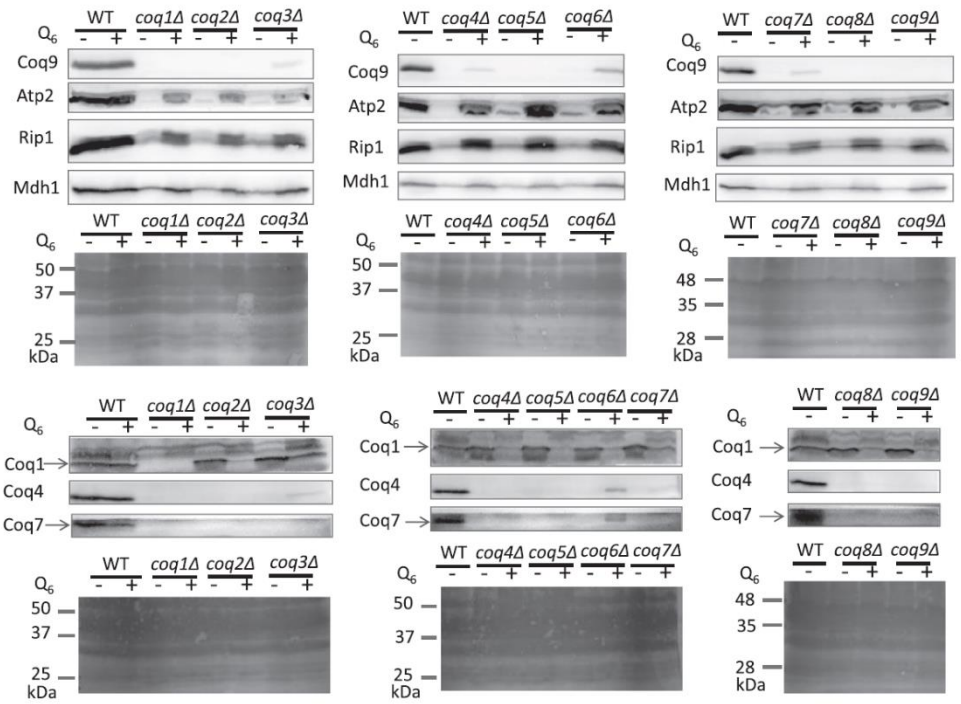

Fig. 7. Inclusion of exogenous Q_6 during culture of *coq1–coq9* null yeast mutants stabilizes certain Coq and mitochondrial polypeptides. Wild type or *coq1–coq9* null mutant yeast were grown in 18 ml of YPD with either 1.5 μl ethanol/ml medium (no Q_6 addition) or the same volume of Q_6 dissolved in ethanol giving a final concentration of 10 μM Q_6 (+Q_6) for 42 h. Yeast cells (10 $A_{600\,nm}$) were collected as pellets. Protein extracts were prepared from the pellets and analyzed by SDS-PAGE and immunoblot. Immunoblots were performed with antibodies against Coq1, Coq4, Coq7, Coq9, Atp2, malate dehydrogenase (Mdh1), or Rieske iron–sulfur protein (Rip1). Ponceau staining was used to detect the total proteins transferred to the membrane and served as the loading control.

supplementation with Q_6 affects other mitochondrial proteins, we investigated the steady-state levels of the beta subunit of the F1 sector of mitochondrial F_1F_0 ATP synthase (Atp2), malate dehydrogenase (Mdh1), and the Rieske iron–sulfur protein (Rip1) of the cytochrome bc_1 complex. The addition of Q_6 increases steady-state levels of Atp2, Mdh1 and Rip1 in each of the *coq* null mutants, suggesting that supplementation with Q_6 may have general protective effects on mitochondria.

The effect of Q_6 supplementation on Q intermediates was assessed in each of the *coq* null mutants. The *coq3–coq9* null mutants accumulate only early stage intermediates HHB and HAB (Fig. 1). However, DMQ_6 is produced in *coq7* null mutants cultured in the presence of exogenous Q_6 [34]. Here, we used HPLC with tandem mass spectrometry to detect Q_6 intermediates in lipid extracts of *coq* null mutants cultured in either the presence or absence of 10 μM exogenous Q_6. We confirmed the accumulation of DMQ_6 in the *coq7* null mutant cultured in exogenous Q_6 (Fig. 8). Since exogenous Q_6 contains a small amount of DMQ_6, $^{13}C_6$-4HB was used to detect de novo synthesis of $^{13}C_6$-DMQ_6. A very small amount of $^{13}C_6$-DMQ_6 was detected in *coq7* null mutant labeled with $^{13}C_6$-4HB. (We note that DMQ_6 is detectable in *coq7* null mutant when lipid extracts are prepared from 30 $A_{600\,nm}$ or more yeast and attribute this to the high sensitivity LC-MS/MS system.) In the presence of 10 μM exogenous Q_6, $^{13}C_6$-DMQ_6 accumulation increased significantly in *coq7* null mutant labeled with $^{13}C_6$-4HB. $^{13}C_6$-DMQ_6 was identified by its retention time of 4.56 min (the same as DMQ_6), and a precursor-to-product ion transition of 567.0/173.0, consistent with the presence of the $^{13}C_6$-ring (Fig. 8).

In addition, exogenous Q_6 led to an increased accumulation of 3-hexaprenyl-4-amino-5-hydroxybenzoic acid (HHAB) in *coq4* (Fig. 9A). This intermediate has a retention time of 2.69 min and a precursor-to-product ion transition of 562.0/166.0 detected with multiple reaction monitoring (MRM). We have previously detected HHAB in lipid extracts of *coq4-1* mutants, harboring a point mutation [17]. Surprisingly, smaller but readily detectable amounts of HHAB (a product of the Coq6 step) were also detected in the *coq6* mutant cultured with exogenous Q_6 (Fig. 9B). In addition to HHAB, 4-AP increased significantly in the *coq6* null mutant cultured with exogenous Q_6 (Fig. 10A). 4-AP was identified by its retention time (2.88 min), precursor-to-product ion transition (518.5/162.2), and fragmentation spectrum (Fig. 10B). 4-AP has been shown to accumulate in certain *coq6* point mutants [16], and in *coq6* and *coq9* null mutants over-expressing Coq8 [17]. The addition of Q_6 caused the accumulation of imino-demethoxy-Q_6 ($IDMQ_6$) in the *coq9* null mutant (Fig. 11A). This intermediate has a retention time of 4.9 min and a precursor-to-product ion transition of 560.5/166.1. Its identity is further confirmed by the fragmentation spectrum (Fig. 11B). In contrast, late-stage Q-intermediates were not detected in the other *coq* null mutants. Thus only *coq4*, *coq6*, *coq7* and *coq9* null mutants accumulate late-stage Q-intermediates upon the addition of Q_6. These data indicate that Q_6 stabilizes the Q-biosynthetic complex and allows later Q-intermediates to accumulate.

4. Discussion

This study examined the location and organization of the yeast mitochondrial Q-biosynthetic complex. We found that over-expression of Coq8, an ancient atypical putative kinase, stabilizes the high molecular mass Coq polypeptide complex in several of the *coq* null mutants.

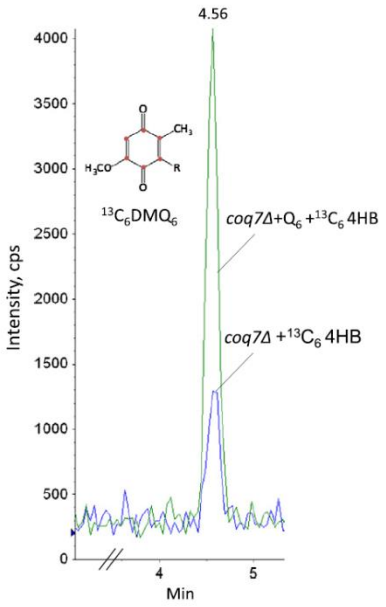

Fig. 8. Exogenous Q_6 increases synthesis of demethoxy-Q_6 (DMQ_6) in *coq7* null mutant. Yeast *coq7* null mutant was cultured in YPD with either 10 μg/ml $^{13}C_6$-4HB and 1.5 μl ethanol/ml medium (no Q_6 addition) or 10 μg/ml $^{13}C_6$-4HB and the same volume of Q_6 dissolved in ethanol giving a final concentration of 10 μM Q_6 (+Q_6) for 42 h. Yeast cells (30 $A_{600\ nm}$) were collected as pellets and washed twice with distilled water. Q_4 (145.4 pmol) was added as internal standard. Lipid extracts were prepared from the pellets and analyzed by RP-HPLC-MS/MS. Multiple reaction monitoring (MRM) detected precursor-to-product ion transitions 567.0/173.0 ($^{13}C_6$-DMQ_6). The *green trace* designates the $^{13}C_6$-DMQ_6 signal in the + Q_6 condition, and the *blue trace* indicates the $^{13}C_6$-DMQ_6 signal in the absence of added Q_6. The peak areas of $^{13}C_6$-DMQ_6 normalized by peak areas of Q_4 are 0.0665 in *coq7Δ* +$^{13}C_6$-4HB and 0.215 in *coq7Δ* + Q_6 +$^{13}C_6$-4HB.

Supplementation of growth medium with exogenous Q_6 restored steady-state levels of Coq polypeptides and enhanced the production of late-stage Q-intermediates in certain *coq* null mutants. Based on the observed interdependence of the Coq polypeptides, the effect of exogenous Q_6, and the requirement for an endogenously produced polyisoprenyl intermediate (summarized in Table 3), we propose a new model for the CoQ-synthome, a Coq multi-subunit polypeptide and lipid complex required for the biosynthesis of Q in yeast (Fig. 12).

The Coq4 and Coq9 polypeptides were used as sensitive indicator polypeptides to monitor the state of high molecular mass Coq polypeptide complexes in digitonin extracts of mitochondria, as assayed by two-dimensional blue native/SDS PAGE. The over-expression of Coq8 in the *coq* null mutants was found to profoundly affect the association of Coq4 and Coq9 in high molecular mass complexes. The Coq4 polypeptide persists at high molecular mass with the over-expression of Coq8 in the *coq3*, *coq5*, *coq6*, *coq7*, and *coq9* null mutants (Figs. 4 and 5). This finding indicates that deletion of any of these Coq polypeptide components has little impact on the association of Coq4 with a high molecular mass Coq complex. The Coq9 polypeptide persists at high molecular mass with the over-expression of Coq8 in the *coq3*, *coq5* and *coq6* null mutants, but is present only at low molecular mass in the *coq4* and *coq7* null mutants upon Coq8 over-expression (Fig. 5). Hence, we propose that Coq4 may be a crucial component through which Coq3, Coq5, Coq6, Coq7, and Coq9 associate to form the CoQ-synthome. Coq7 is an important component through which Coq9 associates with Coq4.

Based on these findings our model depicts Coq4 as a central organizer, and the Coq3, Coq5 and Coq6 polypeptides as more peripheral members of the CoQ-synthome (Fig. 12). In this model Coq4 is depicted as a homodimer, with each monomer harboring a binding site for the polyisoprenyl-tail of Q_6 or a polyisoprenyl-intermediate. This is based on the structure determined for Alr8543, a Coq4 homolog from *Nostoc* sp. *PCC7120*, and the molecular modeling of the highly similar *S. cerevisiae* Coq4 [71]. Each monomer of the Alr8543 homodimer co-crystalized with a geranylgeranyl monophosphate, and Rea et al. [71] proposed that yeast Coq4 may similarly bind to the polyisoprenyl tail of HHB (or HAB), consistent with the idea that Coq4 forms a scaffold organizing the Coq polypeptide complex [19], facilitating the action of the Coq6 hydroxylase, the Coq3 and Coq5 methyltransferases, and the Coq7 hydroxylase. The model is also consistent with the hypothetical branched biosynthetic scheme of Q biosynthesis (Fig. 1). For example, in the presence of hcCOQ8, *coq6* or *coq9* mutants accumulate 4-AP (derived from pABA), and 4-HP (derived from 4HB) [17], indicating that in some cases decarboxylation and hydroxylation at position 1 of the ring (catalyzed by yet to be identified enzymes) might precede the Coq6 hydroxylation step.

The CoQ-synthome represents a minimal schematic model because the total predicted mass based on the sum of the component Coq polypeptides is only 230–240 kDa [31] (Fig. 12); this is well below the 1 MDa size of the complex estimated from blue-native gels. The stoichiometry of the Coq polypeptides in the complex is not known and it is likely that additional components remain to be identified. The model is consistent with the peripheral association of each of the Coq polypeptides to the matrix side of the mitochondrial inner membrane, with the exception of Coq2 (Figs. 2 and 3). In addition to interaction with Coq4, it is possible that the association of Coq polypeptides with the inner mitochondrial membrane may derive from interactions with Q_6 and/or a polyisoprenyl-intermediate. So far, Coq2 is the only integral membrane protein of the Q-biosynthetic proteins. Previous models suggested that Coq2 might serve as an ideal anchor-protein candidate for the Coq complex [12], and blue native/SDS PAGE indicated Coq2 migrated at high molecular mass [21]. However, co-precipitation experiments have so far failed to identify any physical interactions between Coq2 and the other Coq polypeptides (data not shown). Based on this, Fig. 12 shows Coq1 and Coq2 independently generate HHB or HAB, early Q-intermediates that accumulate in each of the *coq3–coq9* null mutants.

Studies in *S. cerevisiae* and *Schizosaccharomyces pombe* have set the stage for understanding Q biosynthesis in animals; many human and mouse COQ homologs have been shown to rescue the corresponding yeast *coq* mutants [14,72]. Expression of human COQ4 has been shown to rescue the *S. cerevisiae coq4* null mutant [73], suggesting that human COQ4 might maintain interactions with yeast Coq polypeptides. However, certain animal Q biosynthetic proteins require specific partner proteins to observe cross complementation of the yeast mutant. For example, Pdss1 and Pdss2 (Coq1 homologs) from *S. pombe*, mouse, and human form heterotetrameric complexes, and must be co-expressed to reconstitute synthesis of the polyisoprene-diphosphate tail [74,75]. Human COQ9 has not yet demonstrated interspecific complementation of the yeast *coq9* mutants [76]; this might be due to interactions of Coq9 with Coq7. Similar to yeast, the function of Coq7 in mouse requires Coq9. A homozygous $Coq9^{X/X}$ mouse, containing a Coq9-R239X stop codon mutation, displayed a severe reduction of Q_9 content, accumulated DMQ_9, and showed a profound decrease in steady state Coq7 polypeptide levels [77]. The $Coq9^{X/X}$ mouse model was patterned after human patients with Q deficiency and mitochondrial encephalomyopathy [76]. The recapitulation of the human disease in the $Coq9^{X/X}$ mouse model suggests that COQ7 hydroxylation of DMQ requires COQ9 in mice and humans. Other interactions between human COQ polypeptides have been reported recently. ADCK4 (a human homolog of yeast Coq8) was shown to interact with COQ6 and COQ7 polypeptides in podocyte cell cultures [78]. Although we have not detected Coq8 in direct association with any of the yeast Coq polypeptides, it is tempting to speculate that human ADCK4 may

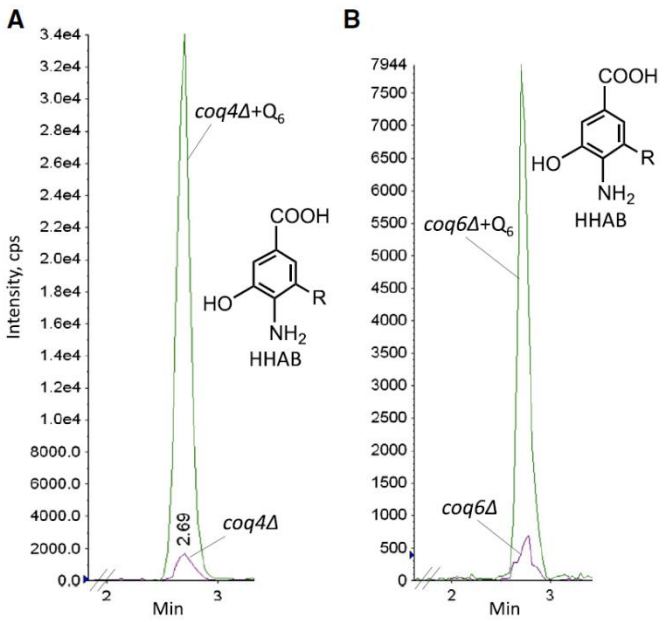

Fig. 9. Exogenous Q_6 increases the accumulation of 3-hexaprenyl-4-amino-5-hydroxybenzoic acid (HHAB) in *coq4* and *coq6* null mutants. Yeast *coq4* and *coq6* null mutants were cultured in YPD with either 1.5 μl ethanol/ml medium (no Q_6 addition) or the same volume of Q_6 dissolved in ethanol giving a final concentration of 10 μM Q_6 (+Q_6) for 42 h. Yeast cells (30 $A_{600\ nm}$) were collected as pellets and washed twice with distilled water. Q_4 (145.4 pmol) was added as internal standard. Lipid extracts were prepared from the pellets and analyzed by RP-HPLC-MS/MS. Multiple reaction monitoring (MRM) detected precursor-to-product ion transitions 562.0/166.0 (HHAB) and 455.4/197.0 (Q_4). The arbitrary units (cps) and the scale is the same for all the traces within the same panel. In panels *A* and *B*, green traces designate the HHAB signal in the + Q_6 condition, and purple traces the HHAB signal in the absence of added Q_6. The peak areas of HHAB normalized by peak areas of Q_4 are 0.01 in *coq4Δ* (A), 0.14 in *coq4Δ* + Q_6 (A), 0.003 in *coq6Δ* (B), and 0.03 in *coq6Δ* + Q_6 (B). The retention times of HHAB are 2.69 min in *coq4Δ* + Q_6 (A), and 2.71 min in *coq6Δ* + Q_6 (B).

recognize COQ6 and COQ7 as potential substrates for phosphorylation. Interestingly, while expression of ADCK4 failed to rescue the yeast *coq8* mutant [78], expression of human ADCK3 did rescue the *coq8* mutant, partially restore Q_6 content as well as phosphorylated forms of yeast Coq3, Coq5, and Coq7 [31].

We investigated the effects of Coq8 over-expression on the Coq4 and Coq9 polypeptides in the *coq10* null mutant. Over-expression of Coq8 in the *coq10* null mutant increases steady-state levels of the Coq4 and Coq9 polypeptides and their association with the high molecular mass Coq complexes (Fig. 6). In the *coq10* null mutant the rate of Q biosynthesis is reduced but may be significantly increased by Coq8 over-expression, or by the expression of a START domain ortholog of Coq10 [38]. These findings are consistent with the model that the Coq10:Q polypeptide ligand complex functions as a chaperone of Q and that Q delivery to the CoQ-synthome is necessary for efficient de novo Q biosynthesis (Fig. 12), and/or for delivery of Q to the N-site of the bc_1 complex [38,79]. It is tempting to speculate that Coq10 may function to chaperone the "inactive" pool of Q (depicted as residing at the midplane of the bilayer [80,81]) to form an "active" pool of Q, consistent with a dedicated subset of Q molecules performing electron transport within the respirasomes [82,83].

Results presented here show that exogenous Q_6 restores the growth of any of the *coq1–coq10* null mutant yeast in medium containing a nonfermentable carbon source. This effect of supplementation with exogenous Q_6 is known to require uptake; Q_6 binds to soluble proteins derived from peptone in the growth medium and is taken up by cells and transported to mitochondria via an endocytic pathway [84]. James et al. [85] identified 16 yeast *ORFs* required for utilization of exogenous Q_4 in a yeast double knockout library (*ΔORFΔcoq2*). We determined the steady-state levels of the Coq4, Coq7, and Coq9 polypeptides as indicators of the CoQ-synthome, and scanned for Q_6-intermediates by HPLC tandem mass spectrometry. Upon the addition of Q_6, the *coq3*, *coq4*, *coq6*, *coq7* and *coq9* null mutants accumulate distinct hexaprenyl Q-intermediates and/or show increased steady-state levels of one or more of the indicator Coq polypeptides (Figs. 8–11 and Table 3). These findings confirm and extend previous studies showing that addition of exogenous Q_6 restored de novo synthesis of DMQ_6 and increased steady-state levels of the Coq4 polypeptide in a *coq7* null mutant [23,34]. These results indicate that Q_6 itself may interact with certain Coq polypeptides and enhance formation of later Q-intermediates. Surprisingly, HHAB (a product of the Coq6 step) was detected in the *coq6* mutant cultured with exogenous Q_6 (Fig. 9B). It is possible that the presence of Q_6 facilitates the function of another hydroxylase; such a scenario has been reported for hydroxylases in *E. coli* Q_8 biosynthesis [86]. However, HHAB as identified in Fig. 9, may actually have the hydroxyl substituent located in another position on the ring. Determination of this will require purification of the intermediate and structural characterization.

In contrast, addition of exogenous Q_6 had no discernable effect on the Coq4, Coq7, or Coq9 indicator polypeptides or late-stage Q-intermediates in the *coq1*, *coq2*, *coq5* or *coq8* null mutants (Fig. 7 and Table 3). The steady-state levels of Coq1, Coq2, and Coq5 polypeptides are not affected by deletions in any of the other *COQ* genes [20]. While Coq8 over-expression in the *coq1* or *coq2* null mutants has little effect, Coq8 over-expression in the *coq5* null mutant allows production of $DDMQ_6$, and enhances steady-state levels of the Coq4, Coq6, Coq9 and Coq7 polypeptides [17]. Because Coq5 physically interacts with the other core-Coq polypeptides, yet the stabilizing effect of exogenous Q_6 on the other Coq polypeptides is not observed in the *coq5* null mutant, the Coq5 polypeptide is required for the interaction of Q_6 with CoQ-

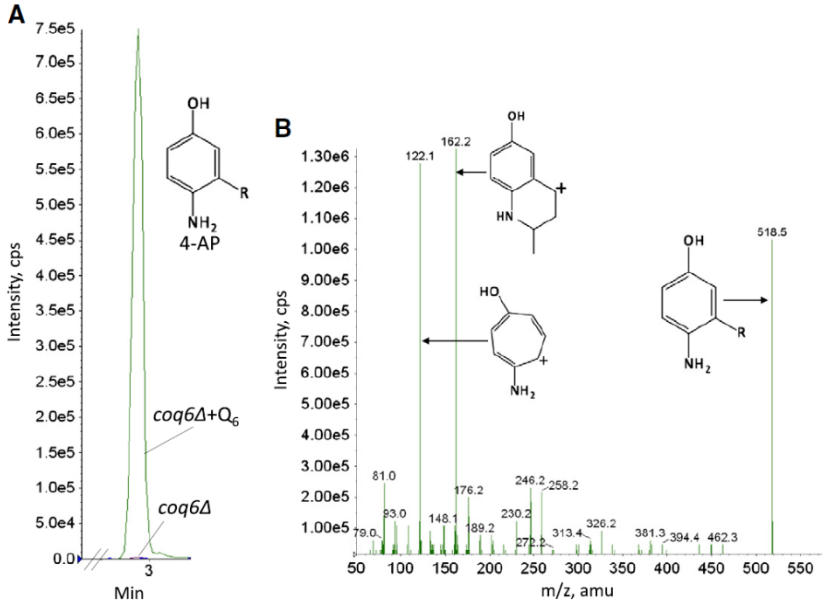

Fig. 10. Exogenous Q_6 increases the accumulation of 3-hexaprenyl-4-aminophenol (4-AP) in the *coq6* null mutant. Lipid extracts were prepared from the cell pellets of *coq6* null mutant yeast following growth in YPD with either the presence ($+Q_6$) or absence of Q_6 and analyzed by RP-HPLC-MS/MS as described in Fig. 9. MRM detected precursor-to-product ion transitions 518.4/122.0 (4-AP) and 455.4/197.0 (Q_4). In panel *A*, the green trace designates the 4-AP signal in the $+Q_6$ condition, and the purple trace designates the 4-AP signal in the absence of added Q_6 (*coq6Δ*). The peak areas of 4AP normalized by peak areas of Q_4 are 0.008 in *coq6Δ* and 2.68 in *coq6Δ* + Q_6. Panel *B* shows the fragmentation spectrum for the 4-AP $[M + H]^+$ precursor ion ($C_{36}H_{56}NO^+$; monoisotopic mass 518.4), the 4-AP tropylium ion $[M]^+$ ($C_7H_8NO^+$; 122.06), and the 4-AP chromenylium ion $[M]^+$ ($C_{10}H_{12}NO^+$; 162.1).

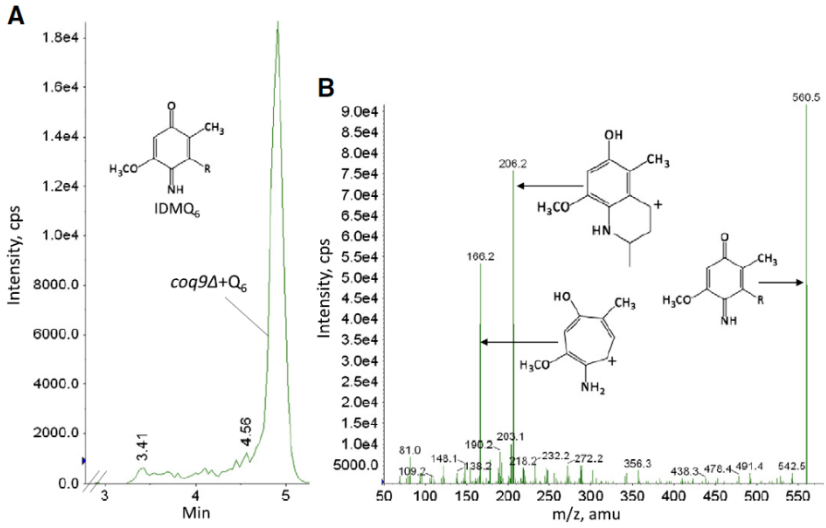

Fig. 11. Exogenous Q_6 leads to the accumulation of imino-demethoxy-Q_6 ($IDMQ_6$) in the *coq9* null mutant. Lipid extracts were prepared from cell pellets of the *coq9* null mutant yeast following growth in YPD with either the presence ($+Q_6$) or absence of Q_6 and analyzed by RP-HPLC-MS/MS as described in Fig. 8. Panel *A* shows the MRM detected precursor-to-product ion transition 560.5/166.2 ($IDMQ_6$). Panel *B*, shows the fragmentation spectrum for the $IDMQ_6$ $[M + H]^+$ precursor ion ($C_{38}H_{58}NO^+$; monoisotopic mass 560.4), the $IDMQ_6$ tropylium ion $[M]^+$ ($C_9H_{12}NO_2^+$; 166.1), and the $IDMQ_6$ chromenylium ion $[M]^+$ ($C_{12}H_{16}NO_2^+$; 206.1).

Table 3
Summary of the effect of over-expressing Coq8 or supplementation with exogenous Q_6.

Strains	No treatment					+hcCOQ8					+10 μM Q_6				
	Polypeptides			Intermediates from:		Polypeptides			Intermediates from:		Polypeptides			Intermediates from:	
	Coq4	Coq7	Coq9	4HB	pABA	Coq4	Coq7	Coq9	4HB	pABA	Coq4	Coq7	Coq9	4HB	pABA
WT	+	+	+	Q_6	Q_6	N/A	N/A	N/A	N/A	N/A	+	+	+	Q_6	Q_6
Coq1Δ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Coq2Δ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Coq3Δ	−	−	−	HHB	HAB	+	+	+	HHB	HAB	+	−	+	HHB	HAB
Coq4Δ	−	−	−	HHB	HAB	−	+	+	HHB	HHAB	−	−	+	HHB	HHAB
Coq5Δ	−	−	−	HHB	HAB	+	+	+	$DDMQ_6$	$DDMQ_6$	−	−	−	HHB	HAB
Coq6Δ	−	−	−	HHB	HAB	+	+	+	4-HP	4-AP	+	+	+	HHB	HHAB, 4-AP
Coq7Δ	−	−	−	HHB	HAB	+	−	+	DMQ_6	DMQ_6	+	−	+	DMQ_6	DMQ_6
Coq8Δ	−	−	−	HHB	HAB	+	+	+	Q_6	Q_6	−	−	−	HHB	HAB
Coq9Δ	−	−	−	HHB	HAB	+	+	−	DMQ_6	$IDMQ_6$	−	−	−	N/A	$IDMQ_6$

synthome (Fig. 12). On the other hand, there is no evidence that the Coq1 or Coq2 polypeptides are physically associated with the CoQ-synthome. In fact steady-state levels of indicator Coq polypeptides in the *coq1* null mutant are restored by expression of diverse polyisoprenyl-diphosphate synthases, including those from species that do not produce Q and hence would not be expected to interact with Coq polypeptides in yeast [36]. These findings support the interpretation that the stabilizing effects of exogenously added Q_6 or over-expression of Coq8 must depend on the synthesis of an endogenously produced polyisoprenyl-intermediate, such as HHB or HAB (Fig. 12).

In this study, steady-state levels of Coq1 are decreased upon supplementation of the *coq* null mutants with exogenous Q_6. It is tempting to speculate that Coq1 may play a regulatory step in the pathway where increases in Q_6 lead to a decrease in Coq1 polypeptide levels. This effect is not observed in wild type perhaps because supplementation with exogenous Q_6 does not have a significant impact on mitochondrial Q_6 content in wild-type yeast [70]. In contrast supplementation with exogenous Q_6 dramatically increases mitochondrial content of Q_6 in the *coq* null mutants, provided steps of endocytosis required for Q_6 uptake and trafficking to mitochondria are retained [70,84].

Dietary supplementation with Q_{10} can be an effective treatment for patients with partial defects in Q biosynthesis [87,88], and also shows benefit in mouse and *C. elegans* models of Q deficiency [89–92], and in cell culture models of mitochondrial diseases [93,94]. In this study we found that inclusion of exogenous Q_6 in the growth medium increased steady-state levels of mitochondrial polypeptides involved in respiratory electron transport and the citric acid cycle, including Rip1 (a subunit of complex III), Atp2 (a subunit of F_1 of complex V), and Mdh1, malate dehydrogenase. Previous studies indicated that supplemented Q_6 in *coq7* null mutant yeast restored steady-state levels of Atp2, cytochromes *c* and c_1, as well as porin, a mitochondrial outer membrane protein [95]. Exogenously supplied Q is converted to QH_2 by the respiratory chain, and QH_2 would exert its well known antioxidant effect on the mitochondrial membrane compartment and associated proteins [6]. Endogenous (hydroquinone) Q-intermediates formed after over-expression of Coq8 might also act as antioxidants. It is also possible that exogenous Q restored mitochondrial protein levels by increasing the content of mitochondria in *coq* null mutants. Q deficiencies may result from mitochondrial mutations affecting other processes; this is consistent with the observed effects of Q deficiency on mitophagy, and the inhibition of mitophagy by Q supplementation [93,94]. The findings presented here suggest that Q supplementation may correct defects in mitochondrial function through its beneficial effects in stabilizing the CoQ-synthome and de novo biosynthesis of Q, as well as contributing to enhanced respiratory electron transport and mitochondrial metabolism.

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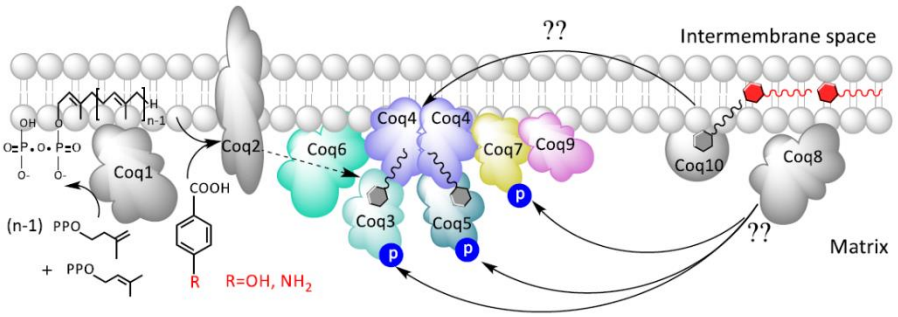

Fig. 12. Proposed model for the yeast CoQ-synthome. This model is consistent with co-precipitation studies in *S. cerevisiae* with tagged Coq polypeptides, and the association of several of the Coq polypeptides in high molecular mass complexes, as assayed in digitonin extracts of mitochondria separated by two-dimensional blue native/SDS PAGE. The over-expression of Coq8, a putative kinase, is required to observe phosphorylated forms of Coq3, Coq5, and Coq7 [31]. Coq10, a START domain polypeptide, binds to Q and is postulated to act as a Q chaperone that delivers Q to the CoQ-synthome and/or the bc_1 complex [38]. Coq4 is denoted as a scaffolding protein, with binding sites for Q or polyisoprenyl-intermediates and serves to organize the high molecular mass Q biosynthetic complexes. See text for additional explanation.

Henn (UTHSCSA) for the Mdh1 antibodies, and C. M. Koehler (UCLA) for the cytochrome *c*, cytochrome b_2, Hsp60, and Atp2 antibodies.

References

[1] F.L. Crane, R. Barr, Chemical structure and properties of coenzyme Q and related compounds, in: G. Lenaz (Ed.), Coenzyme Q: Biochemistry, Bioenergetics and Clinical Applications of Ubiquinone, John Wiley & Sons, New York, NY, 1985, pp. 1–37.

[2] M. Turunen, J. Olsson, G. Dallner, Metabolism and function of coenzyme Q, Biochim. Biophys. Acta 1660 (2004) 171–199.

[3] K.M. Overkamp, B.M. Bakker, P. Kotter, A. van Tuijl, S. de Vries, J.P. van Dijken, J.T. Pronk, In vivo analysis of the mechanisms for oxidation of cytosolic NADH by *Saccharomyces cerevisiae* mitochondria, J. Bacteriol. 182 (2000) 2823–2830.

[4] G. Lenaz, A. De Santis, A survey of the function and specificity of ubiquinone in the mitochondrial respiratory chain, in: G. Lenaz (Ed.), Coenzyme Q Biochemistry, Bioenergetics and Clinical Applications of Ubiquinone, John Wiley & Sons, New York, 1985, pp. 165–199.

[5] T.M. Hildebrandt, M.K. Grieshaber, Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria, FEBS J. 275 (2008) 3352–3361.

[6] M. Bentinger, K. Brismar, G. Dallner, The antioxidant role of coenzyme Q, Mitochondrion 7 (2007) S41–S50(Suppl.).

[7] R. Montero, M. Grazina, E. Lopez-Gallardo, J. Montoya, P. Briones, A. Navarro-Sastre, J.M. Land, I.P. Hargreaves, R. Artuch, Q.D.S.G. Coenzyme, Coenzyme Q10 deficiency in mitochondrial DNA depletion syndromes, Mitochondrion 13 (2013) 337–341.

[8] V. Emmanuele, L.C. Lopez, A. Berardo, A. Naini, S. Tadesse, B. Wen, E. D'Agostino, M. Solomon, S. DiMauro, C. Quinzii, M. Hirano, Heterogeneity of coenzyme Q10 deficiency: patient study and literature review, Arch. Neurol. 69 (2012) 978–983.

[9] S. Sharma, C.S. Moon, A. Khogali, A. Haidous, A. Chabenne, C. Ojo, M. Jelebinkov, Y. Kurdi, M. Ebadi, Biomarkers in Parkinson's disease (recent update), Neurochem. Int. 63 (2013) 201–229.

[10] A.D. Fotino, A.M. Thompson-Paul, L.A. Bazzano, Effect of coenzyme Q10 supplementation on heart failure: a meta-analysis, Am. J. Clin. Nutr. 97 (2013) 268–275.

[11] D.L. Gasser, C.A. Winkler, M. Peng, P. An, L.A. McKenzie, G.D. Kirk, Y. Shi, L.X. Xie, B.N. Marbois, C.F. Clarke, J.B. Kopp, Focal segmental glomerulosclerosis is associated with a PDSS2 haplotype and independently, with a decreased content of coenzyme Q10, Am. J. Physiol. Renal Physiol. 305 (2013) F1228–F1238.

[12] U.C. Tran, C.F. Clarke, Endogenous synthesis of coenzyme Q in eukaryotes, Mitochondrion 7 (2007) S62–S71(Supplement).

[13] F. Pierrel, O. Hamelin, T. Douki, S. Kieffer-Jaquinod, U. Mühlenhoff, M. Ozeir, R. Lill, M. Fontecave, Involvement of mitochondrial ferredoxin and para-aminobenzoic acid in yeast coenzyme Q biosynthesis, Chem. Biol. 17 (2010) 449–459.

[14] Y. Wang, S. Hekimi, Molecular genetics of ubiquinone biosynthesis in animals, Crit. Rev. Biochem. Mol. Biol. 48 (2013) 69–88.

[15] B. Marbois, L.X. Xie, S. Choi, K. Hirano, K. Hyman, C.F. Clarke, para-Aminobenzoic acid is a precursor in coenzyme Q_6 biosynthesis in *Saccharomyces cerevisiae*, J. Biol. Chem. 285 (2010) 27827–27838.

[16] M. Ozeir, U. Muhlenhoff, H. Webert, R. Lill, M. Fontecave, F. Pierrel, Coenzyme Q biosynthesis: Coq6 is required for the C5-hydroxylation reaction and substrate analogs rescue Coq6 deficiency, Chem. Biol. 18 (2011) 1134–1142.

[17] L.X. Xie, M. Ozeir, J.Y. Tang, J.Y. Chen, S.K. Jaquinod, M. Fontecave, C.F. Clarke, F. Pierrel, Overexpression of the Coq8 kinase in *Saccharomyces cerevisiae coq* null mutants allows for accumulation of diagnostic intermediates of the coenzyme Q_6 biosynthetic pathway, J. Biol. Chem. 287 (2012) 23571–23581.

[18] B. Marbois, P. Gin, K.F. Faull, W.W. Poon, P.T. Lee, J. Strahan, J.N. Shepherd, C.F. Clarke, Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q, J. Biol. Chem. 280 (2005) 20231–20238.

[19] B. Marbois, P. Gin, M. Gulmezian, C.F. Clarke, The yeast Coq4 polypeptide organizes a mitochondrial protein complex essential for coenzyme Q biosynthesis, Biochim. Biophys. Acta 1791 (2009) 69–75.

[20] E.J. Hsieh, P. Gin, M. Gulmezian, U.C. Tran, R. Saiki, B.N. Marbois, C.F. Clarke, *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex, Arch. Biochem. Biophys. 463 (2007) 19–26.

[21] A. Tauche, U. Krause-Buchholz, G. Rodel, Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of O-methylase Coq3p depends on Abc1p/Coq8p, FEMS Yeast Res. 8 (2008) 1263–1275.

[22] B.N. Marbois, C.F. Clarke, The *COQ7* gene encodes a protein in *Saccharomyces cerevisiae* necessary for ubiquinone biosynthesis, J. Biol. Chem. 271 (1996) 2995–3004.

[23] U.C. Tran, B. Marbois, P. Gin, M. Gulmezian, T. Jonassen, C.F. Clarke, Complementation of *Saccharomyces cerevisiae coq7* mutants by mitochondrial targeting of the *Escherichia coli* UbiF polypeptide: two functions of yeast Coq7 polypeptide in coenzyme Q biosynthesis, J. Biol. Chem. 281 (2006) 16401–16409.

[24] C.J. Leonard, L. Aravind, E.V. Koonin, Novel families of putative protein kinases in bacteria and archaea: evolution of the "eukaryotic" protein kinase superfamily, Genome Res. 8 (1998) 1038–1047.

[25] W.W. Poon, D.E. Davis, H.T. Ha, T. Jonassen, P.N. Rather, C.F. Clarke, Identification of *Escherichia coli ubiB*, a gene required for the first monooxygenase step in ubiquinone biosynthesis, J. Bacteriol. 182 (2000) 5139–5146.

[26] T.Q. Do, A.Y. Hsu, T. Jonassen, P.T. Lee, C.F. Clarke, A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in *Saccharomyces cerevisiae abc1* mutants, J. Biol. Chem. 276 (2001) 18161–18168.

[27] R. Saiki, Y. Ogiyama, T. Kainou, T. Nishi, H. Matsuda, M. Kawamukai, Pleiotropic phenotypes of fission yeast defective in ubiquinone-10 production. A study from the *abc1Sp* (*coq8Sp*) mutant, Biofactors 18 (2003) 229–235.

[28] C. Lagier-Tourenne, M. Tazir, L.C. Lopez, C.M. Quinzii, M. Assoum, N. Drouot, C. Busso, S. Makri, L. Ali-Pacha, T. Benhassine, M. Anheim, D.R. Lynch, C. Thibault, F. Plewniak, L. Bianchetti, C. Tranchant, O. Poch, S. DiMauro, J.L. Mandel, M.H. Barros, M. Hirano, M. Koenig, ADCK3, an ancestral kinase, is mutated in a form of recessive ataxia associated with coenzyme Q10 deficiency, Am. J. Hum. Genet. 82 (2008) 661–672.

[29] J. Mollet, A. Delahodde, V. Serre, D. Chretien, D. Schlemmer, A. Lombes, N. Boddaert, I. Desguerre, P. de Lonlay, H.O. de Baulny, A. Munnich, A. Rotig, CABC1 gene mutations cause ubiquinone deficiency with cerebellar ataxia and seizures, Am. J. Hum. Genet. 82 (2008) 623–630.

[30] B. Cardazzo, P. Hamel, W. Sakamoto, H. Wintz, G. Dujardin, Isolation of an *Arabidopsis thaliana* cDNA by complementation of a yeast *abc1* deletion mutant deficient in complex III respiratory activity, Gene 221 (1998) 117–125.

[31] L.X. Xie, E.J. Hsieh, S. Watanabe, C.M. Allan, J.Y. Chen, U.C. Tran, C.F. Clarke, Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants, Biochim. Biophys. Acta 1811 (2011) 348–360.

[32] A. Martin-Montalvo, I. Gonzalez-Mariscal, S. Padilla, M. Ballesteros, D.L. Brautigan, P. Navas, C. Santos-Ocana, Respiratory-induced coenzyme Q biosynthesis is regulated by a phosphorylation cycle of Cat5p/Coq7p, Biochem. J. 440 (2011) 107–114.

[33] A. Martin-Montalvo, I. Gonzalez-Mariscal, T. Pomares-Viciana, S. Padilla-Lopez, M. Ballesteros, L. Vazquez-Fonseca, P. Gandolfo, D.L. Brautigan, P. Navas, C. Santos-Ocana, The phosphatase Ptc7 induces coenzyme Q biosynthesis by activating the hydroxylase Coq7 in yeast, J. Biol. Chem. 288 (2013) 28126–28137.

[34] S. Padilla, U.C. Tran, M. Jimenez-Hidalgo, J.M. Lopez-Martin, A. Martin-Montalvo, C.F. Clarke, P. Navas, C. Santos-Ocana, Hydroxylation of demethoxy-Q_6 constitutes a control point in yeast coenzyme Q_6 biosynthesis, Cell. Mol. Life Sci. 66 (2009) 173–186.

[35] M.A. Zampol, C. Busso, F. Gomes, J.R. Ferreira-Junior, A. Tzagoloff, M.H. Barros, Over-expression of *COQ10* in *Saccharomyces cerevisiae* inhibits mitochondrial respiration, Biochem. Biophys. Res. Commun. 402 (2010) 82–87.

[36] P. Gin, C.F. Clarke, Genetic evidence for a multi-subunit complex in coenzyme Q biosynthesis in yeast and the role of the Coq1 hexaprenyl diphosphate synthase, J. Biol. Chem. 280 (2005) 2676–2681.

[37] M.H. Barros, A. Johnson, P. Gin, B.N. Marbois, C.F. Clarke, A. Tzagoloff, The *Saccharomyces cerevisiae COQ10* gene encodes a START domain protein required for function of coenzyme Q in respiration, J. Biol. Chem. 280 (2005) 42627–42635.

[38] C.M. Allan, S. Hill, S. Morvaridi, R. Saiki, J.S. Johnson, W.S. Liau, K. Hirano, T. Kawashima, Z. Ji, J.A. Loo, J.N. Shepherd, C.F. Clarke, A conserved START domain coenzyme Q-binding polypeptide is required for efficient Q biosynthesis, respiratory electron transport, and antioxidant function in *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1831 (2013) 776–791.

[39] T.Z. Cui, M. Kawamukai, Coq10, a mitochondrial coenzyme Q binding protein, is required for proper respiration in *Schizosaccharomyces pombe*, FEBS J. 276 (2009) 748–759.

[40] Y. Shen, S. Goldsmith-Fischman, H.S. Atreya, T. Acton, L. Ma, R. Xiao, B. Honig, G.T. Montelione, T. Szyperski, NMR structure of the 18 kDa protein CC1736 from *Caulobacter crescentus* identifies a member of the START domain superfamily and suggests residues mediating substrate specificity, Proteins 58 (2005) 747–750.

[41] B.J. Clark, The mammalian START domain protein family in lipid transport in health and disease, J. Endocrinol. 212 (2012) 257–275.

[42] H.W. Kang, J. Wei, D.E. Cohen, PC-TP/StARD2: of membranes and metabolism, Trends Endocrinol. Metab. 21 (2010) 449–456.

[43] D. Burke, D. Dawson, T. Stearns, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Plainview, NY, 2000.

[44] R. Daniel Gietz, R.A. Woods, Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method, in: G. Christine, R.F. Gerald (Eds.), Methods Enzymol, vol. 350, Academic Press, 2002, pp. 87–96.

[45] R.J. Barkovich, A. Shtanko, J.A. Shepherd, P.T. Lee, D.C. Myles, A. Tzagoloff, C.F. Clarke, Characterization of the *COQ5* gene from *Saccharomyces cerevisiae*. Evidence for a C-methyltransferase in ubiquinone biosynthesis, J. Biol. Chem. 272 (1997) 9182–9188.

[46] E.J. Hsieh, J.B. Dinoso, C.F. Clarke, A tRNATRP gene mediates the suppression of *cbs2-223* previously attributed to *ABC1/COQ8*, Biochem. Biophys. Res. Commun. 317 (2004) 648–653.

[47] M.P. Yaffe, Analysis of mitochondrial function and assembly, Methods Enzymol. 194 (1991) 627–643.

[48] B.S. Glick, L.A. Pon, Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*, Methods Enzymol. 260 (1995) 213–223.

[49] P. Gin, A.Y. Hsu, S.C. Rothman, T. Jonassen, P.T. Lee, A. Tzagoloff, C.F. Clarke, The *Saccharomyces cerevisiae COQ6* gene encodes a mitochondrial flavin-dependent monooxygenase required for coenzyme Q biosynthesis, J. Biol. Chem. 278 (2003) 25308–25316.

[50] T. Jonassen, M. Proft, F. Randez-Gil, J.R. Schultz, B.N. Marbois, K.D. Entian, C.F. Clarke, Yeast Clk-1 homologue (Coq7/Cat5) is a mitochondrial protein in coenzyme Q synthesis, J. Biol. Chem. 273 (1998) 3351–3357.

[51] E. Jarosch, G. Tuller, G. Daum, M. Waldherr, A. Voskova, R.J. Schweyen, Mrs5p, an essential protein of the mitochondrial intermembrane space, affects protein import into yeast mitochondria, J. Biol. Chem. 271 (1996) 17219–17225.

[52] B.S. Glick, A. Brandt, K. Cunningham, S. Muller, R.L. Hallberg, G. Schatz, Cytochromes c1 and b2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism, Cell 69 (1992) 809–822.

[53] H.A. Kemp, G.F. Sprague Jr., Far3 and five interacting proteins prevent premature recovery from pheromone arrest in the budding yeast *Saccharomyces cerevisiae*, Mol. Cell. Biol. 23 (2003) 1750–1763.

[54] H. Steiner, A. Zollner, A. Haid, W. Neupert, R. Lill, Biogenesis of mitochondrial heme lyases in yeast. Import and folding in the intermembrane space, J. Biol. Chem. 270 (1995) 22842–22849.
[55] M.P. Yaffe, G. Schatz, Two nuclear mutations that block mitochondrial protein import in yeast, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 4819–4823.
[56] D. Leuenberger, N.A. Bally, G. Schatz, C.M. Koehler, Different import pathways through the mitochondrial intermembrane space for inner membrane proteins, EMBO J. 18 (1999) 4816–4822.
[57] Y. Fujiki, A.L. Hubbard, S. Fowler, P.B. Lazarow, Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum, J. Cell Biol. 93 (1982) 97–102.
[58] D.S. Reading, R.L. Hallberg, A.M. Myers, Characterization of the yeast *HSP60* gene coding for a mitochondrial assembly factor, Nature 337 (1989) 655–659.
[59] W.J. Chen, M.G. Douglas, Phosphodiester bond cleavage outside mitochondria is required for the completion of protein import into the mitochondrial matrix, Cell 49 (1987) 651–658.
[60] A. Ohashi, J. Gibson, I. Gregor, G. Schatz, Import of proteins into mitochondria. The precursor of cytochrome c1 is processed in two steps, one of them heme-dependent, J. Biol. Chem. 257 (1982) 13042–13047.
[61] P. Stenmark, J. Grunler, J. Mattsson, P.J. Sindelar, P. Nordlund, D.A. Berthold, A new member of the family of di-iron carboxylate proteins. Coq7 (clk-1), a membrane-bound hydroxylase involved in ubiquinone biosynthesis, J. Biol. Chem. 276 (2001) 33297–33300.
[62] D.A. Berthold, P. Stenmark, Membrane-bound diiron carboxylate proteins, Annu. Rev. Plant Biol. 54 (2003) 497–517.
[63] D. Picot, P.J. Loll, R.M. Garavito, The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1, Nature 367 (1994) 243–249.
[64] K.U. Wendt, A. Lenhart, G.E. Schulz, The structure of the membrane protein squalene–hopene cyclase at 2.0 A resolution, J. Mol. Biol. 286 (1999) 175–187.
[65] E.K. Tuominen, C.J. Wallace, P.K. Kinnunen, Phospholipid-cytochrome c interaction: evidence for the extended lipid anchorage, J. Biol. Chem. 277 (2002) 8822–8826.
[66] S.W. Baba, G.I. Belogrudov, J.C. Lee, P.T. Lee, J. Strahan, J.N. Shepherd, C.F. Clarke, Yeast Coq5 C-methyltransferase is required for stability of other polypeptides involved in coenzyme Q biosynthesis, J. Biol. Chem. 279 (2004) 10052–10059.
[67] G.I. Belogrudov, P.T. Lee, T. Jonassen, A.Y. Hsu, P. Gin, C.F. Clarke, Yeast *COQ4* encodes a mitochondrial protein required for coenzyme Q synthesis, Arch. Biochem. Biophys. 392 (2001) 48–58.
[68] W.W. Poon, R.J. Barkovich, A.Y. Hsu, A. Frankel, P.T. Lee, J.N. Shepherd, D.C. Myles, C.F. Clarke, Yeast and rat Coq3 and *Escherichia coli* UbiG polypeptides catalyze both O-methyltransferase steps in coenzyme Q biosynthesis, J. Biol. Chem. 274 (1999) 21665–21672.
[69] A.M. James, H.M. Cocheme, R.A. Smith, M.P. Murphy, Interactions of mitochondria-targeted and untargeted ubiquinones with the mitochondrial respiratory chain and reactive oxygen species. Implications for the use of exogenous ubiquinones as therapies and experimental tools, J. Biol. Chem. 280 (2005) 21295–21312.
[70] C. Santos-Ocana, T.Q. Do, S. Padilla, P. Navas, C.F. Clarke, Uptake of exogenous coenzyme Q and transport to mitochondria is required for *bc*1 complex stability in yeast *coq* mutants, J. Biol. Chem. 277 (2002) 10973–10981.
[71] S.L. Rea, B.H. Graham, E. Nakamaru-Ogiso, A. Kar, M.J. Falk, Bacteria, yeast, worms, and flies: exploiting simple model organisms to investigate human mitochondrial diseases, Dev. Disabil. Res. Rev. 16 (2010) 200–218.
[72] M. Kawamukai, Biosynthesis and bioproduction of coenzyme Q10 by yeasts and other organisms, Biotechnol. Appl. Biochem. 53 (2009) 217–226.
[73] A. Casarin, J.C. Jimenez-Ortega, E. Trevisson, V. Pertegato, M. Doimo, M.L. Ferrero-Gomez, S. Abbadi, R. Artuch, C. Quinzii, M. Hirano, G. Basso, C.S. Ocana, P. Navas, L. Salviati, Functional characterization of human COQ4, a gene required for Coenzyme Q10 biosynthesis, Biochem. Biophys. Res. Commun. 372 (2008) 35–39.
[74] R. Saiki, A. Nagata, N. Uchida, T. Kainou, H. Matsuda, M. Kawamukai, Fission yeast decaprenyl diphosphate synthase consists of Dps1 and the newly characterized Dlp1 protein in a novel heterotetrameric structure, Eur. J. Biochem. 270 (2003) 4113–4121.
[75] R. Saiki, A. Nagata, T. Kainou, H. Matsuda, M. Kawamukai, Characterization of solanesyl and decaprenyl diphosphate synthases in mice and humans, FEBS J. 272 (2005) 5606–5622.
[76] A.J. Duncan, M. Bitner-Glindzicz, B. Meunier, H. Costello, I.P. Hargreaves, L.C. Lopez, M. Hirano, C.M. Quinzii, M.I. Sadowski, J. Hardy, A. Singleton, P.T. Clayton, S. Rahman, A nonsense mutation in COQ9 causes autosomal-recessive neonatal-onset primary coenzyme Q10 deficiency: a potentially treatable form of mitochondrial disease, Am. J. Hum. Genet. 84 (2009) 558–566.
[77] L. Garcia-Corzo, M. Luna-Sanchez, C. Doerrier, J.A. Garcia, A. Guaras, R. Acin-Perez, J. Bullejos-Peregrin, A. Lopez, G. Escames, J.A. Enriquez, D. Acuna-Castroviejo, L.C. Lopez, Dysfunctional Coq9 protein causes predominant encephalomyopathy associated with CoQ deficiency, Hum. Mol. Genet. 22 (2013) 1233–1248.
[78] S. Ashraf, H.Y. Gee, S. Woerner, L.X. Xie, V. Vega-Warner, S. Lovric, H. Fang, X. Song, D.C. Cattran, C. Avila-Casado, A.D. Paterson, P. Nitschke, C. Bole-Feysot, P. Cochat, J. Esteve-Rudd, B. Haberberger, S.J. Allen, W. Zhou, R. Airik, E.A. Otto, M. Barua, M.H. Al-Hamed, J.A. Kari, J. Evans, A. Bierzynska, M.A. Saleem, D. Bockenhauer, R. Kleta, S. El Desoky, D.O. Hacihamdioglu, F. Gok, J. Washburn, R.C. Wiggins, M. Choi, R.P. Lifton, S. Levy, Z. Han, L. Salviati, H. Prokisch, D.S. Williams, M. Pollak, C.F. Clarke, Y. Pei, C. Antignac, F. Hildebrandt, ADCK4 mutations promote steroid-resistant nephrotic syndrome through CoQ10 biosynthesis disruption, J. Clin. Invest. 123 (2013) 5179–5189.
[79] C. Busso, E.B. Tahara, R. Ogusucu, O. Augusto, J.R. Ferreira-Junior, A. Tzagoloff, A.J. Kowaltowski, M.H. Barros, Saccharomyces cerevisiae coq10 null mutants are responsive to antimycin A, FEBS J. 277 (2010) 4530–4538.
[80] T.H. Haines, Do sterols reduce proton and sodium leaks through lipid bilayers? Prog. Lipid Res. 40 (2001) 299–324.
[81] Y. Roche, P. Peretti, S. Bernard, Influence of the chain length of ubiquinones on their interaction with DPPC in mixed monolayers, Biochim. Biophys. Acta 1758 (2006) 468–478.
[82] E. Lapuente-Brun, R. Moreno-Loshuertos, R. Acin-Perez, A. Latorre-Pellicer, C. Colas, E. Balsa, E. Perales-Clemente, P.M. Quiros, E. Calvo, M.A. Rodriguez-Hernandez, P. Navas, R. Cruz, A. Carracedo, C. Lopez-Otin, A. Perez-Martos, P. Fernandez-Silva, E. Fernandez-Vizarra, J.A. Enriquez, Supercomplex assembly determines electron flux in the mitochondrial electron transport chain, Science 340 (2013) 1567–1570.
[83] M.L. Genova, G. Lenaz, Functional role of mitochondrial respiratory supercomplexes, Biochim. Biophys. Acta 1837 (2014) 427–443.
[84] S. Padilla-Lopez, M. Jimenez-Hidalgo, A. Martin-Montalvo, C.F. Clarke, P. Navas, C. Santos-Ocana, Genetic evidence for the requirement of the endocytic pathway in the uptake of coenzyme Q_6 in *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1788 (2009) 1238–1248.
[85] A.M. James, H.M. Cocheme, M. Murai, H. Miyoshi, M.P. Murphy, Complementation of coenzyme Q-deficient yeast by coenzyme Q analogues requires the isoprenoid side chain, FEBS J. 277 (2010) 2067–2082.
[86] M. Hajj Chehade, L. Loiseau, M. Lombard, L. Pecqueur, A. Ismail, M. Smadja, B. Golinelli-Pimpaneau, C. Mellot-Draznieks, O. Hamelin, L. Aussel, S. Kieffer-Jaquinod, N. Labessan, F. Barras, M. Fontecave, F. Pierrel, ubil, a new gene in *Escherichia coli* coenzyme Q biosynthesis, is involved in aerobic C5-hydroxylation, J. Biol. Chem. 288 (2013) 20085–20092.
[87] G. Montini, C. Malaventura, L. Salviati, Early coenzyme Q10 supplementation in primary coenzyme Q10 deficiency, N. Engl. J. Med. 358 (2008) 2849–2850.
[88] S.F. Heeringa, G. Chernin, M. Chaki, W. Zhou, A.J. Sloan, Z. Ji, L.X. Xie, L. Salviati, T.W. Hurd, V. Vega-Warner, P.D. Killen, Y. Raphael, S. Ashraf, B. Ovunc, D.S. Schoeb, H.M. McLaughlin, R. Airik, C.N. Vlangos, R. Gbadegesin, B. Hinkes, P. Saisawat, E. Trevisson, M. Doimo, A. Casarin, V. Pertegato, G. Giorgi, H. Prokisch, A. Rotig, G. Nurnberg, C. Becker, S. Wang, F. Ozaltin, R. Topaloglu, A. Bakkaloglu, S.A. Bakkaloglu, D. Muller, A. Beissert, S. Mir, A. Berdeli, S. Varpizen, M. Zenker, V. Matejas, C. Santos-Ocana, P. Navas, T. Kusakabe, A. Kispert, S. Akman, N.A. Soliman, S. Krick, P. Mundel, J. Reiser, P. Nurnberg, C.F. Clarke, R.C. Wiggins, C. Faul, F. Hildebrandt, COQ6 mutations in human patients produce nephrotic syndrome with sensorineural deafness, J. Clin. Invest. 121 (2011) 2013–2024.
[89] R. Saiki, A.L. Lunceford, Y. Shi, B. Marbois, R. King, J. Pachuski, M. Kawamukai, D.L. Gasser, C.F. Clarke, Coenzyme Q10 supplementation rescues renal disease in Pdss2kd/kd mice with mutations in prenyl diphosphate synthase subunit 2, Am. J. Physiol. Renal Physiol. 295 (2008) F1535–F1544.
[90] Y. Wang, S. Hekimi, Mitochondrial respiration without ubiquinone biosynthesis, Hum. Mol. Genet. 22 (2013) 4768–4783.
[91] T. Jonassen, B.N. Marbois, K.F. Faull, C.F. Clarke, P.L. Larsen, Development and fertility in Caenorhabditis elegans clk-1 mutants depend upon transport of dietary coenzyme Q8 to mitochondria, J. Biol. Chem. 277 (2002) 45020–45027.
[92] F. Gomez, R. Saiki, R. Chin, C. Srinivasan, C.F. Clarke, Restoring de novo coenzyme Q biosynthesis in *Caenorhabditis elegans* coq-3 mutants yields profound rescue compared to exogenous coenzyme Q supplementation, Gene 506 (2012) 106–116.
[93] D. Cotan, M.D. Cordero, J. Garrido-Maraver, M. Oropesa-Avila, A. Rodriguez-Hernandez, L. Gomez Izquierdo, M. De la Mata, M. De Miguel, J.B. Lorite, E.R. Infante, S. Jackson, P. Navas, J.A. Sanchez-Alcazar, Secondary coenzyme Q10 deficiency triggers mitochondria degradation by mitophagy in MELAS fibroblasts, FASEB J. 25 (2011) 2669–2687.
[94] M. De la Mata, J. Garrido-Maraver, D. Cotan, M.D. Cordero, M. Oropesa-Avila, L.G. Izquierdo, M. De Miguel, J.B. Lorite, E.R. Infante, P. Ybot, S. Jackson, J.A. Sanchez-Alcazar, Recovery of MERRF fibroblasts and cybrids pathophysiology by coenzyme Q10, Neurotherapeutics 9 (2012) 446–463.
[95] S. Padilla, T. Jonassen, M.A. Jimenez-Hidalgo, D.J. Fernandez-Ayala, G. Lopez-Lluch, B. Marbois, P. Navas, C.F. Clarke, C. Santos-Ocana, Demethoxy-Q, an intermediate of coenzyme Q biosynthesis, fails to support respiration in *Saccharomyces cerevisiae* and lacks antioxidant activity, J. Biol. Chem. 279 (2004) 25995–26004.
[96] J.E. McEwen, C. Ko, B. Kloeckner-Gruissem, R.O. Poyton, Nuclear functions required for cytochrome c oxidase biogenesis in *Saccharomyces cerevisiae*. Characterization of mutants in 34 complementation groups, J. Biol. Chem. 261 (1986) 11872–11879.
[97] M.N. Ashby, S.Y. Kutsunai, S. Ackerman, A. Tzagoloff, P.A. Edwards, COQ2 is a candidate for the structural gene encoding para-hydroxybenzoate:polyprenyltransferase, J. Biol. Chem. 267 (1992) 4128–4136.
[98] T.Q. Do, J.R. Schultz, C.F. Clarke, Enhanced sensitivity of ubiquinone-deficient mutants of *Saccharomyces cerevisiae* to products of autoxidized polyunsaturated fatty acids, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7534–7539.
[99] A.Y. Hsu, T.Q. Do, P.T. Lee, C.F. Clarke, Genetic evidence for a multi-subunit complex in the O-methyltransferase steps of coenzyme Q biosynthesis, Biochim. Biophys. Acta 1484 (2000) 287–297.