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

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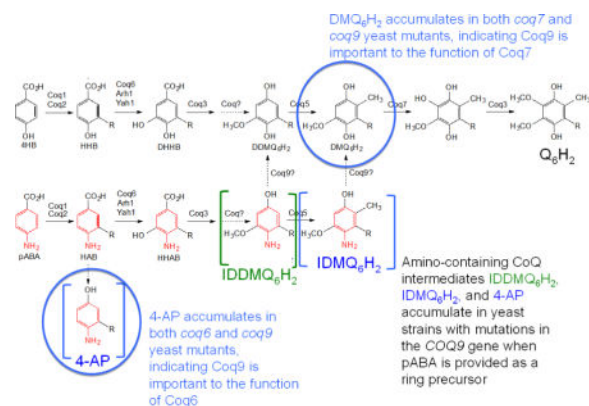
Abstract

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-Q_6 ($IDMQ_6$) 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 $^{13}C_6$-pABA are provided, the *coq5-5* mutant accumulates both $^{13}C_6$-imino-demethyl-demethoxy-Q_6 ($^{13}C_6$-$IDDMQ_6$) and demethyl-demethoxy-Q_6 ($^{13}C_6$-$DDMQ_6$). Deletion of *COQ9* in the yeast *coq5-5* mutant along with Coq8 over-expression and $^{13}C_6$-pABA labeling leads to the absence of $^{13}C_6$-$DDMQ_6$, and the nitrogen-containing intermediates $^{13}C_6$-4-AP and $^{13}C_6$-$IDDMQ_6$ 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 Q_6 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.

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* (Q_6), eight units in *Escherichia coli* (Q_8) and ten units in humans (Q_{10}) [2]. The reversible reduction and oxidation of the quinone/hydroquinone (Q/QH_2) 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 Q_6 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

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[1]The abbreviations used are: 4-AP, 3-hexaprenyl-4-aminophenol; $DDMQ_6$, demethyl-demethoxy; DMQ_6, demethoxy-Q_6; HAB, 3-hexaprenyl-4-aminobenzoic acid; 4HB, 4-hydroxybenzoic acid; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; 4-HP, 3-hexaprenyl-4-hydroxyphenol; $IDDMQ_6$, imino-demethyldemethoxy Q_6; $IDMQ_6$, imino-demethoxy-Q_6; mc*COQ8*, 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, Q_6, Q_6-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, $^{13}C_6$-DMQ$_6$ accumulates in both yeast *coq9* and *coq7* null mutants when $^{13}C_6$-4HB was provided as an aromatic ring precursor [10, 16]. However, when the same strains were provided with $^{13}C_6$-pABA the yeast *coq9* null mutant with Coq8 over-expression accumulated $^{13}C_6$-imino-demethoxy Q$_6$ ($^{13}C_6$-IDMQ$_6$), while under the same labeling conditions, the yeast *coq7* null mutant with Coq8 over-expression still produced $^{13}C_6$-DMQ$_6$ [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 Q_6 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 $^{13}C_6$-4-HP (upon labeling with $^{13}C_6$-4HB) and $^{13}C_6$-4-AP (upon labeling with $^{13}C_6$-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% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$, 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 (mc*COQ8*), 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′-CTCGAGCCGGGTTCAGAGGTAAAAGG-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 $MgCl_2$, and 0.6–0.8 mM $MnCl_2$. 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 Q_6-intermediates by HPLC and tandem mass spectrometry

The designated strains of *coq5*-5 yeast mutants were labeled with $^{13}C_6$-pABA followed by lipid analysis. Labeling media were prepared with 50 μg/ml $^{13}C_6$-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 $^{13}C_6$ 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 Q_4 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 Q_6 content and determine *de novo* synthesis of Q_6-intermediates in temperature-sensitive yeast mutants at permissive (25 °C) and non-permissive (37 °C) temperatures, yeast cells were labeled with $^{13}C_6$-pABA followed by lipid analysis as described above. Labeling media were prepared with 10 μg/ml $^{13}C_6$-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. Q_4 was added (145 pmol) to each cell pellet as an internal standard. The exact amounts (total pmol) of ^{12}C-Q_6 and $^{13}C_6$-Q_6 were calculated by normalizing the peak areas of $^{12}C_6$-Q_6 (sum of oxidized and reduced) and $^{13}C_6$-Q_6 (sum of oxidized and reduced) by the peak areas of Q_4 (sum of oxidized and reduced); the pmol amounts were then determined from the Q_6 standard curve. After the pmol of ^{12}C-Q_6 and $^{13}C_6$-Q_6 was calculated, they were further normalized by the wet weight of yeast pellets. Chemical standards for Q-intermediates 4-AP, $IDMQ_6$ and DMQ_6 are not available. To quantify these intermediates, the peak areas for each were normalized by the recovery of Q_4 (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 $1000 \times g$ 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 NaH_2PO_4, and 1 mM EDTA [pH 7.7]) as described [26]. Blots were crossed-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 H_2O, 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-^{32}P] 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−^{32}P] ATP (3,000 Ci/mmol; 10 μCi/μl; Perkin Elmer), and 4.8 μl of distilled H_2O. 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 $IDMQ_6$ to DMQ_6 (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 $DDMQ_6$ as a late-stage Q-intermediate [28]. To examine the effect of *COQ9* on the $DDMQ_6$ 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 $^{13}C_6$-pABA for 6 hours and used HPLC with tandem mass spectrometry to detect Q_6 intermediates in the yeast lipid extracts. We found that both *coq5-5* and *coq5-5:mcCOQ8* strains accumulated predominant amounts of $^{13}C_6$-$DDMQ_6$ as well as readily detectable levels of $^{13}C_6$-$IDDMQ_6$ (blue and green traces in Fig. 2A and 2B). However, in the double mutant *coq5-5 coq9:mcCOQ8*, $^{13}C_6$-$DDMQ_6$ disappeared (Fig. 2B), while intermediates containing the nitrogen group, $^{13}C_6$-IDDMQ and $^{13}C_6$-4-AP, accumulated (red traces in Fig. 2A and 2C). $^{13}C_6$-$DDMQ_6$ was identified by its retention time (4.59 min), precursor-to-product ion transition (553.4/159.0), and fragmentation spectrum [28]. $^{13}C_6$-$IDDMQ_6$ was identified by its retention time (4.41 min), precursor-to-product ion transition (552.4/158.0), and fragmentation spectrum (Fig. 3). $^{13}C_6$-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−93Guanine (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 Q_6 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 Q_6 and Q_6-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 $^{13}C_6$-pABA for five hours at 25 °C or 37 °C. At 37 °C there was a two-fold decrease in *de novo* synthesized $^{13}C_6$-Q_6 in WT, but there was not significant change of ^{12}C-Q_6. In contrast, there was a marked decrease of both ^{12}C-Q_6 and $^{13}C_6$-Q_6 in *coq9*:TS19 at the non-permissive temperature (Fig.7A). Therefore, both the content of ^{12}C-Q_6 and the synthesis of de novo $^{13}C_6$-Q_6 are dramatically decreased by high-temperature incubation in the *coq9*:TS19 mutant.

The nitrogen containing compounds, 4-AP and $IDMQ_6$ accumulated in the *coq9:TS19* mutant. For example, ^{12}C- and $^{13}C_6$-4-AP were uniquely present in the *coq9:TS19* mutant at 37 °C (Fig. 7B) and levels of $^{13}C_6$-$IDMQ_6$ and $^{12}C_6$-$IDMQ_6$ increased six- and two-fold, respectively, at the non-permissive temperature (Fig. 7C). In contrast, WT had decreased amount of $^{13}C_6$-$IDMQ_6$ at the non-permissive temperature (Fig. 7C). Because Coq9 appears to be required to convert $IDMQ_6$ to DMQ_6, we also measured the amount of DMQ_6. We found that both $^{12}C_6$- and $^{13}C_6$-DMQ_6 were increased significantly in WT at the non-permissive temperature. However the amount of $^{13}C_6$-DMQ_6 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 Q_6 and accumulation of the nitrogen containing compounds, 4-AP and $IDMQ_6$, that derive from $^{13}C_6$-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 $IDMQ_6$ accumulates in a yeast *coq9* null mutant overexpressing *COQ8* [10] suggested that Coq9 is required for the deamination of $IDMQ_6$. Here we identified an earlier and new imino-intermediate in the pathway, $IDDMQ_6$. We discovered this intermediate when the *coq5-5* point mutant, defective in the *C*-methyltransferase step, was fed $^{13}C_6$-pABA. We showed that the *coq5-5*

mutant fed $^{13}C_6$-pABA accumulated both ^{13}C-DDMQ$_6$ and $^{13}C_6$-IDDMQ$_6$ (Fig. 2A and 2B). We speculated that the deamination of IDDMQ$_6$ 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 $^{13}C_6$-DDMQ$_6$, but still accumulated $^{13}C_6$-IDDMQ$_6$ (Fig. 2A and 2B). The data are consistent with the idea that Coq9 is required for removal of the nitrogen substituent for IDDMQ$_6$ to form DDMQ$_6$. The results also suggest that normally, the Coq5 *C*-methyltransferase acts prior to Coq9 and methylates IDDMQ$_6$ to form IDMQ$_6$. In the event that Coq5 activity is slow (or defective) then Coq9 is able to process IDDMQ$_6$ to DDMQ$_6$. 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 $^{13}C_6$-4-AP depended on the presence of $^{13}C_6$-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 $^{13}C_6$-IDMQ$_6$. While we have postulated potential pathways linking 4-AP, IDDMQ$_6$ and IDMQ$_6$ to the production of Q$_6$ (Fig. 1 and [7]), none have yet been proved to be productive intermediates in the pathway leading to Q$_6$.

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 DMQ$_6$ 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 DMQ$_9$ [34]. Human cells with Coq9 defects accumulate an intermediate slightly more polar than Q$_{10}$; based on studies in yeast and mice this seems likely to be DMQ$_{10}$ [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 Q_6 content and the accumulation of intermediates containing the nitrogen group, $IDMQ_6$ 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 Q_6 was decreased, while the level of de novo synthesized $^{13}C_6$-DMQ_6 increased (Fig. 7). This observation is consistent with impaired Coq7 function at the elevated temperature. The phosphorylation state of Coq7 affects Q_6 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 or 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 destablized (Fig. 5A).

According to a model proposed by Gonzalez-Mariscal, Coq7 is recruited to the precomplex to catalyze the conversion of DMQ_6 to Q_6 [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 DMQ_6 [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 DMQ_6 accumulated in the temperature sensitive mutant at non-permissive temperature, but very little Q_6 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 Q_6 and increased DMQ_6. It would be interesting to determine the effect of these mutations on the accumulation of $IDMQ_6$.

In conclusion, we found that yeast Coq9 is required for the deamination of 4-AP, $IDDMQ_6$ and $IDMQ_6$. 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 Q_6, 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

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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 Q_6 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.
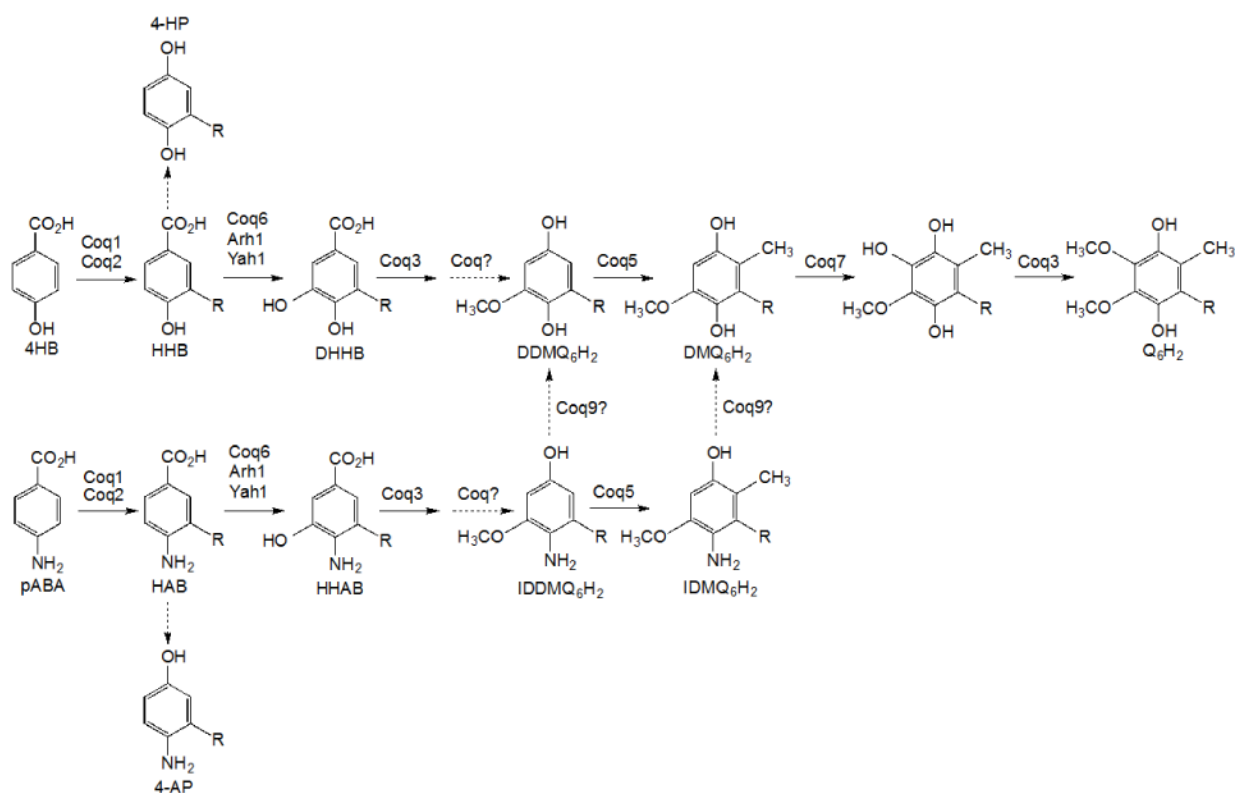
**Fig. 1. Biosynthesis of Q in *S. cerevisiae* from 4HB or pABA**

S. cerevisiae uses either 4-hydroxybenzoic acid (4HB) or *para*-aminobenzoic acid (pABA) as an aromatic ring precursor for Q_6 biosynthesis. Coq1 synthesizes the hexaprenyl-diphosphate tail and Coq2 attaches it to either ring. Coq6 performs the C5-hydoxylation 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-Q_6 (IDDMQ$_6$) or imino-demethoxy-Q_6 (IDMQ$_6$). Coq9 is also required for Coq7 to catalyze the penultimate hydroxylation step, and for efficient C5-hydroxylation by Coq6.
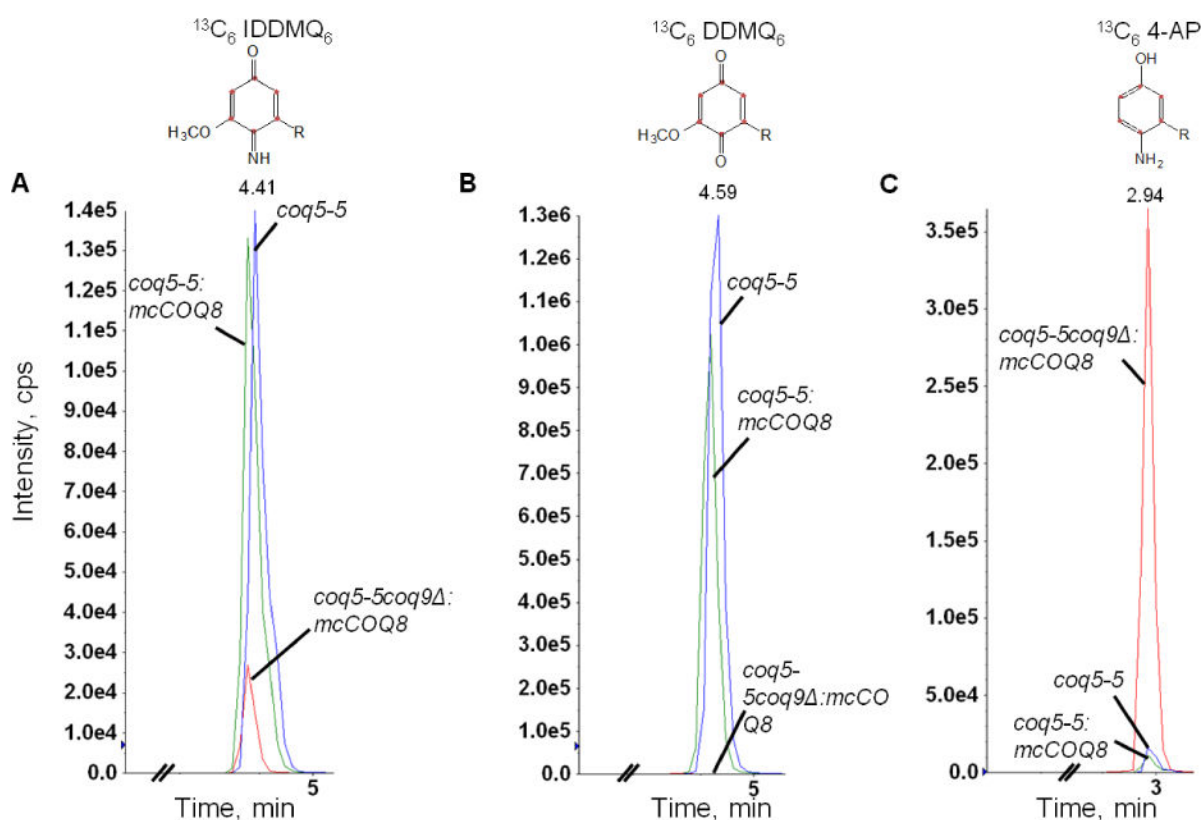
Fig. 2. The deletion of *COQ9* in a yeast *coq5-5* point mutant leads to the accumulation of 3-hexaprenyl-4-aminophenol (4-AP) and the disappearance of demethyl-demethoxy-Q$_6$ (DDMQ$_6$), but imino-demethyl-demethoxy-Q$_6$ (IDDMQ$_6$) is still present

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 (*coq5-5 coq9 :mcCOQ8*), were cultured in SD complete or SD–Ura with 50 μg/ml $^{13}C_6$-pABA and 2 μl ethanol/ml medium at 0.5 A$_{600nm}$/ml and collected after 6 hours. Q$_4$ (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 ($^{13}C_6$ –IDDMQ$_6$), 553.4/159.0 ($^{13}C_6$-DDMQ$_6$), and 524.4/128.0 ($^{13}C_6$-4-AP). Just the oxidized forms of IDDMQ$_6$ and DDMQ$_6$ were detected, while only the reduced form of 4-AP was present. $^{13}C_6$-DDMQ$_6$ accumulates in the *coq5-5* and *coq5-5:mcCOQ8* yeast mutants (*B*), and $^{13}C_6$-IDDMQ$_6$ is readily detected (*A*). $^{13}C_6$-IDDMQ$_6$ and $^{13}C_6$-4-AP accumulate in *coq5-5 coq9 :mcCOQ8* (*A* and *C*), but $^{13}C_6$-DDMQ$_6$ 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 *coq5-5 coq9 :mcCOQ8*.
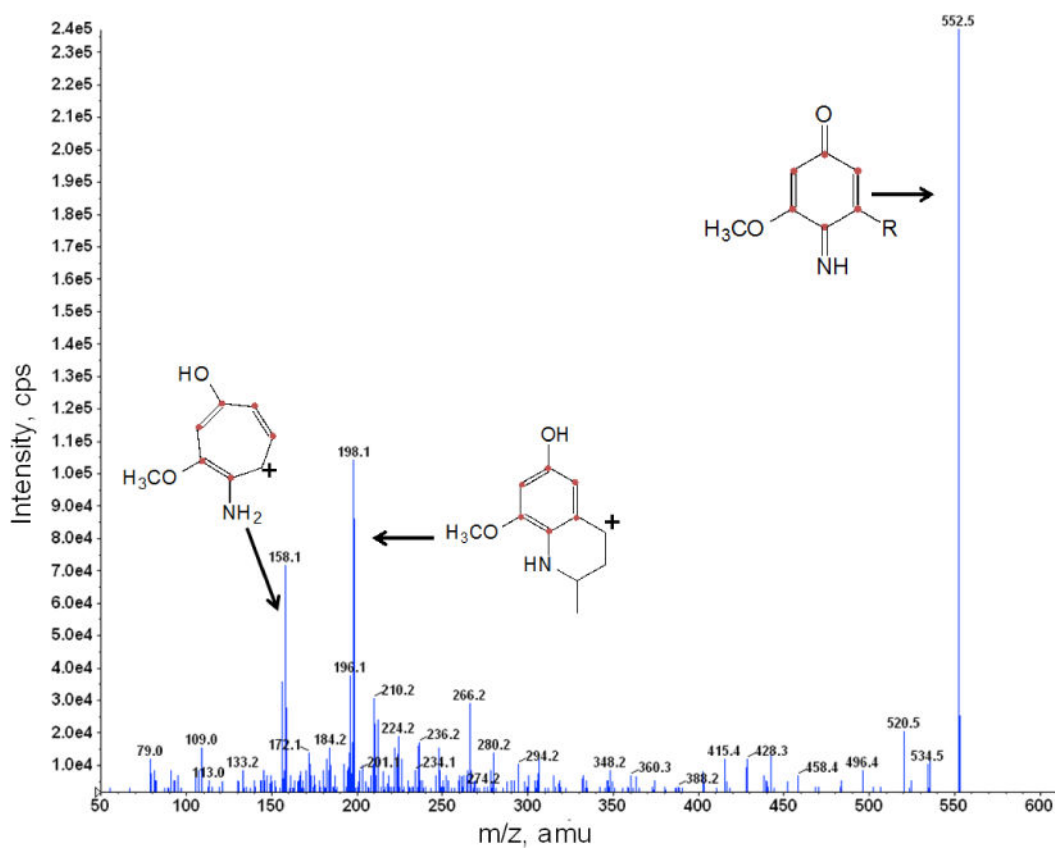
**Fig. 3. Identification of** *de novo* **imino-demethyl-demethoxy-Q$_6$ (^{13}C$_6$-IDDMQ$_6$)**
Yeast *coq5-5 coq9 :mcCOQ8* was cultured and labeled with 50 μg/ml ^{13}C$_6$ pABA as described in Figure 2. Total lipids were extracted and analyzed by RP-HPLC-MS/MS. The fragmentation spectra are shown for ^{13}C$_6$-IDDMQ$_6$ [M+H]$^+$ precursor ion (^{13}C$_6$ ^{12}C$_{31}$H$_{56}$NO$_2^+$; monoisotopic mass 552.4), the ^{13}C$_6$-IDDMQ$_6$ tropylium ion [M]$^+$ (^{13}C$_6$ ^{12}C$_2$H$_{10}$NO$_2^+$; 158.1), and the ^{13}C$_6$-IDDMQ$_6$ chromenylium ion [M]$^+$(^{13}C$_6$ ^{12}C$_5$H$_{14}$NO$_2^+$; 198.1).
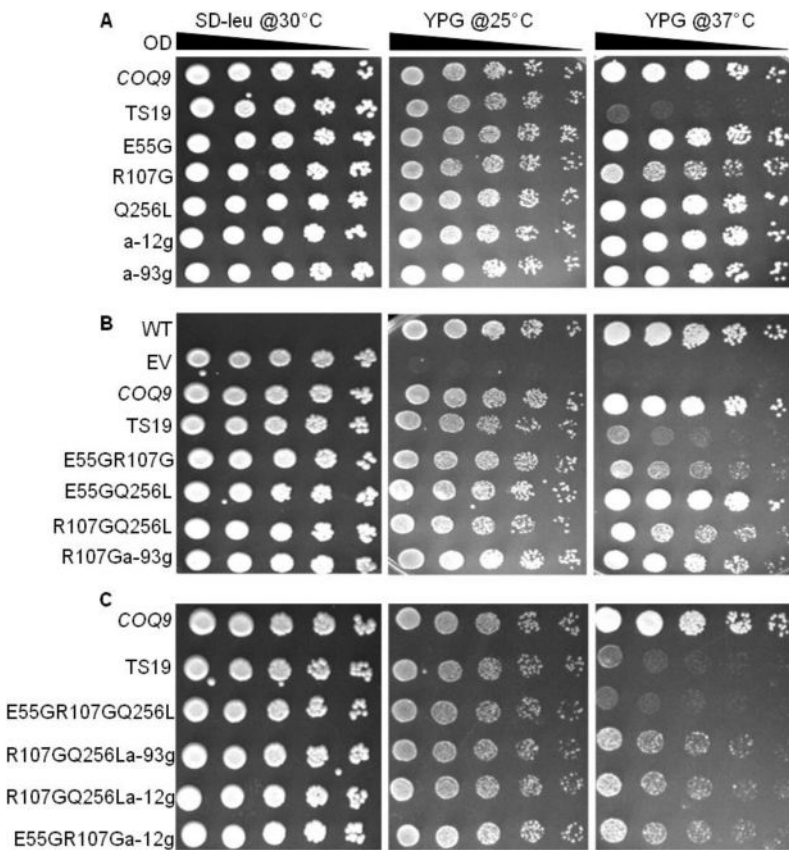
Fig. 4. Characterization of the mutations responsible for temperature sensitivity of the Coq9-TS19 polypeptide

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 μl of 1:5 serial dilutions were spotted onto SD–Leu and YPG plate media and incubated at the specified temperatures for 3 days.
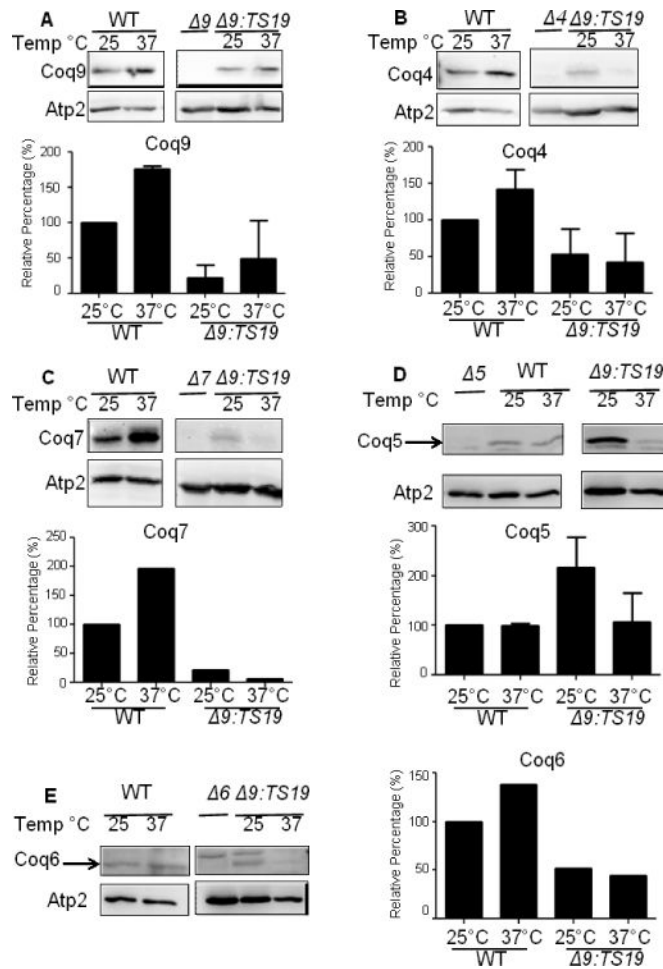
Fig. 5. Expression of Coq9-ts19 polypeptide affects steady state levels of other yeast Coq polypeptides in response to different growth temperatures

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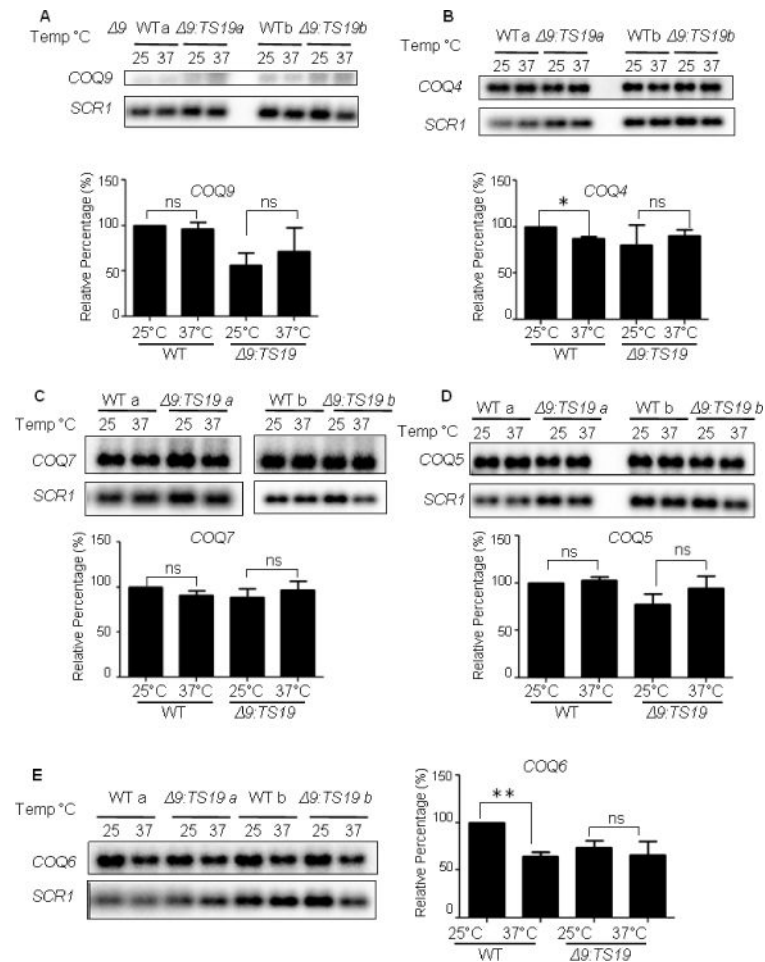
Fig. 6. The observed changes in Coq polypeptide levels with temperature do not correspond to alterations in *COQ* RNA content

The total RNA of W3031B (WT) and W303 ΔCOQ9 harboring the temperature-sensitive plasmid TS19 (Δ9: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 ΔCOQ9 (Δ9), W303 ΔCOQ7 (Δ7), and W303 ΔCOQ4 (Δ4) after yeast were grown overnight at 30 °C. Aliquots of RNA (5 μg) 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.
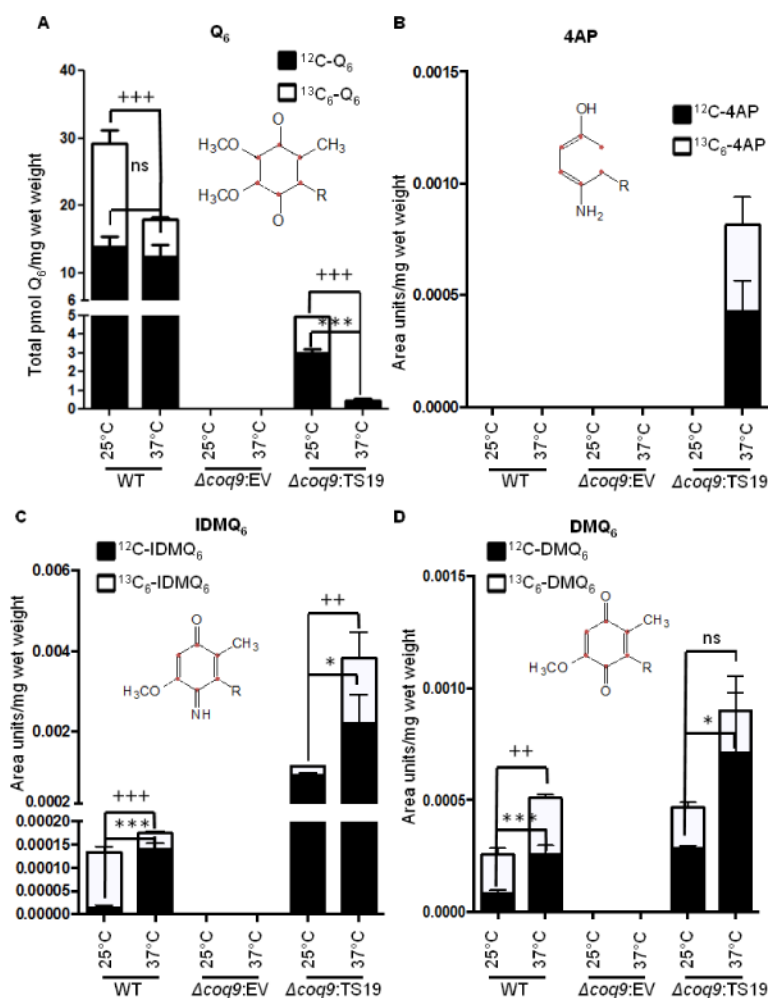
Fig. 7. Yeast strains expressing Coq9-ts19 have decreased Q$_6$ content and accumulate higher levels of nitrogen-containing Q-intermediates at the restrictive temperature

Yeast W3031B (WT), W303 ΔCOQ9 harboring pRS315 empty vector (Δcoq9: EV), and W303 ΔCOQ9 harboring the temperature-sensitive plasmid TS19 (Δcoq9: TS19) were seeded in 20 ml of SD-Complete (WT) or SD–Leu (Δcoq9: EV and Δcoq9: TS19) at 0.01 A$_{600nm}$/ml and grown for 18.5 hours at 25 °C or 37 °C. $^{13}C_6$-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 ^{12}C-compounds and white bars represent the amount of $^{13}C_6$-compounds. For Q$_6$, IDMQ$_6$, and DMQ$_6$, the total amounts of the ^{12}C- and $^{13}C_6$-compounds represent the sum of reduced and oxidized forms; ^{12}C-AP and $^{13}C_6$-4AP, were present only in the reduced form. Significant changes in the amounts of Q$_6$ and Q$_6$ intermediates at different temperatures were determined with the Student's two-tailed t-test. The *symbols represent ^{12}C-compounds in samples at 37 °C compared to ^{12}C-compounds at 25 °C; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. The + symbols represent $^{13}C_6$-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".
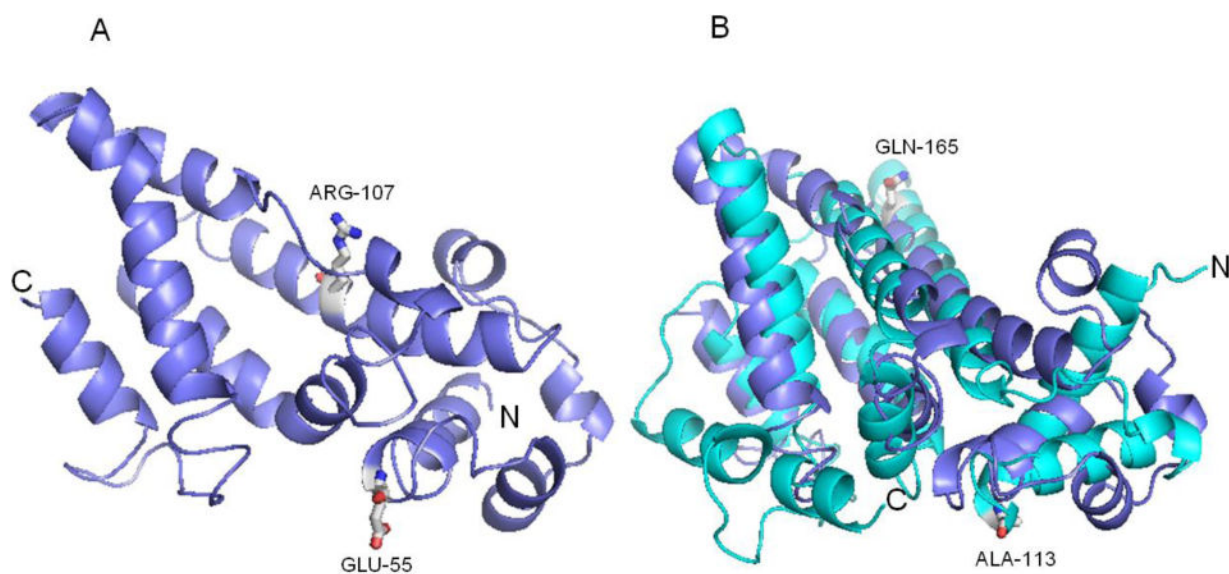
Fig. 8. A homology model of yeast Coq9

(*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 *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303 coq4	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq4::TRP1*	[41]
W303 coq7	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq7::LEU2*	[42]
W303 coq9	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq9::URA3*	[15]
BY4741 coq9	MAT **a** *coq9 ::kanMX4 his3 1 leu2 0 lys2 0 ura3 0*	[43][b]
CH316-6B	MAT α *coq5-5 trp1-1 ura3-1*	[27]
CH316-6B coq9	MAT α *coq5-5 trp1-1 ura3-1 coq9::kanMX4*	This study

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

[b]European *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 *ABC1/COQ8*	Multicopy	[45]
pRS315COQ9 (*COQ9*)	Yeast *COQ9*	Low copy	This work
TS19	Yeast *COQ9*	Low copy	This work
E55G	Yeast *COQ9*	Low copy	This work
R107G	Yeast *COQ9*	Low copy	This work
Q256L	Yeast *COQ9*	Low copy	This work
a-12g	Yeast *COQ9*	Low copy	This work
a-93g	Yeast *COQ9*	Low copy	This work
E55GR107G	Yeast *COQ9*	Low copy	This work
E55GQ256L	Yeast *COQ9*	Low copy	This work
R107GQ256L	Yeast *COQ9*	Low copy	This work
R107GQ256L	Yeast *COQ9*	Low copy	This work
E55GR107GQ256L	Yeast *COQ9*	Low copy	This work
R107GQ256La-93g	Yeast *COQ9*	Low copy	This work
R107GQ256La-12g	Yeast *COQ9*	Low copy	This work
E55GR107Ga-12g	Yeast *COQ9*	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]

[a]Dr. 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′-GTTTGTTCCCGGGCACGGTTTCTCT-3′
R107Gf	5′-GGGTTGATTCCTTCAGTTAAACGATACCCTTTATCTACC-3′
R107Gr	5′-GGTAGATAAAGGGTATCGTTTAACTGAAGGAATCAACCC-3′
Q256Lf	5′-CCCCTAACTAATAGAGATTTGATTAAATTTA 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′-TGTTGATTTCTTCACGGATCGTTCG-3′
COQ5T3R	5′-AATTAACCCTCACTAAAGGGAGCCAGCAGATTTGAATCCTGCCTTC-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′