Published in final edited form as:

Biochim Biophys Acta. 2015 September ; 1851(9): 1227–1239. doi:10.1016/j.bbalip.2015.05.003.

Yeast Coq9 controls deamination of coenzyme Q intermediates that derive from para-aminobenzoic acid*

Cuiwen H. He (何翠雯)[¶], Dylan S. Black[¶], Theresa P. T. Nguyen[¶], Charles Wang[¶], Chandra Srinivasan[§], and Catherine F. Clarke[¶]

[¶]Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, CA USA 90095–1569

[§]Department of Chemistry and Biochemistry, California State University, Fullerton, Fullerton CA USA 92834

Abstract

Coq9 is a polypeptide subunit in a mitochondrial multi-subunit complex, termed the CoQ-synthome, required for biosynthesis of coenzyme Q (ubiquinone or Q). Deletion of *COQ9* results in dissociation of the CoQ-synthome, but over-expression of Coq8 putative kinase stabilizes the CoQ-synthome in the *coq9* null mutant and leads to the accumulation of two nitrogen containing Q-intermediates, imino-demethoxy-Q₆ (IDMQ₆) and 3-hexaprenyl-4-aminophenol (4-AP) when para-aminobenzoic acid (pABA) is provided as a ring precursor. To investigate whether Coq9 is responsible for deamination steps in Q biosynthesis, we utilized the yeast *coq5-5* point mutant. The yeast *coq5-5* point mutant is defective in the C-methyltransferase step of Q biosynthesis, but retains normal steady-state levels of the Coq5 polypeptide. Here we show that when high amounts of ¹³C₆-pABA are provided, the *coq5-5* mutant accumulates both ¹³C₆-imino-demethyl-demethoxy-Q₆ (¹³C₆-IDDMQ₆) and demethyl-demethoxy-Q₆ (¹³C₆-DDMQ₆). Deletion of *COQ9* in the yeast *coq5-5* mutant along with Coq8 over-expression and ¹³C₆-pABA labeling leads to the absence of ¹³C₆-DDMQ₆, and the nitrogen-containing intermediates ¹³C₆-4-AP and ¹³C₆-IDDMQ₆ persist. We describe a *coq9* temperature sensitive mutant and show that at the non-permissive temperature, steady state polypeptide levels of Coq9-ts19 increased, while Coq4, Coq5, Coq6, and Coq7 decreased. The *coq9-ts19* mutant had decreased Q₆ content and increased levels of nitrogen-containing intermediates. These findings identify Coq9 as a multi-functional protein that is required for the function of Coq6 and Coq7 hydroxylases, for removal of the nitrogen substituent from pABA-derived Q-intermediates, and is an essential component of the CoQ synthome.

*This work was supported by the National Science Foundation Grant MCB-1330803 (C.F.C.), and the National Institutes of Health Grant T32 GM 008496 and the Whitcome Pre-doctoral Training Program (UCLA Molecular Biology Institute) supported C. H. H. The authors thank and the General Biochemistry Laboratory course students (fall 2013 and spring 2013) at California State University Fullerton for their technical assistance in contributing towards the generation of some of the mutants used in this study.

[¶]Corresponding author: Catherine F. Clarke, UCLA Department of Chemistry and Biochemistry, 607 Charles E Young Dr E, Box 156905, Los Angeles, CA USA 90095-1569. Tel (310) 825-0771; Fax (310) 206-5213; cathy@chem.ucla.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Graphical abstract

Keywords

Temperature-sensitive mutant; coenzyme Q; mitochondrial metabolism; Q biosynthetic intermediates; *Saccharomyces cerevisiae*; mass spectrometry (MS)

1. Introduction

Coenzyme Q (ubiquinone or Q)¹ is a polyprenylated benzoquinone lipid essential in cellular energy metabolism [1]. Q has a redox active benzoquinone ring connected to a polyisoprenoid side chain, and is anchored to the mitochondrial inner membrane by the polyisoprenyl tail. The polyisoprenyl chain contains six units in *Saccharomyces cerevisiae* (Q₆), eight units in *Escherichia coli* (Q₈) and ten units in humans (Q₁₀) [2]. The reversible reduction and oxidation of the quinone/hydroquinone (Q/QH₂) enables its function as an electron and proton carrier in the mitochondrial respiratory chain and as a lipid-soluble antioxidant present in cellular membranes and in lipoproteins [1].

Q biosynthesis in *S. cerevisiae* requires nine Coq polypeptides (Coq1–Coq9), ferredoxin (Yah1) and ferredoxin reductase (Arh1) [3]. In addition, a Q-binding protein (Coq10) is required for efficient Q biosynthesis and for Q function as an electron carrier in respiratory electron transport [4]. 4-hydroxybenzoic acid (4HB) and *para*-aminobenzoic acid (pABA) both function as aromatic ring precursors for Q₆ biosynthesis in *S. cerevisiae* [3, 5] (Fig. 1). Coq1 synthesizes the hexaprenyl diphosphate tail, which Coq2 attaches to ring precursors. Coq3 performs two *O*-methylation steps, Coq5 catalyzes *C*-methylation, and Coq6 and Coq7 catalyze hydroxylation steps. The proteins responsible for several steps in the Q biosynthesis pathway remain unknown, and the functional roles of the Coq4, Coq8, and Coq9 polypeptides still need further characterization (Fig. 1).

Coq9 is a polypeptide subunit in the Q biosynthetic complex. Similar to the other Coq polypeptides (with the exception of Coq2, an integral membrane protein), Coq9 is

¹The abbreviations used are: 4-AP, 3-hexaprenyl-4-aminophenol; DDMQ₆, demethyl-demethoxy; DMQ₆, demethoxy-Q₆; HAB, 3-hexaprenyl-4-aminobenzoic acid; 4HB, 4-hydroxybenzoic acid; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; 4-HP, 3-hexaprenyl-4-hydroxyphenol; IDDMQ₆, imino-demethyl-demethoxy Q₆; IDMQ₆, imino-demethoxy-Q₆; mcCOQ8, multi-copy COQ8; pABA, *para*-aminobenzoic acid; RP-HPLC-MS/MS; reverse phase-HPLC-MS/MS; Q, Coenzyme Q.

peripherally associated to the inner mitochondrial membrane facing the matrix side [6, 7]. Coq9 co-migrates with Coq3 and Coq4 at high molecular mass and HA tagged Coq9 co-purifies with Coq4, Coq5, Coq6 and Coq7 [6, 7]. Recovery of tagged versions of Coq3, Coq6, or Coq9 from digitonin-extracts of yeast mitochondria results in the recovery of the CoQ-synthome, a multi-subunit Q-biosynthetic complex, containing Coq3–Coq9 polypeptides, Q₆, Q₆-intermediates, as well as other partner proteins, including the newly identified Coq11 [8]. Deletion of any one of the *COQ3–COQ9* genes leads to the decreased steady state of several of the other Coq polypeptides and to the accumulation of two early Q-intermediates, 3-hexaprenyl-4-hydroxybenzoic acid (HHB) and 3-hexaprenyl-4-aminobenzoic acid (HAB) [6, 9]. Sensitive Coq polypeptides were stabilized and late-stage Q-intermediates accumulated in some of the *coq3–coq9* null mutants that over-expressed Coq8, a putative kinase [10]. Conserved kinase motifs in Coq8 are essential for the phosphorylation of Coq3, Coq5, and Coq7 [11, 12], and Coq8 over-expression stabilized the Q biosynthetic complex in yeast [7]. These studies suggest that Coq8 over-expression might stabilize the complex by phosphorylation. Recent work identified auto-phosphorylation and ATPase activity in ADCK3, a human ortholog of yeast Coq8 [13, 14].

Several studies suggest that yeast Coq9 is important for formation or stability of the CoQ synthome [7]. Coq8 over-expression suppressed the Q-less phenotype of the *coq9* point mutant yeast strain C92 [15]. C92 has a nonsense point mutation in the *coq9* gene causing an early stop codon; Coq8 over-expression increased the steady-state level of the Coq9 polypeptide in the C92 mutant [6]. Other work utilizing Coq8 over-expression showed that yeast Coq9 is important for correct function of Coq7 [10]. When Coq8 is over-expressed, intermediates that accumulate in the yeast *coq9* null mutant were also found to accumulate in the *coq7* null mutant. For example, with Coq8 over-expression, ¹³C₆-DMQ₆ accumulates in both yeast *coq9* and *coq7* null mutants when ¹³C₆-4HB was provided as an aromatic ring precursor [10, 16]. However, when the same strains were provided with ¹³C₆-pABA the yeast *coq9* null mutant with Coq8 over-expression accumulated ¹³C₆-imino-demethoxy Q₆ (¹³C₆-IDMQ₆), while under the same labeling conditions, the yeast *coq7* null mutant with Coq8 over-expression still produced ¹³C₆-DMQ₆ [10]. This finding suggests that Coq9 is required for Coq7 function, but is also required for deamination of Q-intermediates when pABA is used as a ring precursor. While pABA is utilized to generate Q₆ in yeast, it is not a ring precursor for Q biosynthesis in human, mouse, *Arabidopsis thaliana* or *E. coli* [17, 18]. Therefore, the important role that Coq9 plays in the deamination of Q-intermediates might be unique to yeast Coq9. Coq9 is also necessary for correct function of Coq6, because in the presence of Coq8 over-expression, both *coq6* null and *coq9* null mutants accumulate ¹³C₆-4-HP (upon labeling with ¹³C₆-4HB) and ¹³C₆-4-AP (upon labeling with ¹³C₆-pABA) [10]. In this study, we examined the role of yeast Coq9 in mediating the deamination of other nitrogen-containing Q intermediates and employed a temperature sensitive mutant to further clarify its role in stabilizing the CoQ synthome.

2. Materials and Methods

2.1 Yeast strains and growth media

S. cerevisiae strains used in this study are listed in Table 1. Growth media used in this study were prepared as described [19], and included: YPD (2% glucose, 1% yeast extract, 2% peptone), YPEG (1% yeast extract, 2% peptone, 2% ethanol and 3% glycerol), YPGal (1% yeast extract, 2% peptone, 2% galactose, 0.1% dextrose), and YPG (3% glycerol, 1% yeast extract, 2% peptone). Synthetic Dextrose/Minimal medium (SD-complete) consisted of 0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH₂PO₄, 0.5% (NH₄)₂SO₄, and amino acids were added to final concentrations as described [20]. Selective SD/Minimal medium lacking uracil or leucine (SD-Ura, or SD-Leu) were similarly prepared. Agar plate media were prepared as described above and included 2% bacto agar (Fisher).

2.2 Disruption of COQ9 in W3031B and CH316-6B (coq5-5 point mutant) yeast strains

A PCR product containing the KanMX4 gene was amplified with the genomic DNA isolated from BY4741 *coq9* (used as template) and with primers that annealed to 100 bp upstream and downstream of the *COQ9* ORF. The sequences of the primers utilized were: 5'-TTTGGGCCTACATAAGGTACTTC-3' and 5'-CGCACAGACCAATAAATCTGCC-3'. The PCR product was then transformed into the yeast W3031B to create W303 *coq9K*. Transformants that grew on YPD + 200 µg/ml G418 (Geneticin) were selected. Proteins were extracted from these transformants as described [21] and separated by SDS-PAGE with a 10% polyacrylamide gel. Proteins were transferred to an Immobilon-P transfer membrane (Millipore) and analyzed by immunoblotting as described [7]. The primary antibody against Coq9 was used at a 1:1000 dilution and the secondary antibody, goat anti-rabbit IgG H&L chain specific peroxidase conjugate (Calbiochem), at a 1:10,000 dilution. The absence of Coq9 polypeptide confirmed that *COQ9* was replaced with KanMX4. The resulting null mutant is W303 *coq9K*. To generate the double mutant, *coq5* point mutant with *coq9* knocked out, PCR product was transformed into the yeast *coq5-5* point mutant strain (CH316-6B) and transformants were screened and verified as described above. The resulting double mutant is CH316-6B *coq9*.

2.3 Construction of plasmids

Plasmids used in this study are listed in Table 2. Over-expression of Coq8 made use of the p4HN4 plasmid (mcCOQ8), which contains the *COQ8* gene in pRS426, a multi-copy yeast shuttle vector [22]. To construct the plasmid pRS315COQ9 (*COQ9*), the genomic DNA of W3031B was isolated using the Wizard Genomic DNA purification kit (Promega). The *COQ9* gene was then amplified with Taq polymerase and primers XhoI400upCoq9F (5'-CTCGAGCCGGGTTTCAGAGGTAAAGG-3' -400 to -380 of *COQ9* with XhoI restriction site at the 5' end) and BamHI240downCoq9R (5'-GGATCCGGGACAAGCAGGAAGAACTA-3' +220 to +240 with BamHI restriction site at the 5' end). PCR products were inserted into the TOPO vector using the TOPO TA Cloning kit (Invitrogen) resulting in a plasmid named TOPOCOQ9. pRS315 and TOPOCOQ9 were digested with the restriction digestion enzymes XhoI and BamHI (New England BioLabs) and separated by gel electrophoresis. DNA fragments that contained the digested pRS315 or

COQ9 were purified from agarose gel using the Purelink quick DNA gel extraction kit (Invitrogen) and then ligated with T4 DNA Ligase (New England BioLabs) resulting in pRS315COQ9. The correct nucleotide sequence of the *COQ9* ORF in pRS315COQ9 was verified (Laragen, Los Angeles). pRS315COQ9 was shown to rescue growth of W303 *COQ9* on medium containing a non-fermentable carbon source, YPG.

2.4 Construction of *coq9* temperature-sensitive mutants using polymerase chain reaction (PCR) mutagenesis

Temperature-sensitive *coq9* yeast strains were generated using error-prone PCR, followed by *in vivo* homologous recombination [23]. *COQ9* with 400 bp 5'- and 240 bp 3'-flanking regions was cloned into pRS315, resulting in the plasmid pRS315COQ9. *COQ9* was amplified using PCR with primers designed 180 bp upstream (5'-ACTGGAAAGCGGGCAGTGA-3') and 240 downstream (5'-CAAGTGTAGCGGTCACGCTG-3') of the multiple cloning region in the presence of 0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP, 0.05 mM dATP, 0.8 mM MgCl₂, and 0.6–0.8 mM MnCl₂. The amplified fragments were purified and co-transformed with linearized pRS315 into the yeast null mutant W303 *coq9*. Leu⁺ transformants were then selected and screened for growth at 25°C and 37°C on YPEG plates. One of the plasmids generated (TS19) using the method outlined here was used in this study.

2.5 Site-Directed Mutagenesis of *S. cerevisiae* COQ9

Mutagenesis of the wild-type yeast *COQ9* was carried out with either the QuikChange or QuikChange Lightning site-directed mutagenesis kit from Agilent following the manufacturer's protocol. The primers used to generate single mutant plasmids are listed in Table 4: for E55G mutant, forward primer E55Gf and reverse primer E55Gr; for R107G mutant, forward primer R107Gf and reverse primer R107Gr; for Q256L mutant, forward primer Q256Lf and reverse primer Q256Lr; for a-12g mutant, forward primer a-12gf and reverse primer a-12gr; for a-93g mutant, forward primer a-93gf and reverse primer a-93gr. Single mutant plasmids were transformed into *E. coli* as described in the manufacturer's protocol (Agilent) and then were purified from 3 ml cultures. The identities of the mutations were verified by DNA sequencing (Laragen). To generate secondary point mutations, single mutant plasmids were used as templates: E55GR107G mutant was generated using E55G as template and the R107Gf and R107Gr as primers; E55GQ256L mutant was generated using E55G as template and the Q256Lf and Q256Lr as primers; R107GQ256L mutant was generated using R107G as template and the Q256Lf and Q256Lr as primers. To generate tertiary point mutations, double mutant plasmids were used as templates: E55GR107GQ256L mutant was generated using R107GQ256L as template and the E55Gf and E55Gr as primers; R107GQ256La-12g mutant was generated using R107GQ256L as template and the a-12gf and a-12gr as primers; R107GQ256La-93g mutant was generated using R107GQ256L as template and the a-93gf and a-93gr as primers; E55GR107Ga-12g mutant was generated using E55GR107G as template and the a-12gf and a-12gr as primers. The identities of these mutations were verified by DNA sequencing (Laragen).

2.6 Lipid extraction and detection of Q₆-intermediates by HPLC and tandem mass spectrometry

The designated strains of *coq5-5* yeast mutants were labeled with ¹³C₆-pABA followed by lipid analysis. Labeling media were prepared with 50 µg/ml ¹³C₆-pABA dissolved in ethanol. The final concentration of ethanol in the medium was 0.2%. Yeast mutants without plasmids or harboring p4HN4 (mcCOQ8), were grown in 100 ml of SD complete or SD –Ura, respectively. To label cells, yeast cultures were diluted to 0.5 A_{600nm}/ml in 100 ml of fresh SD complete or SD-URA with ¹³C₆ pABA and labeled for 6 hours. The final cell density was 2–3 A_{600nm}/ml. For lipid extraction cells were collected by centrifugation and 145 pmol Q₄ was added to each cell pellet to serve as an internal standard. Lipid extracts were analyzed by RP-HPLC-MS/MS [10]. Briefly, a phenyl-hexyl column (Luna 5u, 100 × 4.60 mm, 5-µm, Phenomenex) was used for liquid chromatography. The mobile phase includes Solvent A (methanol/isopropanol, 95:5, with 2.5 mM ammonium formate) and Solvent B (isopropanol, 2.5 mM ammonium formate). From 0 to 6 min, Solvent B was increased linearly from 0 to 5% and the flow rate was increased from 600 to 800 µl/min. At 7 min, the flow rate and mobile phase were changed back to 100% Solvent A and a flow rate of 600 µl/min. The 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (Foster City, CA) was used for multiple reaction monitoring mode (MRM) analysis. Data were processed with Analyst version 1.4.2 software (Applied Biosystems).

To quantify Q₆ content and determine *de novo* synthesis of Q₆-intermediates in temperature-sensitive yeast mutants at permissive (25 °C) and non-permissive (37 °C) temperatures, yeast cells were labeled with ¹³C₆-pABA followed by lipid analysis as described above. Labeling media were prepared with 10 µg/ml ¹³C₆-pABA dissolved in ethanol (ethanol was 0.2% final concentration). Cells were collected (a total of 30 A_{600nm}) as pellets after 5 hours of labeling. Q₄ was added (145 pmol) to each cell pellet as an internal standard. The exact amounts (total pmol) of ¹²C-Q₆ and ¹³C₆-Q₆ were calculated by normalizing the peak areas of ¹²C-Q₆ (sum of oxidized and reduced) and ¹³C₆-Q₆ (sum of oxidized and reduced) by the peak areas of Q₄ (sum of oxidized and reduced); the pmol amounts were then determined from the Q₆ standard curve. After the pmol of ¹²C-Q₆ and ¹³C₆-Q₆ was calculated, they were further normalized by the wet weight of yeast pellets. Chemical standards for Q-intermediates 4-AP, IDMQ₆ and DMQ₆ are not available. To quantify these intermediates, the peak areas for each were normalized by the recovery of Q₄ (sum of oxidized and reduced peaks). Finally, calculated values were further normalized by the wet weight of yeast pellets.

2.7 Mitochondrial isolation and immunoblot analyses with temperature-sensitive mutants

To study the protein levels in temperature-sensitive mutants, mitochondria were isolated from yeast cells and analysed by immunoblot. Yeast cultures were grown to 3–4 A_{600nm} in YPGal medium at different temperatures (W3031B and W303 *coq9*:TS19 were grown at 25°C and 37°C for 18.5 hours; W303 COQ9, W303 COQ7, and W303 COQ4 were grown at 30°C overnight). Crude mitochondria were isolated from a total volume of 1 L of culture as described [24]. Next, crude mitochondria were further purified with an OptiPrep discontinuous iodixanol gradient and then solubilized with digitonin as described in [7]. Solubilized mitochondria (15 µg based on total protein measured by the bicinchoninic acid assay from Thermo) were separated by SDS-PAGE with 10% polyacrylamide gels. Proteins

were transferred to Immobilon-P transfer membranes (Millipore) and analyzed by immunoblotting as described [7]. The source and use of primary antibodies is described in Table 3. The secondary antibody used was goat anti-rabbit IgG H&L chain specific peroxidase conjugate (Calbiochem), 1:10,000.

2.8 RNA extraction and Northern blot analyses with temperature-sensitive mutants

To determine the *COQ* mRNA levels in temperature-sensitive mutants, samples of yeast total RNA were analysed by Northern blot. Yeast cells were grown to 0.5 A_{600nm} in YPGal; W3031B and W303 *coq9*:TS19 were grown at 25 °C and 37 °C for 18.5 hours; W303 *COQ9*, W303 *COQ7*, and W303 *COQ4* were grown at 30 °C overnight. Aliquots (25 ml) of each culture were harvested by centrifugation at 1000 × *g* for 5 min at 4°C, and cell pellets were washed with water and frozen in liquid nitrogen. RNA was extracted as described in [25] with some modifications. Briefly, 500 µl of RNA-SDS buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, pH 8.0, 2% SDS), 400 µl of acid-washed glass beads (Sigma), and 500 µl RNA Phenol-Chloroform (Fisher) were added. The tubes were vortexed for 1 min and incubated for 6 min at 65 °C. An aliquot (450 µl) of the aqueous top layer was added to 450 µl fresh RNA Phenol-Chloroform for the second extraction. RNA was precipitated with 1 ml ethanol and 40 µl 3 M sodium acetate (pH 5.2). The RNA pellets were then washed with 450 µl 70% ethanol (v/v) and resuspended in distilled water.

Samples of RNA (5 µg) were denatured at 55 °C for one hour with 5 volumes of glyoxal buffer. (Glyoxal buffer contains 60.9% DMSO (v/v) (SIGMA), 20.3% deionized glyoxal (v/v) (Fluka), 4.87% glycerol (v/v), 0.04 mg/ml ethidium bromide, and 12.2% 10× BPTE (v/v)). 10× BPTE contains 100 mM PIPES (SIGMA), 300 mM BIS-TRIS (SIGMA) and 10 mM EDTA. Samples of denatured RNA were separated by 1.2% agarose-1× BPTE gels and transferred to Hybond N⁺ nylon membranes (GE Healthcare) in 10× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]) as described [26]. Blots were cross-linked and hybridized with Church hybridization buffer containing probes as described [25].

Probes were generated as described [25], with some modifications. Briefly, PCR products were generated with genomic DNA extracted from W3031B and the primers annealed to the ORF of *COQ4*, *COQ5*, *COQ6*, *COQ7* or *COQ9* as listed in Table 5. Next, 4 µl of PCR products (100–200 ng/µl) were used as a template and mixed with 2.1 µl of distilled H₂O, 4 µl of the mixture of rATP, rCTP, and Rgtp (2.5 mM each), 2.4 µl of 100 µM rUTP, 2 µl of 100 mM DTT, 1.25 µl of [α-³²P] UTP (6,000 Ci/mmol; 40 µCi/µl; Perkin Elmer), 4 µl of 5× Promega transcription buffer, and 0.25 µl of T3 RNA polymerase (Promega). The in vitro transcription reaction was incubated at 37 °C for 1 h. To generate the *SCR1* probe, 0.2 µl of 5'-GTCTAGCCGCGAGGAAGG-3' oligo (100 µM) was mixed with 1 µl of T4 polynucleotide kinase (PNK), 1 µl of 10x PNK buffer, 3 µl of [γ-³²P] ATP (3,000 Ci/mmol; 10 µCi/µl; Perkin Elmer), and 4.8 µl of distilled H₂O. The reaction was incubated at 37 °C for 30 min.

3. Results

3.1 The deletion of COQ9 in *coq5-5* point mutant yeast leads to the accumulation of unique nitrogen-containing Q-intermediates

In addition to Coq6 and Coq7 function, Coq9 also appears to be necessary to convert IDMQ₆ to DMQ₆ (Fig. 1). To investigate whether Coq9 may act to remove the amino/imino group from other Q-intermediates, we utilized the CH316-6B yeast strain that harbors the *coq5-5* point mutation. This mutant lacks C-methyltransferase activity but retains steady state levels of Coq5 and other Coq polypeptides [27] and accumulates DDMQ₆ as a late-stage Q-intermediate [28]. To examine the effect of *COQ9* on the DDMQ₆ intermediate, we deleted the *COQ9* gene in CH316-6B yeast to generate a double mutant strain (*coq5-5 coq9*), and overexpressed Coq8 to stabilize the CoQ-synthome. We labeled the *coq5-5*, *coq5-5:mcCOQ8*, and *coq5-5 coq9:mcCOQ8* yeast strains with ¹³C₆-pABA for 6 hours and used HPLC with tandem mass spectrometry to detect Q₆ intermediates in the yeast lipid extracts. We found that both *coq5-5* and *coq5-5:mcCOQ8* strains accumulated predominant amounts of ¹³C₆-DDMQ₆ as well as readily detectable levels of ¹³C₆-IDDMQ₆ (blue and green traces in Fig. 2A and 2B). However, in the double mutant *coq5-5 coq9:mcCOQ8*, ¹³C₆-DDMQ₆ disappeared (Fig. 2B), while intermediates containing the nitrogen group, ¹³C₆-IDDMQ and ¹³C₆-4-AP, accumulated (red traces in Fig. 2A and 2C). ¹³C₆-DDMQ₆ was identified by its retention time (4.59 min), precursor-to-product ion transition (553.4/159.0), and fragmentation spectrum [28]. ¹³C₆-IDDMQ₆ was identified by its retention time (4.41 min), precursor-to-product ion transition (552.4/158.0), and fragmentation spectrum (Fig. 3). ¹³C₆-4-AP was identified by its retention time (2.94 min), precursor-to-product ion transition (524.4/128.0), and fragmentation spectrum [7]. The results suggest that Coq9 is essential for converting the amino or imino group to a hydroxyl group in Q intermediates derived from pABA.

3.2 Characterization of a *coq9* temperature-sensitive mutant (*coq9-ts19*)

To better understand the role of Coq9 in Q biosynthesis, we generated conditional *coq9* mutants. Using error-prone PCR and *in vivo* recombination, we mutagenized the cloned *COQ9* gene. One clone, TS19, was selected for further analysis (Fig. 4). At the permissive temperature of 25 °C, the yeast *coq9* null mutant harboring TS19 grew as well as wild type, while at the non-permissive temperature of 37 °C, it grew poorly when compared to either the wild type at 37 °C, or to growth at the permissive temperature, 25 °C.

The TS19 clone was sequenced to determine the mutations that caused the temperature-sensitive (ts) phenotype. Five mutations were detected in TS19: Adenine-12→Guanine (a-12g), Adenine-93→Guanine (a-93g), Glu55→Gly (E55G), Arg107→Gly (R107G), and Gln256→Leu (Q256L). The first two mutations are upstream of the *COQ9* ORF and the remaining three are within the *COQ9* ORF. To identify the amino acids that were critical for Coq9 function, *coq9* alleles were generated that contained single, double, or triple mutations. The plasmids generated were then transformed into the yeast *coq9* null mutant (W303 *COQ9*) and subjected to plate dilution assay. Serial dilutions were plated on SD -Leu to confirm the presence of the plasmid. WT was not transformed with any plasmid, so it had no growth on SD-Leu, but it grew well on YPG at both permissive and non-

permissive temperatures. W303 *COQ9* harboring empty vector (EV) showed no growth on YPG at either temperature as expected. W303 *COQ9* harboring wild-type *COQ9* (COQ9) was included to provide a positive control for growth on YPG at the different temperatures. Yeast *coq9* mutants harboring plasmids containing single mutations, E55G and Q256L, did not show altered growth at the non-permissive temperature, but the presence of the R107G single mutation did produce slightly defective growth at 37 °C (Fig. 4A). Yeast *coq9* mutants harboring plasmids containing double mutations in combination with R107G had defective growth at 37 °C, but not with E55G Q256L (Fig. 4B). The combination of three amino acid substitution mutations E55G R107G Q256L recapitulated the TS19 phenotype (Fig. 4C). The presence of two mutations upstream of *COQ9* start codon had no effect on yeast growth at non-permissive temperature (Fig. 4). Therefore, the full temperature sensitive phenotype of *coq9*:TS19 requires the presence of E55G, R107G, and Q256L mutations.

3.3 Temperature-sensitive mutations in *COQ9* lead to the destabilization of other Coq polypeptides at non-permissive temperature

Deletion of the yeast *COQ9* gene leads to the decreased steady state of other Coq polypeptides, especially Coq4 and Coq7 [6]. To determine whether the Coq9-ts19 polypeptide impacts other Coq polypeptide levels, we grew wild type (WT) and *coq9*:TS19 (9:TS19) yeast in YPGal for 18.5 hours at 25 °C or 37 °C, and then isolated mitochondria. The steady-state levels of Coq9, Coq4, Coq7, Coq5 and Coq6 in isolated mitochondria were analyzed by immunoblotting (Fig. 5). In wild-type mitochondria, Coq9, Coq4, and Coq7 levels were increased at 37 °C (Fig. 5A, B and C). Expression of certain genes in *S. cerevisiae* can be induced or repressed in response to environmental changes, such as heat shock [29]. Therefore, the increased steady-state levels of Coq polypeptides at the non-permissive temperature might be a stress response. Coq5 and Coq6 levels were not changed at higher temperature (Fig. 5D and E). In the *coq9*:TS19 mutant, steady state polypeptide levels of Coq9-ts19 were increased at the non-permissive temperature (Fig. 4A), while Coq4, Coq7, Coq5, and Coq6 tended to be decreased at 37 °C (Fig. 5B, C, D, and E). The results suggest that at high temperature the expression of Coq9-ts19 causes destabilization of the Coq polypeptide complex.

3.4 Changes in *COQ* RNA levels do not correspond to the observed changes in Coq polypeptide levels

To investigate whether the change of Coq polypeptides at different temperatures is a response at gene expression level or protein level, we analyzed the mRNA levels of *COQ4*, *COQ5*, *COQ6*, *COQ7* and *COQ9* in WT and 9:TS19 yeast grown at either the permissive (25 °C) or non-permissive temperatures (37 °C). The mRNA levels of *COQ4*, *COQ5*, *COQ6*, *COQ7* and *COQ9* were not changed in the 9:TS19 mutant at different temperatures (Fig. 6). This was also the case for the mRNA levels of *COQ5*, *COQ7* and *COQ9* in wild type (Fig. 6A, C and D). The mRNA levels of *COQ4* and *COQ6* in wild-type yeast were decreased at non-permissive temperature (Fig. 6B and E). Thus, the increase observed in the steady state polypeptide levels of Coq9, Coq4, Coq7, and Coq6 in wild-type mitochondria at 37°C (Fig. 5A, B and C) cannot be attributed to corresponding changes in mRNA content.

Therefore, it seems most likely that changes in steady state Coq polypeptide levels observed in Fig. 5 are instead due to the stabilization or destabilization of the CoQ-synthome.

3.5 Incubation at the non-permissive temperature leads to decreased Q₆ and the accumulation of nitrogen-containing intermediates in the yeast *coq9* mutant harboring TS19

The results in Figs. 4 and 5 suggest that the Coq9 polypeptide harboring the TS19 mutations (*Coq9-ts19*) is functionally impaired at the non-permissive temperature, 37 °C. To gain further insight into the nature of the *Coq9-ts19* temperature sensitive defects, we analyzed the *de novo* synthesis of Q₆ and Q₆-intermediates in the *coq9:TS19* mutant. Wild type (WT) and *coq9* null mutant harboring empty vector (*coq9: EV*) were included as controls. Yeast were grown in selective liquid media for 18.5 hours at 25 °C or 37 °C followed by labeling with 10 µg/ml ¹³C₆-pABA for five hours at 25 °C or 37 °C. At 37 °C there was a two-fold decrease in *de novo* synthesized ¹³C₆-Q₆ in WT, but there was not significant change of ¹²C-Q₆. In contrast, there was a marked decrease of both ¹²C-Q₆ and ¹³C₆-Q₆ in *coq9:TS19* at the non-permissive temperature (Fig.7A). Therefore, both the content of ¹²C-Q₆ and the synthesis of *de novo* ¹³C₆-Q₆ are dramatically decreased by high-temperature incubation in the *coq9:TS19* mutant.

The nitrogen containing compounds, 4-AP and IDMQ₆ accumulated in the *coq9:TS19* mutant. For example, ¹²C- and ¹³C₆-4-AP were uniquely present in the *coq9:TS19* mutant at 37 °C (Fig. 7B) and levels of ¹³C₆-IDMQ₆ and ¹²C₆-IDMQ₆ increased six- and two-fold, respectively, at the non-permissive temperature (Fig. 7C). In contrast, WT had decreased amount of ¹³C₆-IDMQ₆ at the non-permissive temperature (Fig. 7C). Because Coq9 appears to be required to convert IDMQ₆ to DMQ₆, we also measured the amount of DMQ₆. We found that both ¹²C₆- and ¹³C₆-DMQ₆ were increased significantly in WT at the non-permissive temperature. However the amount of ¹³C₆-DMQ₆ was not changed in the *coq9:TS19* mutant at 37 °C (Fig. 7D). In conclusion, incubation of the *coq9:TS19* mutant at 37 °C led to a dramatic decline in the amount of Q₆ and accumulation of the nitrogen containing compounds, 4-AP and IDMQ₆, that derive from ¹³C₆-pABA.

4. Discussion

Q plays a crucial role in mitochondrial electron transport and also serves as an important lipid soluble antioxidant. Despite the obvious importance of Q in human health and mitochondrial disease, many questions remain regarding its biosynthesis. Although the Coq9 polypeptide is one of eleven polypeptides essential for Q biosynthesis in yeast and human cells, the functional role Coq9 plays in the Q biosynthetic pathway remains an outstanding question.

In this study we examined the role of Coq9 in removing amino/imino groups from yeast Q-intermediates derived from pABA (Fig. 1). The finding that IDMQ₆ accumulates in a yeast *coq9* null mutant overexpressing *COQ8* [10] suggested that Coq9 is required for the deamination of IDMQ₆. Here we identified an earlier and new imino-intermediate in the pathway, IDDMQ₆. We discovered this intermediate when the *coq5-5* point mutant, defective in the C-methyltransferase step, was fed ¹³C₆-pABA. We showed that the *coq5-5*

mutant fed $^{13}\text{C}_6$ -pABA accumulated both ^{13}C -DDMQ₆ and $^{13}\text{C}_6$ -IDDMQ₆ (Fig. 2A and 2B). We speculated that the deamination of IDDMQ₆ would depend on Coq9. Therefore, we analyzed the intermediates that accumulated in the *coq5-5*, *coq9* double mutant over-expressing Coq8 (*coq5-5 coq9:mcCOQ8*). We found that this yeast strain lacked $^{13}\text{C}_6$ -DDMQ₆, but still accumulated $^{13}\text{C}_6$ -IDDMQ₆ (Fig. 2A and 2B). The data are consistent with the idea that Coq9 is required for removal of the nitrogen substituent for IDDMQ₆ to form DDMQ₆. The results also suggest that normally, the Coq5 C-methyltransferase acts prior to Coq9 and methylates IDDMQ₆ to form IDMQ₆. In the event that Coq5 activity is slow (or defective) then Coq9 is able to process IDDMQ₆ to DDMQ₆. There are likely to be profound differences between the yeast and human enzymes at these steps, because human cells are unable to convert pABA to Q [17]

Yeast Coq9 also plays an important role in supporting the activity of Coq6. This is evident because the dysfunction of Coq9 leads to the accumulation of 4-AP (Fig. 2C and 6B), which is an intermediate found in *coq6* null yeast mutants over-expressing *COQ8* [10]. In both the *coq6* and *coq9* mutants, the accumulation of $^{13}\text{C}_6$ -4-AP depended on the presence of $^{13}\text{C}_6$ -pABA and on Coq8 over-expression. Thus, even though Coq9 appears to be essential in removing the nitrogen groups from Q-intermediates, we have not demonstrated that Coq9 is the enzyme that catalyzes the deamination step directly. Indeed, based on the accumulation of 4-AP, it is likely that Coq6 may play an important role in mediating the deamination step(s). It is important to note that there is some Coq6 activity present in the *coq9* null mutant over-expressing Coq8, because Q-intermediates accumulate that harbor the Coq6-mediated hydroxyl-group, such as $^{13}\text{C}_6$ -IDMQ₆. While we have postulated potential pathways linking 4-AP, IDDMQ₆ and IDMQ₆ to the production of Q₆ (Fig. 1 and [7]), none have yet been proved to be productive intermediates in the pathway leading to Q₆.

While yeast Coq6 does not function very well in the absence of Coq9, several lines of evidence suggest that Coq7 is completely inactive in the absence of Coq9. Coq7 is a di-iron containing hydroxylase that catalyzes the hydroxylation of DMQ [30, 31]. Mutations in *COQ7* result in the accumulation of DMQ in *S. cerevisiae*, *C. elegans* and mice [10, 32, 33]. Yeast *coq7* and *coq9* null mutants over-expressing *COQ8* both accumulate DMQ₆ when 4-HB is provided as the ring precursor [10]. In the yeast *coq9* strain, Coq8 over-expression only slightly increases the steady state level of Coq7, while Coq8 over-expression restored Coq9 polypeptide to wild-type level in *coq7*. Purification of HA-tagged yeast Coq9 captures the yeast Coq7, Coq4, Coq6 and Coq5 polypeptides [6]. In addition, purification of tagged forms of Coq3 and Coq6 also capture Coq4, Coq5, Coq7, Coq8, and Coq9 [8]. These results indicate that yeast Coq9 and Coq7 are in a complex, together with other polypeptides and Q and Q-intermediates, termed the CoQ-synthome [7, 8]. Such Coq polypeptide biosynthetic complexes also appear to play a role in Q biosynthesis in the mouse. The lack of a functional Coq9 protein in homozygous Coq9 mutant mice causes a severe reduction in the Coq7 protein and accumulation of DMQ₉ [34]. Human cells with Coq9 defects accumulate an intermediate slightly more polar than Q₁₀; based on studies in yeast and mice this seems likely to be DMQ₁₀ [35]. Thus it seems likely that the function of Coq9 in enhancing Coq7 function is conserved from yeast to humans.

To gain further insight into the function of Coq9 we created a temperature-sensitive yeast *coq9* allele (*coq9-ts19*). We showed that amino acid substitution mutations E55G, R107G and Q256L recapitulated the YPG growth phenotype of the *coq9-ts19* mutant. At non-permissive temperature (37 °C), the presence of the Coq9-ts19 polypeptide led to a trend of decreased steady-state polypeptide levels of Coq4, Coq5, Coq6, and Coq7 in isolated mitochondria (Fig. 5). These observed changes in Coq polypeptide levels did not result from changes in the corresponding *COQ* RNA levels (Fig. 6). Lipid extracts prepared from the *coq9-ts19* mutant grown at non-permissive temperature showed a drastic decrease in Q₆ content and the accumulation of intermediates containing the nitrogen group, IDMQ₆ and 4-AP (Fig. 7). Taken together, these findings indicate that at the restrictive temperature, the *coq9-ts19* mutant loses the ability to support the activities catalyzed by Coq6 and Coq7, and that the CoQ-synthome is destabilized.

In contrast, when wild-type yeast cells were subjected to the same temperature shift, steady state polypeptide levels of the Coq4, Coq7 and Coq6 polypeptides were increased (Fig. 5). Again, these changes were not paralleled by changes in the corresponding *COQ* RNA levels (Fig. 6). In contrast to the increase in the Coq polypeptide content at high temperature, the level of *de novo* synthesized Q₆ was decreased, while the level of *de novo* synthesized ¹³C₆-DMQ₆ increased (Fig. 7). This observation is consistent with impaired Coq7 function at the elevated temperature. The phosphorylation state of Coq7 affects Q₆ biosynthesis and the status of respiratory metabolism can cause Coq7 become dephosphorylated or phosphorylated [36]. It would be interesting to compare the phosphorylation state of Coq7 at permissive and non-permissive temperatures. It is also possible that certain Coq enzyme activities may be sensitive to high temperatures. It was shown that Coq3 homologs from either *C. elegans* or *S. cerevisiae* rescue the *E. coli ubiG* mutant at 30 °C, but not at 37 °C [37].

It is important to note that the recent study by Lohman et al. [38] provides insights into the possible effects of these yeast Coq9-ts19 amino acid substitutions. Lohman et al. solved the structure of human Coq9 and identified it as a member of an ancient protein family TFR (TetR family of regulators) with a canonical amino terminal helix-turn-helix (HTH) domain. Two human Coq9 polypeptides crystallized as a dimer and formed a hydrophobic interface that binds lipids, including phospholipids and Q [38]. Another separate surface patch of Coq9 was shown to be key to the ability to bind human Coq7. Intriguingly, the authors identified key amino acid residues in the human Coq9 polypeptide that affected binding with Coq7. We used the human Coq9 structure to predict the structure of yeast Coq9 with the protein homology/analogy recognition engine (Phyre 2) [39]. The predicted yeast structure is comprised of residues P40 to L231, so only the E55G and R107G can be evaluated (Fig. 8A). In the predicted structure, residue E55 is at the end of α helix one and R107 is in α helix five (Fig. 8A). When compared to the human Coq9 structure, yeast E55 corresponds to human A113, which resides at the C-terminus of α helix one, and is part of the HTH domain (Fig. 8B). The HTH motif is structurally similar to the TFR family of bacterial transcriptional regulators, but is predicted to lack DNA-binding capacity [38]. The presence of the E55G on its own did not affect growth on at the non-permissive temperature (Fig. 4). This is similar to the observation that single mutations introduced into the HTH domain of

yeast Coq9 did not affect respiration competence [38]. Yeast R107 aligns with Q165 in α helix four of human Coq9 (Fig 8B). We found that the R107G substitution on its own produced a moderate temperature sensitive phenotype, but the combination of either E55G or Q256L in combination with R107G gave a much more pronounced temperature sensitivity. In the human Coq9 structure, Q165 resides in a region that is neither highly conserved nor is shown to be important in lipid binding or interaction with human Coq7 [38]. Our immunoblot analyses show that at non-permissive temperature, the steady state level of Coq9-ts19 is increased, while other Coq polypeptides are destabilized (Fig. 5A).

According to a model proposed by Gonzalez-Mariscal, Coq7 is recruited to the precomplex to catalyze the conversion of DMQ₆ to Q₆ [40]. They proposed that Coq9 plays an important structural role to stabilize the Q-biosynthetic complex, and is recruited to form a 700 kDa precomplex as part of the nucleation process initiated by Coq4 binding to HHB [40]. Hence it seems plausible that the temperature-sensitive mutations cause misfolding of yeast Coq9-ts19 at high temperature and prevent its proper function or interaction with other Coq polypeptides and lead to the destabilization of the precomplex. There might be a small amount of functional Coq9-ts19, so some of the 700 kDa precomplex is able to form and produces DMQ₆ [40]. Although their model did not depict the interaction of Coq9 and Coq7 as the means of Coq7's recruitment, the results presented here, and the Coq9 structure by Lohman et al suggest that this may be the case. There is a large amount of DMQ₆ accumulated in the temperature sensitive mutant at non-permissive temperature, but very little Q₆ was produced (Fig. 7). In this scenario, the mutations in Coq9-ts19 disrupt the interaction of Coq7 and Coq9, so Coq7 cannot bind to the pre-complex and perform its function. Lohman et al. [38] noted that several residues predicted to affect the interaction of yeast Coq9 with Coq7 resulted in decreased Q₆ and increased DMQ₆. It would be interesting to determine the effect of these mutations on the accumulation of IDMQ₆.

In conclusion, we found that yeast Coq9 is required for the deamination of 4-AP, IDDMQ₆ and IDMQ₆. At the non-permissive temperature, the coq9-ts19 mutant has low steady state levels of Coq4, Coq5, Coq6 and Coq7 polypeptides, shows defective growth on non-fermentable carbon source and a drastic decrease in the content of Q₆, and accumulates imino/amino Q-intermediates. The results presented here identify Coq9 as a multi-functional protein that is required for the function of Coq6 and Coq7, for removal of the nitrogen substituent from pABA-derived Q-intermediates, and is an essential component of the CoQ synthome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Carla Koehler (UCLA) for her advice on generating the temperature-sensitive mutant plasmid, TS19, and for the Atp2 antibody. We thank Dr. A. Tzagoloff (Columbia University) for the original yeast *coq* mutant strains. We thank Kevin Roy for advice and assistance with RNA analyses, and the members of the Clarke lab for their helpful discussions and feedback on the manuscript.

References

1. Turunen M, Olsson J, Dallner G. Metabolism and function of coenzyme Q. *Biochim Biophys Acta*. 2004; 1660:171–199. [PubMed: 14757233]
2. Crane, F.; Barr, R. Chemical structure and properties of coenzyme Q and related compounds, Coenzyme Q biochemistry, bioenergetics and clinical applications of ubiquinone. John Wiley & Sons; Chichester, United Kingdom: 1985. p. 1-37.
3. Pierrel F, Hamelin O, Douki T, Kieffer-Jaquinod S, Mühlenhoff U, Ozeir M, Lill R, Fontecave M. Involvement of Mitochondrial Ferredoxin and *para*-Aminobenzoic Acid in Yeast Coenzyme Q Biosynthesis. *Chem Biol*. 2010; 17:449–459. [PubMed: 20534343]
4. Allan CM, Hill S, Morvaridi S, Saiki R, Johnson JS, Liao WS, Hirano K, Kawashima T, Ji Z, Loo JA, Shepherd JN, Clarke CF. 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*. 2013; 1831:776–791. [PubMed: 23270816]
5. Marbois B, Xie LX, Choi S, Hirano K, Hyman K, Clarke CF. *para*-Aminobenzoic acid is a precursor in coenzyme Q₆ biosynthesis in *Saccharomyces cerevisiae*. *J Biol Chem*. 2010; 285:27827–27838. [PubMed: 20592037]
6. Hsieh EJ, Gin P, Gulmezian M, Tran UC, Saiki R, Marbois BN, Clarke CF. *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex. *Arch Biochem Biophys*. 2007; 463:19–26. [PubMed: 17391640]
7. He CH, Xie LX, Allan CM, Tran UC, Clarke CF. 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*. 2014; 1841:630–644. [PubMed: 24406904]
8. Allan CM, Awad AM, Johnson JS, Shirasaki DI, Wang C, Blaby-Haas CE, Merchant SS, Loo JA, Clarke CF. Identification of Coq11, a new coenzyme Q biosynthetic protein in the CoQ-synthome in *Saccharomyces cerevisiae*. *J Biol Chem*. 2015; 290 In press.
9. Tran UC, Clarke CF. Endogenous synthesis of coenzyme Q in eukaryotes. *Mitochondrion*. 2007; 7(Supplement):S62–S71. [PubMed: 17482885]
10. Xie LX, Ozeir M, Tang JY, Chen JY, Jaquinod SK, Fontecave M, Clarke CF, Pierrel F. Overexpression 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*. 2012; 287:23571–23581. [PubMed: 22593570]
11. Tauche A, Krause-Buchholz U, Rodel G. Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of O-methylase Coq3p depends on Abc1p/Coq8p. *FEMS Yeast Res*. 2008; 8:1263–1275. [PubMed: 18801050]
12. Xie LX, Hsieh EJ, Watanabe S, Allan CM, Chen JY, Tran UC, Clarke CF. Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants. *Biochim Biophys Acta*. 2011; 1811:348–360. [PubMed: 21296186]
13. Stefely JA, Reidenbach AG, Ulbrich A, Oruganty K, Floyd BJ, Jochem A, Saunders JM, Johnson IE, Minogue CE, Wrobel RL, Barber GE, Lee D, Li S, Kannan N, Coon JJ, Bingman CA, Pagliarini DJ. Mitochondrial ADCK3 employs an atypical protein kinase-like fold to enable coenzyme Q biosynthesis. *Molecular cell*. 2015; 57:83–94. [PubMed: 25498144]
14. Wheeler B, Jia Z. Preparation and characterization of human ADCK3, a putative atypical kinase. Protein expression and purification. 2015; 108:13–17. [PubMed: 25540914]
15. Johnson A, Gin P, Marbois BN, Hsieh EJ, Wu M, Barros MH, Clarke CF, Tzagoloff A. *COQ9*, a new gene required for the biosynthesis of coenzyme Q in *Saccharomyces cerevisiae*. *J Biol Chem*. 2005; 280:31397–31404. [PubMed: 16027161]
16. Padilla S, Tran UC, Jimenez-Hidalgo M, Lopez-Martin JM, Martin-Montalvo A, Clarke CF, Navas P, Santos-Ocana C. Hydroxylation of demethoxy-Q₆ constitutes a control point in yeast coenzyme Q₆ biosynthesis. *Cell Mol Life Sci*. 2009; 66:173–186. [PubMed: 19002377]
17. Xie LX, Williams KJ, He CH, Weng E, Khong S, Rose TE, Kwon O, Bensinger SJ, Marbois BN, Clarke CF. Resveratrol and *para*-coumarate serve as ring precursors for coenzyme Q biosynthesis. *J Lipid Res*. 2015; 56 In press.

18. Block A, Widhalm JR, Fatihi A, Cahoon RE, Wamboldt Y, Elowsky C, Mackenzie SA, Cahoon EB, Chapple C, Dudareva N, Basset GJ. The Origin and Biosynthesis of the Benzenoid Moiety of Ubiquinone (Coenzyme Q) in *Arabidopsis*. *The Plant cell*. 2014; 26:1938–1948. [PubMed: 24838974]
19. Burke, D.; Dawson, D.; Stearns, T. *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*. Cold Spring Harbor Laboratory Press; Plainview, NY: 2000.
20. Barkovich RJ, Shtanko A, Shepherd JA, Lee PT, Myles DC, Tzagoloff A, Clarke CF. Characterization of the *COQ5* gene from *Saccharomyces cerevisiae*. Evidence for a C-methyltransferase in ubiquinone biosynthesis. *J Biol Chem*. 1997; 272:9182–9188. [PubMed: 9083049]
21. Yaffe MP, Schatz G. Two nuclear mutations that block mitochondrial protein import in yeast. *Proc Natl Acad Sci U S A*. 1984; 81:4819–4823. [PubMed: 6235522]
22. Hsieh EJ, Dinoso JB, Clarke CF. A tRNA(Trp) gene mediates the suppression of *chs2-223* previously attributed to *ABC1/COQ8*. *Biochem Biophys Res Commun*. 2004; 317:648–653. [PubMed: 15063807]
23. Muhlrad D, Hunter R, Parker R. A rapid method for localized mutagenesis of yeast genes. *Yeast*. 1992; 8:79–82. [PubMed: 1561838]
24. Padilla-Lopez S, Jimenez-Hidalgo M, Martin-Montalvo A, Clarke CF, Navas P, Santos-Ocana C. Genetic evidence for the requirement of the endocytic pathway in the uptake of coenzyme Q₆ in *Saccharomyces cerevisiae*. *Biochim Biophys Acta*. 2009; 1788:1238–1248. [PubMed: 19345667]
25. Sayani S, Chanfreau GF. Sequential RNA degradation pathways provide a fail-safe mechanism to limit the accumulation of unspliced transcripts in *Saccharomyces cerevisiae*. *RNA*. 2012; 18:1563–1572. [PubMed: 22753783]
26. Al-Hadid Q, Roy K, Munroe W, Dzialo MC, Chanfreau GF, Clarke SG. Histidine methylation of yeast ribosomal protein Rpl3p is required for proper 60S subunit assembly. *Molecular and cellular biology*. 2014; 34:2903–2916. [PubMed: 24865971]
27. Baba SW, Belogradov GI, Lee JC, Lee PT, Strahan J, Shepherd JN, Clarke CF. Yeast Coq5 C-methyltransferase is required for stability of other polypeptides involved in coenzyme Q biosynthesis. *J Biol Chem*. 2004; 279:10052–10059. [PubMed: 14701817]
28. Nguyen TPT, Casarin A, Desbats MA, Doimo M, Trevisson E, Santos-Ocana C, Navas P, Clarke CF, Salvati L. Molecular characterization of the human COQ5 C-methyltransferase in Coenzyme Q biosynthesis. *Biochim Biophys Acta*. 2014; 1841:1628–1638. [PubMed: 25152161]
29. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO. Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell*. 2000; 11:4241–4257. [PubMed: 11102521]
30. Behan RK, Lippard SJ. The aging-associated enzyme CLK-1 is a member of the carboxylate-bridged diiron family of proteins. *Biochemistry*. 2010; 49:9679–9681. [PubMed: 20923139]
31. Lu TT, Lee SJ, Apfel UP, Lippard SJ. Aging-associated enzyme human clock-1: substrate-mediated reduction of the diiron center for 5-demethoxyubiquinone hydroxylation. *Biochemistry*. 2013; 52:2236–2244. [PubMed: 23445365]
32. Hsu AY, Poon WW, Shepherd JA, Myles DC, Clarke CF. 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*. 1996; 35:9797–9806. [PubMed: 8703953]
33. Wang Y, Hekimi S. Molecular genetics of ubiquinone biosynthesis in animals. *Crit Rev Biochem Mol Biol*. 2013; 48:69–88. [PubMed: 23190198]
34. Garcia-Corzo L, Luna-Sanchez M, Doerrier C, Garcia JA, Guaras A, Acin-Perez R, Bullejos-Peregrin J, Lopez A, Escames G, Enriquez JA, Acuna-Castroviejo D, Lopez LC. Dysfunctional Coq9 protein causes predominant encephalomyopathy associated with CoQ deficiency. *Hum Mol Genet*. 2013; 22:1233–1248. [PubMed: 23255162]
35. Duncan AJ, Bitner-Glindzic M, Meunier B, Costello H, Hargreaves IP, Lopez LC, Hirano M, Quinzii CM, Sadowski MI, Hardy J, Singleton A, Clayton PT, Rahman S. 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.* 2009; 84:558–566. [PubMed: 19375058]
36. Martin-Montalvo A, Gonzalez-Mariscal I, Padilla S, Ballesteros M, Brautigan DL, Navas P, Santos-Ocana C. Respiratory-induced coenzyme Q biosynthesis is regulated by a phosphorylation cycle of Cat5p/Coq7p. *Biochemical J.* 2011; 440:107–114.
  37. Gomez F, Saiki R, Chin R, Srinivasan C, Clarke CF. Restoring de novo coenzyme Q biosynthesis in *Caenorhabditis elegans coq-3* mutants yields profound rescue compared to exogenous coenzyme Q supplementation. *Gene.* 2012; 506:106–116. [PubMed: 22735617]
  38. Lohman DC, Forouhar F, Beebe ET, Stefely MS, Minogue CE, Ulbrich A, Stefely JA, Sukumar S, Luna-Sanchez M, Jochem A, Lew S, Seetharaman J, Xiao R, Wang H, Westphall MS, Wrobel RL, Everett JK, Mitchell JC, Lopez LC, Coon JJ, Tong L, Pagliarini DJ. Mitochondrial COQ9 is a lipid-binding protein that associates with COQ7 to enable coenzyme Q biosynthesis. *Proc Natl Acad Sci U S A.* 2014; 111:E4697–4705. [PubMed: 25339443]
  39. Kelley LA, Sternberg MJ. Protein structure prediction on the Web: a case study using the Phyre server. *Nature protocols.* 2009; 4:363–371. [PubMed: 19247286]
  40. Gonzalez-Mariscal I, Garcia-Teston E, Padilla S, Martin-Montalvo A, Pomares-Viciano T, Vazquez-Fonseca L, Gandolfo-Dominguez P, Santos-Ocana C. Regulation of coenzyme Q biosynthesis in yeast: a new complex in the block. *IUBMB life.* 2014; 66:63–70. [PubMed: 24470391]
  41. Hsu AY, Do TQ, Lee PT, Clarke CF. Genetic evidence for a multi-subunit complex in the O-methyltransferase steps of coenzyme Q biosynthesis. *Biochim Biophys Acta.* 2000; 1484:287–297. [PubMed: 10760477]
  42. Marbois BN, Clarke CF. The *COQ7* gene encodes a protein in *saccharomyces cerevisiae* necessary for ubiquinone biosynthesis. *J Biol Chem.* 1996; 271:2995–3004. [PubMed: 8621692]
  43. Winzler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Dow SW, El Bakkoury M, Foury F, Friend SH, Gentale E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Liebundguth N, Lockhart DJ, Lucau-Danila A, Lussier M, M'Rabet N, Menard P, Mittmann M, Pai C, Rebischung C, Revuelta JL, Riles L, Roberts CJ, Ross-MacDonald P, Scherens B, Snyder M, Sookhai-Mahadeo S, Storms RK, Veronneau S, Voet M, Volckaert G, Ward TR, Wysocki R, Yen GS, Yu K, Zimmermann K, Philippsen P, Johnston M, Davis RW. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science.* 1999; 285:901–906. [PubMed: 10436161]
  44. Claypool SM, Boonthung P, McCaffery JM, Loo JA, Koehler CM. The cardiolipin transacylase, tafazzin, associates with two distinct respiratory components providing insight into Barth syndrome. *Mol Biol Cell.* 2008; 19:5143–5155. [PubMed: 18799610]
  45. Gietz RD, Woods RA. Yeast transformation by the LiAc/SS Carrier DNA/PEG method. *Methods Mol Biol.* 2006; 313:107–120. [PubMed: 16118429]
  46. Belogrudov GI, Lee PT, Jonassen T, Hsu AY, Gin P, Clarke CF. Yeast *COQ4* encodes a mitochondrial protein required for coenzyme Q synthesis. *Arch Biochem Biophys.* 2001; 392:48–58. [PubMed: 11469793]
  47. Gin P, Hsu AY, Rothman SC, Jonassen T, Lee PT, Tzagoloff A, Clarke CF. The *Saccharomyces cerevisiae COQ6* gene encodes a mitochondrial flavin-dependent monooxygenase required for coenzyme Q biosynthesis. *J Biol Chem.* 2003; 278:25308–25316. [PubMed: 12721307]
  48. Tran UC, Marbois B, Gin P, Gulmezian M, Jonassen T, Clarke CF. 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. *The Journal of biological chemistry.* 2006; 281:16401–16409. [PubMed: 16624818]

Highlights

- For the first time, a temperature sensitive *coq9* mutant, *coq9-ts19* was isolated.
- Coq4, Coq5, Coq6, and Coq7 polypeptides were destabilized in *coq9-ts19* at 37 °C.
- The *coq9-ts19* has decreased Q₆ and elevated imino-/amino-Q-intermediates at 37°C.
- Coq9 is required to remove the nitrogen group from pABA-derived Q intermediates.
- Coq9 is required for the function of Coq6 and Coq7 in coenzyme Q biosynthesis.

Fig. 1. Biosynthesis of Q in *S. cerevisiae* from 4HB or pABA

S. cerevisiae uses either 4-hydroxybenzoic acid (4HB) or *para*-aminobenzoic acid (pABA) as an aromatic ring precursor for Q₆ biosynthesis. Coq1 synthesizes the hexaprenyl-diphosphate tail and Coq2 attaches it to either ring. Coq6 performs the C5-hydroxylation reaction in concert with ferredoxin (Yah1) and ferredoxin reductase (Arh1). Coq3 catalyzes the two *O*-methylation steps and Coq5 catalyzes the *C*-methylation step. Coq9 is the putative deaminase that removes the amino groups on imino-demethyl-demethoxy-Q₆ (IDDMQ₆) or imino-demethoxy-Q₆ (IDMQ₆). Coq9 is also required for Coq7 to catalyze the penultimate hydroxylation step, and for efficient C5-hydroxylation by Coq6.

Fig. 2. The deletion of *COQ9* in a yeast *coq5-5* point mutant leads to the accumulation of 3-hexaprenyl-4-aminophenol (4-AP) and the disappearance of demethyl-demethoxy-Q₆ (DDMQ₆), but imino-demethyl-demethoxy-Q₆ (IDDMQ₆) is still present

Yeast *coq5-5* point mutants with *COQ8* over-expressed (*coq5-5:mcCOQ8*), without *COQ8* over-expressed (*coq5-5*), or with the *COQ9* gene deleted and *COQ8* over-expressed (*coq5-5coq9Δ:mcCOQ8*), were cultured in SD complete or SD-Ura with 50 μg/ml $^{13}\text{C}_6$ -pABA and 2 μl ethanol/ml medium at 0.5 A_{600nm}/ml and collected after 6 hours. Q₄ (145.4 pmol) was added prior to extraction to serve as an internal standard. Lipid extracts prepared from the cell pellets were analyzed by RP-HPLC-MS/MS. Multiple reaction monitoring (MRM) detected precursor-to-product ion transitions 552.4/158.0 ($^{13}\text{C}_6$ -IDDMQ₆), 553.4/159.0 ($^{13}\text{C}_6$ -DDMQ₆), and 524.4/128.0 ($^{13}\text{C}_6$ -4-AP). Just the oxidized forms of IDDMQ₆ and DDMQ₆ were detected, while only the reduced form of 4-AP was present. $^{13}\text{C}_6$ -DDMQ₆ accumulates in the *coq5-5* and *coq5-5:mcCOQ8* yeast mutants (B), and $^{13}\text{C}_6$ -IDDMQ₆ is readily detected (A). $^{13}\text{C}_6$ -IDDMQ₆ and $^{13}\text{C}_6$ -4-AP accumulate in *coq5-5coq9Δ:mcCOQ8* (A and C), but $^{13}\text{C}_6$ -DDMQ₆ is not detected (B). In all panels, the blue traces designate the Q-intermediate signals in *coq5-5* and green traces designate the Q-intermediate signals in *coq5-5:mcCOQ8*, and the red traces indicate the Q-intermediate signals in *coq5-5coq9Δ:mcCOQ8*.

Fig. 3. Identification of *de novo* imino-demethyl-demethoxy-Q₆ (¹³C₆-IDDMQ₆)

Yeast *coq5-5 coq9 :mcCOQ8* was cultured and labeled with 50 µg/ml ¹³C₆ pABA as described in Figure 2. Total lipids were extracted and analyzed by RP-HPLC-MS/MS. The fragmentation spectra are shown for ¹³C₆-IDDMQ₆ [M+H]⁺ precursor ion (¹³C₆ ¹²C₃₁H₅₆NO₂⁺; monoisotopic mass 552.4), the ¹³C₆-IDDMQ₆ tropylium ion [M]⁺ (¹³C₆ ¹²C₂H₁₀NO₂⁺; 158.1), and the ¹³C₆-IDDMQ₆ chromenylium ion [M]⁺ (¹³C₆ ¹²C₅H₁₄NO₂⁺; 198.1).

Fig. 4. Characterization of the mutations responsible for temperature sensitivity of the Coq9-TS19 polypeptide

The yeast *coq9* null mutant (W303 *COQ9*) was transformed with plasmids containing the designated *coq9* mutations. pRS315 empty vector (EV), and yeast wild-type *COQ9* in pRS315 (*COQ9*), were included as controls. Yeast wild-type W3031B and the transformants were cultured over-night at 30 °C in YPD or SD–Leu media, respectively. Cell cultures were diluted to 0.2 based on A_{600nm} readings and 2 μ l of 1:5 serial dilutions were spotted onto SD–Leu and YPG plate media and incubated at the specified temperatures for 3 days.

Fig. 5. Expression of Coq9-ts19 polypeptide affects steady state levels of other yeast Coq polypeptides in response to different growth temperatures

The mitochondria of W3031B (WT) and W303 COQ9 harboring the temperature-sensitive plasmid TS19 (9:TS19) were isolated after yeast were grown for 18.5 hours at either 25°C or 37°C. Mitochondria were also isolated from the null control strains W303 COQ9 (9), W303 COQ7 (7), and W303 COQ4 (4) after yeast were grown overnight at 30 °C. Purified mitochondria (15 µg protein) were separated by SDS-PAGE and analyzed by immunoblot. Immunoblots were performed with antibodies against the designated polypeptides: Coq4, Coq5, Coq6, Coq7, Coq9 and Atp2. Images presented within a given panel were derived from the same gel, and thus the band intensities corresponding to the same antisera can be directly compared. Densitometry quantification was conducted with the software ImageJ (NIH) and plotted as graphs. The signals of *COQ4*, *COQ5*, *COQ6*, *COQ7*, and *COQ9* were each normalized to the signals of *ATP2*. Normalized values were then compared to WT at 25 °C to get the relative percentage. The immunoblot depicted is representative of two independent blots performed with two different preparations of purified mitochondria. (A, B, D) Bars designate the average signal + S.D. (n=2); (C, E) Each bar represents one measurement (n=1) because signals for Coq6 and Coq7 were detectable at 37 °C in only one set of the immunoblots.

Fig. 6. The observed changes in Coq polypeptide levels with temperature do not correspond to alterations in *COQ* RNA content

The total RNA of W3031B (WT) and W303 COQ9 harboring the temperature-sensitive plasmid TS19 ($\Delta 9$:TS19) were extracted after yeast were grown for 18.5 hours at 25°C or 37°C. RNA were also extracted from the null control strains W303 COQ9 ($\Delta 9$), W303 COQ7 ($\Delta 7$), and W303 COQ4 ($\Delta 4$) after yeast were grown overnight at 30 °C. Aliquots of RNA (5 μ g) were separated by 1.2% agarose gel and analyzed by Northern blot. Hybridizations were performed with probes against the designated RNA: *COQ4*, *COQ5*, *COQ6*, *COQ7*, *COQ9* and *SCR1*. Northern blot assay signals were quantified with the Quantity One software from the Bio-Rad FX Plus Phosphorimaging System. The quantified signals of *COQ4*, *COQ5*, *COQ6*, *COQ7*, and *COQ9* were each normalized by the signals of *SCR1*. Normalized values were then compared to WT at 25°C to get the relative percentage. Each bar represents a total of two measurements from two independent samples (n=2). Significant changes in the amounts of mRNA at different temperatures were determined with the Student's two-tailed *t*-test. The * symbols represent mRNA in samples at 37 °C compared to mRNA at 25 °C; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Fig. 7. Yeast strains expressing Coq9-ts19 have decreased Q₆ content and accumulate higher levels of nitrogen-containing Q-intermediates at the restrictive temperature

Yeast W3031B (WT), W303 COQ9 harboring pRS315 empty vector (coq9: EV), and W303 COQ9 harboring the temperature-sensitive plasmid TS19 (coq9: TS19) were seeded in 20 ml of SD-Complete (WT) or SD-Leu (coq9: EV and coq9: TS19) at 0.01 A_{600nm}/ml and grown for 18.5 hours at 25 °C or 37 °C. ¹³C₆-pABA (10 μg/ml) was added to yeast cultures and incubations were continued at either 25 °C or 37 °C. After labeling for 5 h, yeast cells (30 A_{600nm}) were collected as cell pellets from which lipids were extracted and analyzed by RP-HPLC-MS/MS. Each bar represents a total of four measurements from two independent samples each with two injections. Black bars represent the amount of the designated ¹²C-compounds and white bars represent the amount of ¹³C₆-compounds. For Q₆, IDMQ₆, and DMQ₆, the total amounts of the ¹²C- and ¹³C₆-compounds represent the sum of reduced and oxidized forms; ¹²C-AP and ¹³C₆-4AP, were present only in the reduced form. Significant changes in the amounts of Q₆ and Q₆ intermediates at different temperatures were determined with the Student's two-tailed *t*-test. The *symbols represent ¹²C-compounds in samples at 37 °C compared to ¹²C-compounds at 25 °C; *, *p*<0.05, **, *p*<0.01, ***, *p*<0.001. The + symbols represent ¹³C₆-labeled compounds in

samples at 37°C were compared to samples at 25°C; +, $p<0.05$, ++, $p<0.01$, +++, $p<0.001$; *ns* designates “non-significant”.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 8. A homology model of yeast Coq9

(A) The yeast Coq9 structure was predicted based on the crystal structure of human Coq9 by protein homology/analogy recognition engine (Phyre 2). There are 163 aligned residues, 13% identity and 99.54% confidence. The positions of two of the temperature sensitive amino acid substitution mutations are shown: E55 in α helix one, and R107 in α helix five.

(B) The predicted yeast Coq9 structure (purple) is shown aligned with human Coq9 structure (cyan) [38]. Yeast E55 corresponds to human A113 (both are in a helix one in the HTH domain) and yeast R107 aligns with Q165 in α helix four of human Coq9.

Table 1

Genotype and Source of Yeast Strains

Strain	Genotype	Source
W3031B	MAT α <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i>	R. Rothstein ^a
W303 coq4	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq4::TRP1</i>	[41]
W303 coq7	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq7::LEU2</i>	[42]
W303 coq9	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq9::URA3</i>	[15]
BY4741 coq9	MAT a <i>coq9 ::kanMX4 his3 1 leu2 0 lys2 0 ura3 0</i>	[43] ^b
CH316-6B	MAT α <i>coq5-5 trp1-1 ura3-1</i>	[27]
CH316-6B coq9	MAT α <i>coq5-5 trp1-1 ura3-1 coq9::kanMX4</i>	This study

^aDr. Rodney Rothstein, Department of Human Genetics, Columbia University

^bEuropean *S. cerevisiae* Archive for Functional Analysis (EUROSCARF), available on-line

Table 2

Plasmid constructs used in this study

Plasmid	Relevant genes	Copy number	Source
pRS315	Yeast shuttle vector	Low copy	[44]
pRS426	Yeast shuttle vector	Multicopy	[43, 44]
p4HN4 (mcCOQ8)	Yeast <i>ABC1/COQ8</i>	Multicopy	[45]
pRS315COQ9 (<i>COQ9</i>)	Yeast <i>COQ9</i>	Low copy	This work
TS19	Yeast <i>COQ9</i>	Low copy	This work
E55G	Yeast <i>COQ9</i>	Low copy	This work
R107G	Yeast <i>COQ9</i>	Low copy	This work
Q256L	Yeast <i>COQ9</i>	Low copy	This work
a-12g	Yeast <i>COQ9</i>	Low copy	This work
a-93g	Yeast <i>COQ9</i>	Low copy	This work
E55GR107G	Yeast <i>COQ9</i>	Low copy	This work
E55GQ256L	Yeast <i>COQ9</i>	Low copy	This work
R107GQ256L	Yeast <i>COQ9</i>	Low copy	This work
R107GQ256L	Yeast <i>COQ9</i>	Low copy	This work
E55GR107GQ256L	Yeast <i>COQ9</i>	Low copy	This work
R107GQ256La-93g	Yeast <i>COQ9</i>	Low copy	This work
R107GQ256La-12g	Yeast <i>COQ9</i>	Low copy	This work
E55GR107Ga-12g	Yeast <i>COQ9</i>	Low copy	This work

Table 3

Description and Source of Antibodies

Antibody	Working Dilution	Source
Atp2	1:4000	Carla M. Koehler ^a
Coq4	1:250	[46]
Coq5	1:5000	[27]
Coq6	1:250	[47]
Coq7	1:1000	[48]
Coq9	1:1000	[6]

^aDr. Carla M. Koehler, Department of Chemistry and Biochemistry, UCLA

Table 4 Primer sequences (site-directed mutagenesis of *S. cerevisiae COQ9*)

Primer name	Sequence
E55Gf	5'-AGAGAAACCGTGCCCG GGAACAAAC-3'
E55Gr	5'-GTTTGTCCCGGGCACGGTTTCTCT-3'
R107Gf	5'-GGGTTGATTTCCTTCAGTTAAACGATACCCTTTATCTACC-3'
R107Gr	5'-GGTAGATAAAGGGTATCGTTTAACTGAAGGAATCAACCC-3'
Q256Lf	5'-CCCCTAACTAATAGAGATTTGATTAAATTTC CCGTAGACA-3'
Q256Lr	5'-TGTCTACGGTAAATTTAATCAAATCTC TATTAGTTAGGGG-3'
a-12gf	5'-GAGATAACAGAGTCTTTACCGCATTATAAATC-3'
a-12gr	5'-GATTTATAATGCGGTAAAGACTCTGTTATCTC-3'
a-93gf	5'-GCAATAACAATAGTAAGAAACGATAATACGGGG-3'
a-93gr	5'-CCCCGTATTATCGTTTCTTACTATTGTTATTGC-3'

Table 5

Primer sequences (Riboprobe generation)

Primer name	Sequence
COQ4F	5'-ACAGCTACTTTGCCAGTGAAATGCC-3'
COQ4T3R	5'-AATTAACCCTCACTAAAGGGAAGTCGTGGCTCGTTTCTGTGAGTTGT-3'
COQ5F	5'-TGTTGATTTCTTCACGGATCGTTTCG-3'
COQ5T3R	5'-AATTAACCCTCACTAAAGGGAGCCAGCAGATTGAATCCTGCCTTC-3'
COQ6F	5'-CAGGATTGTCAGTGTTACGCCTAGATC-3'
COQ6T3R	5'-AATTAACCCTCACTAAAGGGAGGGCAACTCTATCAGTGCAATAACGA-3'
COQ7F	5'-GCAGAGGCTTTTCCGTCTTATCATCT-3'
COQ7T3R	5'-AATTAACCCTCACTAAAGGGAGCCATATACGAATCATGCTTGATAGCGG-3'
COQ9F	5'-ATGCTTTGTCGCAATACTGCCAGAACG-3'
COQ9T3R	5'-AATTAACCCTCACTAAAGGGATACTCACCCAAACGCATGACCCTA-3'