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Research Article

Hydroxylation of demethoxy-Q_6 constitutes a control point in yeast coenzyme Q_6 biosynthesis

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Abstract. Coenzyme Q is a lipid molecule required for respiration and antioxidant protection. Q biosynthesis in *Saccharomyces cerevisiae* requires nine proteins (Coq1p–Coq9p). We demonstrate in this study that Q levels are modulated during growth by its conversion from demethoxy-Q (DMQ), a late intermediate. Similar conversion was produced when cells were subjected to oxidative stress conditions. Changes in Q_6/DMQ_6 ratio were accompanied by changes in *COQ7* gene mRNA levels encoding the protein responsible for the DMQ hydroxylation, the penultimate step in Q biosynthesis pathway. Yeast *coq* null mutant failed to accumulate any Q late biosynthetic intermediate. However, in *coq7* mutants the addition of exogenous Q produces the DMQ synthesis. Similar effect was produced by over-expressing *ABC1/COQ8*. These results support the existence of a biosynthetic complex that allows the DMQ_6 accumulation and suggest that Coq7p is a control point for the Q biosynthesis regulation in yeast.

Keywords. Ubiquinone, coenzyme Q, mitochondria, yeast regulation.

Introduction

Coenzyme Q (ubiquinone, Q) is an isoprenylated benzoquinone involved in respiration [1], and in the defense against oxidative stress [2]. The location of Q biosynthesis may depends on the organism analyzed. It is synthesized in mitochondria and then distributed among the other membranes of the cell through the endomembrane system pathway in human cells [3]. *Saccharomyces cerevisiae* shares with human cells the same location based on the presence of mitochondrial import signals and also its mitochondrial localization by immunolocalization analysis [4]. However, other locations have been reported for mammalian cells [5, 6] and in pathogenic fungus such as Pneumocystis sp. [7, 8]. The number of isoprenoid units (n) in the polyprenyl tails of Q_n varies depending on the species; humans produce Q_{10}, *Saccharomyces cerevisiae* produce Q_6, and *Caenorhabditis elegans* produce Q_9. Addition of exogenous Q with different tail lengths can augment respiration, but may also induce oxidative stress in mitochondria [3] and impact longevity [9–11], indicating that Q may affect a variety of cellular pathways [12].

At least nine genes are involved in Q biosynthesis in the yeast *S. cerevisiae* (*COQ1–COQ9*) [13, 14]. As is shown in Figure 1, the protein products of some of

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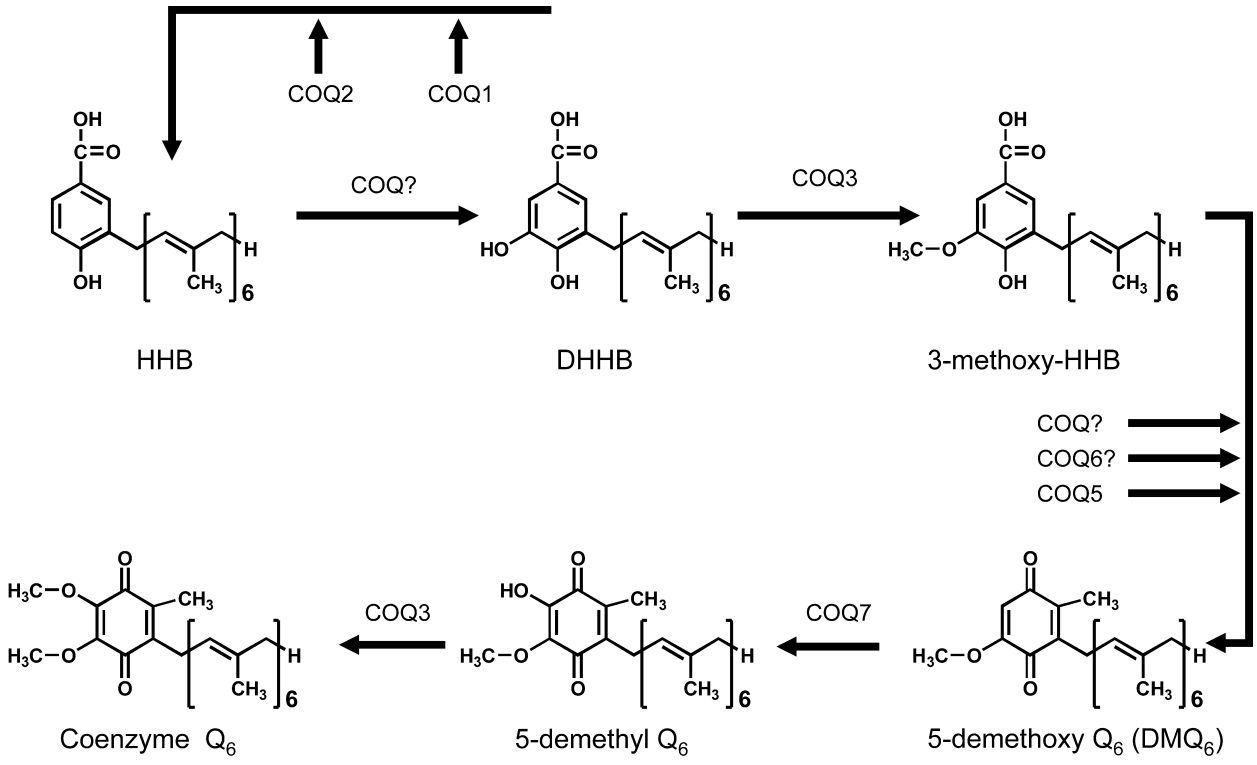

Figure 1. Scheme of coenzyme Q_6 biosynthesis. HHB (3-hexaprenyl-4-hydroxybenzoic acid) is produced by the action of *COQ1* and *COQ2* proteins. HHB is converted to 3,4-dihydroxy-5-hexaprenylbenzoic acid (DHHB) by an unidentified enzyme. The O-methyltransferase encoded by *COQ3* catalyzes the next step to produce 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid. Several modifications of the head group results in 5-demethoxyubiquinone (DMQ_6). Coq7p, a monooxygenase, converts DMQ_6 to 5-demethylubiquinone, which is methylated again by the Coq3p O-methyltransferase to produce Q_6.

these genes have known catalytic functions in eukaryotic Q biosynthesis, but the function of Coq4, Coq6, Abc1/Coq8, and Coq9 still require characterization [13, 15–17].

In yeast *coq* null mutants (*coq3-coq9*), 3-hexaprenyl-4-hydroxybenzoic acid (HHB) is the only Q biosynthetic intermediate detected [13, 15, 18, 19]. It is possible that this result can be explained because most of the other Q-intermediates are rapidly degraded, but a growing body of evidence suggests interactions among Coq proteins lead to formation of a Q-biosynthetic complex [20–22, 23]. However, little is known about the regulation of this pathway in eukaryotic cells. A decrease of the antioxidant α-tocopherol induces Q biosynthesis in rat liver [24], and calorie restriction maintains the Q_{10}/Q_9 ratio in long-lived rats compared to those fed *ad libitum* [25]. Nevertheless, molecular mechanisms involved in this regulation are still unknown [17].

Yeast *coq7* null mutants accumulate HHB, but yeast strains harboring *coq7* point mutations that lead to amino acid substitutions $G_{104}D$ or $E_{194}K$ also produce 2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone (demethoxy-Q_6, or DMQ_6) [26, 27]. This intermediate is also accumulated in *C. elegans clk-1* mutants [28] and *clk-1/coq-7* knockout mice [29]. Both mutants fail to complete development, although dietary Q partially rescues *C. elegans clk-1* mutants [30, 31]. DMQ_6 fails to support both respiration and antioxidant functions in *S. cerevisiae*, but because a small amount of this intermediate is accumulated in wild-type yeast, it may have a specific function in the Q biosynthetic pathway [32].

Here we show that DMQ_6 is accumulated during early log phase of growth of *S. cerevisiae.* We investigate mechanisms responsible for changes in the cellular content of Q_6 and DMQ_6 during growth and in response to oxidative stress. The findings presented here support a model describing two phases of Q biosynthesis. The first phase requires the interactions of several of the Coq polypeptides, and Q_6 itself, to produce DMQ_6. The second phase is defined by the conversion of DMQ_6 to Q_6, and this conversion is regulated by growth phase and by linolenic acid-induced oxidative stress.

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

Strain	Genotype	Source
CEN.PK2–1C	MATa, his3-Δ1, leu2–3,112, trp1–289, ura3–52, MAL2–8c, MAL3, SUC2	[56]
CEN.MP3–1A	CEN.PK2–1C, coq7::HIS3	[56]
CEN coq3	CEN.PK2–1C, coq3::LEU2	[23]
CEN abc1	CEN.PK2–1C, abc1::HIS3	[23]

Materials and Methods

Yeast strains and growth media. Yeast strains used in this study are shown in Table 1. Growth media for yeast was prepared as described [33] and included YPD (1% yeast extract, 2% peptone, 2% dextrose), YPD10 (YPD containing 10% glucose instead of 2%), YPG (1% yeast extract, 2% peptone, 3% glycerol), SDC (0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$, and complete amino acid supplement), and SD-Ura (SDC, but amino acid supplement lacks uracil). Yeasts were grown at 30 °C with shaking (200 rpm).

Plasmid pMCOQ4 was created by digesting the plasmid pTL4 [15] with BamHI and ligating the fragment to pRS426 that had been similarly treated. Plasmid pMABC1 was created by digesting the plasmid p3HN4 [16] with SalI and NotI and ligating the fragment with pRS426 that had been similarly treated. pMCOQ4 and pMABC1 plasmids were used to transform the CEN.MP3–1A yeast strain using the lithium acetate-PEG method [34].

Oxidative stress treatment. Yeast cells were cultured in YPD10 overnight (OD_{600nm} = 9–10), washed and resuspended in sterile 100 mM sodium phosphate buffer, pH 6.2, with 0.2% glucose, at 180 x 10^6 cells/ml. Cell suspensions were incubated with 1 mM H_2O_2, 750 μM oleic acid, or 750 μM linolenic acid for four hours.

In vivo labeling of Q_6 and Q_6-intermediates. p-[U-^{14}C]-hydroxybenzoic acid ([^{14}C]-pHB) was synthesized from L-[U-^{14}C]-tyrosine as previously described [3]. Yeast strains were grown in YPD at 30 °C with shaking (200 rpm) for 48 h in the presence of [^{14}C]-pHB (17 nM or 10^5 ^{14}C-dpm/ml of medium).

Identification of Q_6 and Q_6-intermediates by HPLC/ECD. Lipid extractions of whole cells and quantification of Q_6 and Q_6-intermediates were performed as described [35]. Coenzyme Q_9 was used as an internal standard; 1 μg Q_9 was added per g wet weight cell pellet. Lipid components were separated with a Beckmann 166–126 HPLC system equipped with a 15 cm Kromasil C-18 column in a column oven set to 40 °C, with a flow rate of 1 ml/min and a mobile phase containing 88/24/10 methanol/ethanol/2-propanol and 13.4 mM lithium perchlorate. Quinones were quantified with an ESA Coulochem III electrochemical detector (ECD) and a 5010 analytical cell (E1, –500mV; E2, +500mV). Hydroquinones present in samples were oxidized with a pre-column cell set in oxidizing mode (E +500mV). Q_6 and Q_9 were quantified with external standards.

RPHPLC-APCI-MS/MS multiple reaction monitoring analysis. One gram wet weight pellets of yeast strains as indicated were harvested at 44 hours (14–26 OD/ml) harvested from YPgal media: (2% galactose, 0.1% dextrose) and collected by centrifugation at 1000 x *g* in 50 ml tared borosilicate glass conical tubes with Teflon-lined caps. These were stored at –20 °C until extraction. Samples were thawed on ice, and 183 pmol of internal standard was added (Q_4, Sigma) to samples and standards. This was followed by 1 ml water and 16 ml hexane/2-propanol (3/2, v/v) [36]. After shaking at 200 rpm for 10 minutes the samples were vortexed vigorously for ten seconds and the phases were allowed to separate while standing at room temperature. The upper phase was removed to a 50 ml amber glass vial. The lower phase was re-extracted with hexane:2-propanol; 7:2 (8 ml) twice and the pooled organic phases were concentrated by vacuum centrifugation and stored dry at –20 °C. The four point standard curve (7.8 fmol/μl to 1.01 pmol/μl) was extracted simultaneously with the cell pellet samples.

Dried samples and standards organic extracts were resuspended in 400 μl neat ethanol. These crude lipids were separated (50 μl/injection) by a reverse phase column Thermo Hypersil (Keystone Scientific Operations) BetaBasic 18, 4.6 x 250 mm, 5 μ particle size) equilibrated in water/acetonitrile (50/50 (v/v); solvent A) and eluted (1 ml/min μl/m) with an increasing concentration of 100% acetonitrile; solvent B (min/% acetonitrile: 0/90; 3/90; 7/100; 30/100). The eluate was passed directly into an atmospheric pressure chemical ionization source (APCI, nebulizing gas, "zero"-grade air produced by a Zero Air Generator (Peak Scientific, Chicago, Il) at 2–3 L/s, nebulizer 450 °C, orifice 50 volts) attached to a Perkin-Elmer Sciex (Thornhill,

Canada) API III triple quadrupole mass spectrometer operated in the tandem mass spectrometric (MS/MS) mode (99.999 % argon collision gas at an instrumental GCT setting of 200). The mass spectrometer had been previously tuned and calibrated by flow injection (100 µl/min) of a mixture of polypropylene glycol (PPG) 425, 1000 and 2000 (3.3×10^{-5}, 1×10^{-4} and 2×10^{-4} M, respectively) in water/methanol (1/1, v/v) containing 2 mM ammonium formate and 0.1 % acetonitrile. Calibration across the mass range was made using the singly charged PPG/NH_4^+ ions at m/z 58.99, 326.25, 906.67, 1254.92, 1545.13 and 1863.34 with instrument resolution set so the ^{13}C isotope satellites were resolved with 10–40 % valley.

Quantitative data was collected in the multiple reaction monitoring (MRM) mode by recording the $MH^+ \rightarrow$ tropylium ion transitions (Q_6, m/z 591.4 → 197.0; Q_4, m/z 455.2 →197.0 and DMQ_6, m/z 561.4 → 167.0) with dwell set to 1000 ms, a pause time of 0.3 ms and a total scan time of 4.0 seconds. Standards (DMQ_3 and Q_3) were fragmented and used to confirm the identity of the ions. Synthesis of DMQ_3 was as described [22].

MRM peak areas were measured by instrument manufacturer supplied software (MacSpec™, version 3.3, *PE Sciex*, Ontario, Canada). The calibration curve from the extracted set of standards (ordinate, Q_6/Q_4 peak area ratio; abscissa, Q_6 amount) had correlation coefficient (r^2) of 0.99 with slope = 4.68 (intercept forced to zero). Concentrations of Q_6 and DMQ_6 (in Q_6 equivalents) present in the extracts were directly interpolated from the calibration curve, and quantities in each fraction were calculated after correction for dilution and amount assayed. Lipid extractions and analyses were completed on independent replicate yeast pellets and were injected twice. The data presented represent independent replicate cell pellets and duplicate LC/MS-MS analyses.

RNA isolation and cDNA synthesis. Cells (200×10^6) were harvested by centrifugation and washed twice in DEPC-treated water. Cells were resuspended in 200 µl 20 mM Tris-HCl, pH 7.4, with 30 % glycerol, frozen in N_2(l) and stored at -80 °C until use. For total RNA isolation, cells were thawed and washed with DEPC-treated water. Each cell pellet was resuspended in 500 µl cell-wall-digestion buffer (50 mM potassium phosphate, pH 7.4, 10 mM DTT) and incubated 45 min at 30 °C in the presence of 10 µl Zymolyase 20T, 10 mg/ml (Seikagaku Corporation, Japan). Spheroplasts were collected by centrifugation and total RNA was isolated (Eppendorf Perfect RNA Eukaryotic Mini kit) and quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DL USA). cDNA was synthesized from 1 µg of total RNA in a 20 µl reaction containing Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase buffer, 40 U M-MuLV, 1 mM dNTP mix, 20 U RNase inhibitor (Fermentas Life Sciences, Vilnius, Lithuania) and 500 ng oligo(dT) from MWG, Ebersberg, Germany. Each reaction was performed for 1 h at 42 °C according to manufacturer's directions.

Primers and DNA competitors. Primers were designed with Primer Premier 5.00 software (Premier Biosoft International, Palo Alto, CA, USA) and the available sequence information from SGD yeast database (Table 2). For each gene analyzed, a competitor fragment was generated with recombinant PCR. The competitor fragment was amplified from genomic DNA [33] with the competitor primers (i.e. ABC1-ComF and ABC1-ComR). These primers contain the 5' and 3' specific ends of the sequence used as competitor and a tail corresponding to the sequence of primers used to amplify the target gene (i.e. ABC1-U210 and ABC1-L1260). The competitor was cloned in a T-hang vector such as pMBL (Dominion-MBL SL, Córdoba, Spain) and sequenced to confirm the construct (MWG, Ebersberg, Germany). The target gene fragment was also cloned in the same vector.

For analyses of the *COQ* genes, the *ACT1* gene was used as a competitor. For analyses of the *ACT1* gene (used as calibrator) the *COQ5* gene was used as competitor. The competitor required for expression analyses was amplified by PCR with the appropriate target primers, extracted from agarose (Eppendorf Perfect Gel Cleanup), and quantified by absorbance spectrophotometry. The designated competitor DNA products were prepared and purified freshly for each set of experiments.

Amplification kinetics and determination of the optimal concentration of the competitor DNAs. To test the quality of competitor, each target-competitor pair (0.1 attomol) were used as template for the PCR in the presence of target primers. Samples were removed at the specified cycle number (from cycles numbered 23 to 30), and resolved by agarose gel electrophoresis (Fig. 2A). Both competitor and target specific DNA fragments were quantified with a gel analyzer (Gel Doc 1000, Bio-Rad Laboratories, Hercules, CA, USA). Data obtained were subjected to linear regression analysis validating the effectiveness of each competitor (Fig. 2B). In order to determine the optimal amount of competitor 10-fold serial dilutions of competitor (100–0.1 attomol) were amplified together with constant aliquots (2 µl) of cDNA from the wild-type strain CEN.PK2-1C with target primers and PCR samples were resolved by agarose gel electrophoresis (Fig. 2C). Band in-

Table 2. Primers used in this work.

Name	Sequence (5'→3')
COQ3-U3	GGGATTCATAATGTTGTT
COQ3-L926	AAATAATTACCGACATCG
COQ3-ComF	GGGATTCATAATGTTGTTATTGATAACGGTTCTGGTATGTG
COQ3-ComR	AAATAATTACCGACATCGGTACTTTCTTTCTGGAGGAGCA
COQ4-U25	TCAACAGCTACTTTGCCAGTG
COQ4-L1002	TGGAGTCGTGGCTCGTTT
COQ4-ComF	TCAACAGCTACTTTGCCAGTGATTGATAACGGTTCTGGTATGTG
COQ4-ComR	TGGAGTCGTGGCTCGTTTGTACTTTCTTTCTGGAGGAGCA
COQ5-U61	AGGTGTTTTACGCAAGCTACA
COQ5-L836	TACCTGCCTTCTCAATCATGG
COQ5-ComF	AGGTGTTTTACGCAAGCTCACGAAGCTCCAATGAACCCT
COQ5-ComR	ATCCTGCCTTCTCAATCATGGCCGGCAGATTCCAAACCC
COQ7-U22	CGAGAGTTTTATTCTTGTGAA
COQ7-L709	ATGCTTGATAGCGGTGTCTAG
COQ7-ComF	CGAGCGTTTTATTCTTGTGAAGAAGCTCCAATGAACCCT
COQ7-ComR	ATGCTTGATAGCGGTGTCTAGCCGGCAGATTCCAAACCC
ABC1-U210	ACCAACTTCACGAATATCGAG
ABC1-L1260	GGTTCACCAAGTGTAAGTACA
ABC1-ComF	ACCAACTTCACGAATATCGAGTTCTGAGGTTGCTGCTTTGG
ABC1-ComR	GGTTCACCAAGTGTAAGTACAACTTTCGTCGTATTCTTGTTTTGA
ACT1-U28	ATTGATAACGGTTCTGGTATGTG
ACT1-L1011	GTACTTTCTTTCTGGAGGAGCA
ACT1-ComF	ATTGATAACGGTTCTGGTATGTGAGGTGTTTTACGCAAGCTCAC
ACT1-ComR	GTACTTTCTTTCAGGAGGAGCAATCCTGCCTTCTCAATCATGG
ACT1-F	TTCTGAGGTTGCTGCTTTGG
ACT1-R	ACTTTCGTCGTATTCTTGTTTTGAG

tensities were quantified and expressed as arbitrary units (AU). After a correction depending of the fragment size, the base-10 logarithm of the target/competitor ratio was calculated. The ratios were plotted against the logarithm of the amount of competitor added (Fig. 2D). This plot was used to calculate the equivalence point, the point in which the logarithm of the ratio target/competitor is equal to 0 (target concentration equal to competitor concentration). The measured concentration of the competitor determined the concentration to be used in the expression analysis experiments.

Competitive RT-PCR expression analyses. Samples of yeast cDNA (2 µl from a 20 µl reaction containing 1 µg of total yeast RNA) were amplified together with constant amounts of competitor in a volume of 25 µl. PCR reaction mixtures were resolved by agarose gel electrophoresis and the band intensities were used to calculate the logarithm of the ratio target/competitor. Differences in these ratios indicate differences in expression. Competitive RT-PCR expression method was performed as was described with minor modifications [37, 38].

Results

DMQ_6 is a predominant Q_6-intermediate in log phase yeast cultures. To study the regulation of Q_6 biosynthesis in yeast, we first analyzed the concentrations of both DMQ_6 and Q_6 throughout the growth in both non-fermentable (YPG) (Fig. 3A) and fermentable (YPD) media (Fig. 3B). The Q_6/DMQ_6 ratio was significantly decreased (from 4.0 to 0.5) during the first 10 h culture in YPG culture, due to the transient accumulation of DMQ_6 (Fig. 3A and 3C). The amount of DMQ_6 was surprisingly persistent; even after 24 and 48 hours of culture the content of DMQ_6 remained high. Yeast grown in YPD showed also a transient increase in DMQ_6 content; the Q_6/DMQ_6 ratio decreased from 4.4 to 2.0 over the first 10 h. This

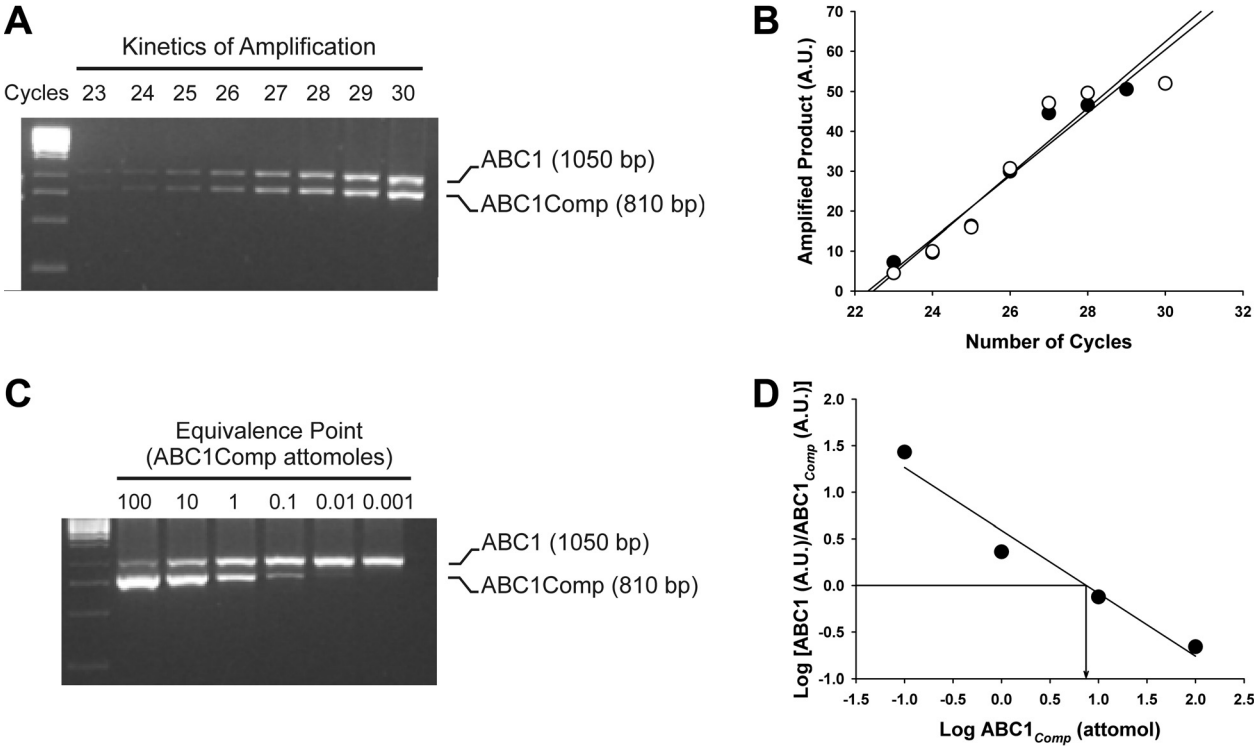

Figure 2. Design of a competitor for the *ABC1/COQ8* gene. The complete procedure is described in *Experimental Procedures*. In this example, a 768 bp fragment of the *ACT1* gene was amplified from genomic DNA with the primers ABC1-ComF/ABC1-ComR to produce an 810 bp fragment corresponding to the *ACT1* gene plus a flanking region recognized by the primers ABC1-U210/ABC-L1260. This fragment is the *ABC1* competitor. The primers ABC1-U210/ABC-L1260 amplify a 1050 bp fragment from yeast genomic DNA corresponding to the *ABC1/COQ8* gene. Both fragments (1050 and 810 bp) were purified and 0.1 attomol of each were used separately as template for PCR with the ABC1-U210/ABC-L1260 primers. PCR samples were removed at the specified cycle number (23 to 30 cycles) and resolved by electrophoresis on a 1% agarose TBE gel (*A*). The band intensities were measured and plotted against the number of cycles to determine the efficiency of each reaction (*B*; closed and open circles represent the competitor and *ABC1*, respectively). In order to determine the optimal amount of competitor DNA, 10-fold serial dilutions of competitor (100–0.001 attomol) were added to a constant amount of cDNA from the wild-type strain CEN.PK2–1C (2 µl from a 20 µl RT-PCR reaction made with 1 µg total RNA). The primers ABC1-U210/ABC-L1260 were added, PCR performed, and the DNA products resolved by agarose gel electrophoresis (*C*). The logarithm of the ratio of the *ABC1* fragment and the *ABC1* competitor fragment was plotted against the logarithm of *ABC1* competitor (*D*). The equivalence point corresponds to the competitor concentration that produces a ratio equal to zero. The amount indicated is the amount of competitor added in subsequent experiments to quantify *ABC1/COQ8* RNA levels.

trend was then reversed by the increase in Q_6 content yielding a ratio of 5.0 by 24 h (Fig. 3B).

Changes in COQ RNA levels as a function of growth phase and culture. Results showed above suggest the existence of a biochemical mechanism through yeast cells could regulate Q_6 biosynthesis by modulation of DMQ_6 to Q_6 transformation rate. To further investigate this process mRNA levels of several genes involved in Q_6 biosynthesis pathway (COQ genes) were determined. Seven of the nine COQ genes products in yeast (Coq3-Coq9) are required for ring modification of Q_6 molecule [20, 21, 23] and Coq7p is the enzyme involved in DMQ_6 hydroxylation (Fig. 1). Therefore, mRNA levels of *COQ3*, *COQ4*, *COQ5*, *COQ7* and *ABC1* genes were measured in yeast grown in YPG and YPD media (Fig. 3A and 3B). In general *COQ* RNA levels are higher in yeast grown in YPG (Fig. 4A). *COQ7, ABC1/COQ8, COQ5,* and *COQ3* RNA levels tend to increase (from 2- to 7-fold) with time in culture. Changes in *COQ* RNA levels as a function of growth phase in YPD media are fairly modest (Fig. 4B), however, *COQ7* RNA levels were significantly elevated as cells transit from log to stationary phase. These results suggest that there are at least two phases in Q_6 biosynthesis; one leading to DMQ_6 biosynthesis driven by a slow increase of the expression of *COQ* genes, and a second phase, driven by the expression of *COQ7*, where the final ring hydroxylation and methylation steps produce Q_6.

The conversion of DMQ_6 to Q_6 responds to oxidative stress. Previous studies have demonstrated antioxidant properties of coenzyme Q. Yeast strains that are unable to synthesize Q_6 or producing only DMQ_6 are very sensitive to oxidative stress induced by H_2O_2 and linolenic acid [32, 39]. Therefore, yeast cells under oxidative stress conditions could potentially activate

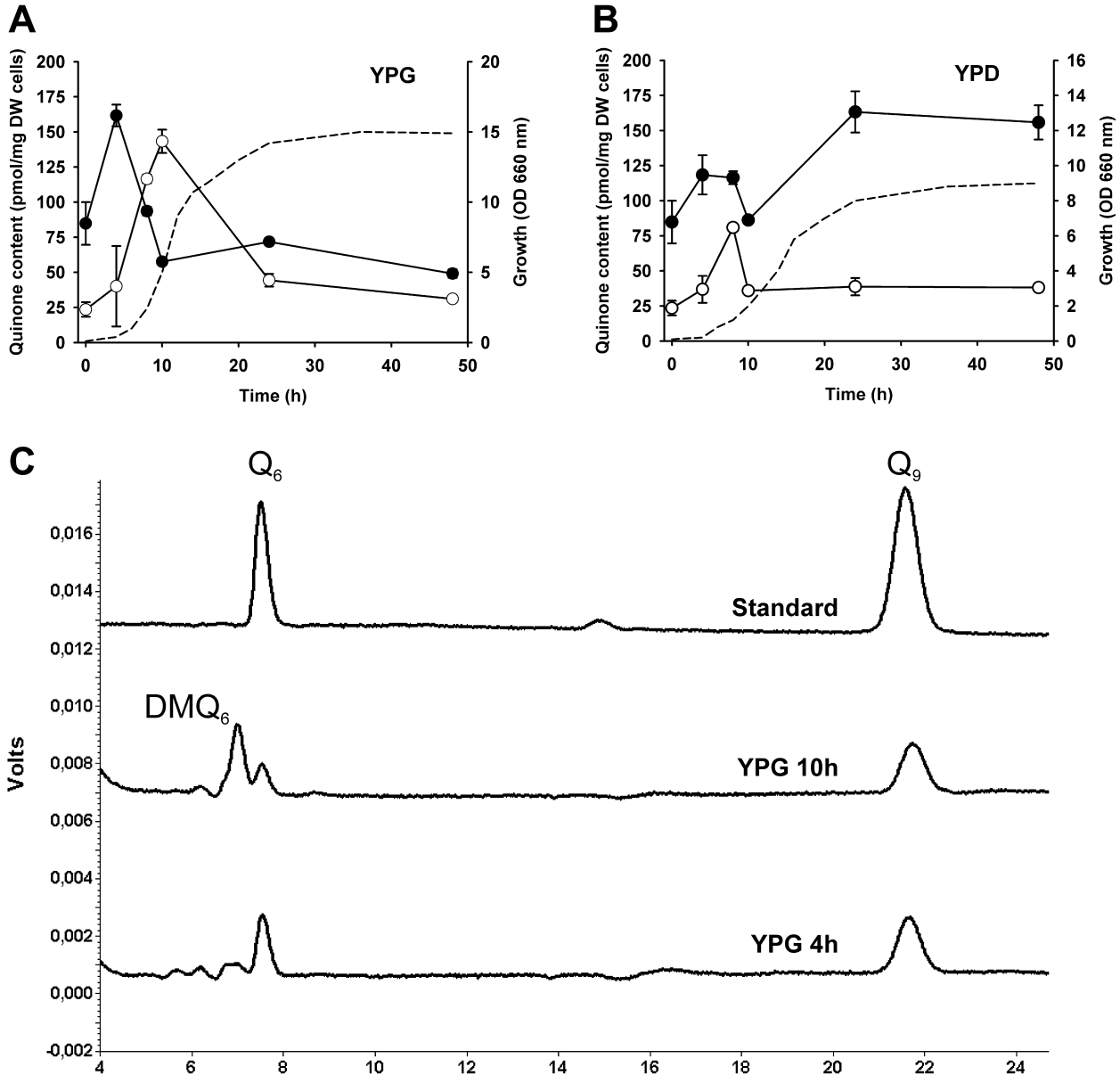

Figure 3. Yeast cell content of Q_6 and DMQ_6 during growth in YPG and YPD. CEN.PK2–1C cells (0.1 OD_{660nm}/ml) were grown in YPG (*A*) and YPD (*B*) media. At the indicated times samples of 1 g wet weight cells were subjected to a lipid extraction and separation by HPLC-ECD to quantify Q_6 and DMQ_6. Data are expressed as the average of three injections from two samples obtained in the same culture ± SD. Closed and open circles designate Q_6 and DMQ_6, respectively. Cell growth was inserted as the dashed line. In panel *C* are shown two chromatograms corresponding to YPG culture at 4 and 10 hours.

Q_6 synthesis in order to increase protection from oxidative damage. To test this hypothesis, in yeast subjected to oxidative stress we have analyzed the content of Q_6 and DMQ_6 in the wild-type CEN.PK2–1C yeast strain treated with H_2O_2, oleic acid, or linolenic acid. This wild-type strain was highly resistant to treatment with either 1 mM H_2O_2 or 750 μM linolenic acid [32].

Figure 5A shows that the Q_6/DMQ_6 ratio was increased (from 0.3 to 0.8) in yeast treated with linolenic acid for 4 h. That increase was due mainly to a shift of DMQ_6 intermediate to Q_6, because total amount of quinones remained unchanged. H_2O_2 did not affect the Q_6/DMQ_6 ratio and even produced a decrease in total quinone content. It is likely that the protective response elicited by H_2O_2 treatment is distinct from that elicited by linolenic acid. Oleic acid was also tested because its provision as a carbon source has been demonstrated to increase the expression of *COQ5*. The promoter of the *COQ5* gene contains an oleic acid response element (ORE) that may boost Q_6 synthesis [40]. However, when provided at the same

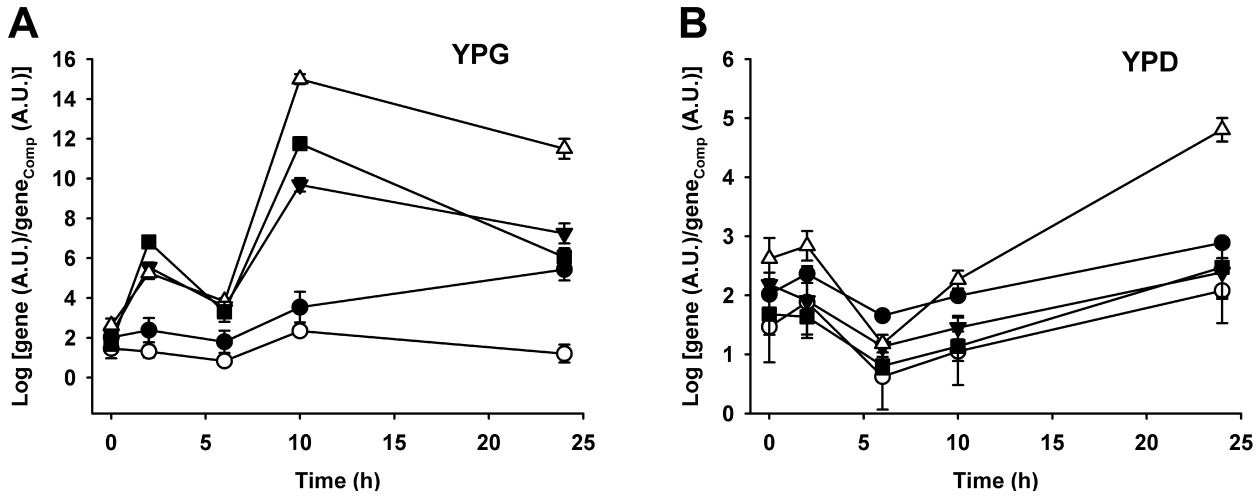

Figure 4. Expression of *COQ* genes during growth in YPG and YPD media. CEN.PK2–1C cells (0.1 OD_{660nm}/ml) were grown in YPG and YPD culture media. At the indicated times samples of 200 x 10^6 cells were subjected to total RNA purification. Levels of *COQ* RNAs were measured as described in *Experimental Procedures* and Figure 2. The amount and size of competitor DNA added to each PCR reaction are as follows: 500 attomol *ACT1*, 840 bp; 60 attomol *COQ3*, 352 bp; *9* attomol *COQ4*, 701 bp; 2 attomol *COQ5*, 542 bp; 2 attomol *COQ7*, 542; and 8 attomol *ABC1*, 810 bp. Target size were 983 bp *ACT1*, 588 bp *COQ3*, 977 bp *COQ4*, 796 bp *COQ5*, 708 bp *COQ7*, and 1050 bp *ABC1*. PCR samples from YPD cultures (Fig. S1A) and YPG cultures (Figure S1B) were resolved by electrophoresis on a 1 % TBE agarose gel, and densitometry analyses were performed according to *Experimental Procedures*. Expression data for YPG (*A*) and YPD (*B*). Symbols correspond to *COQ3* (●), *COQ4* (○), *COQ5* (▼), *COQ7* (△) and *ABC1* (■). *ACT1* RNA levels were used to normalize each of the *COQ* RNAs, and corrections were made to account for effects of fragment size. Data correspond to the average of two independent PCR reaction ± SD using the same total RNA sample. This experiment is representative of a set of three.

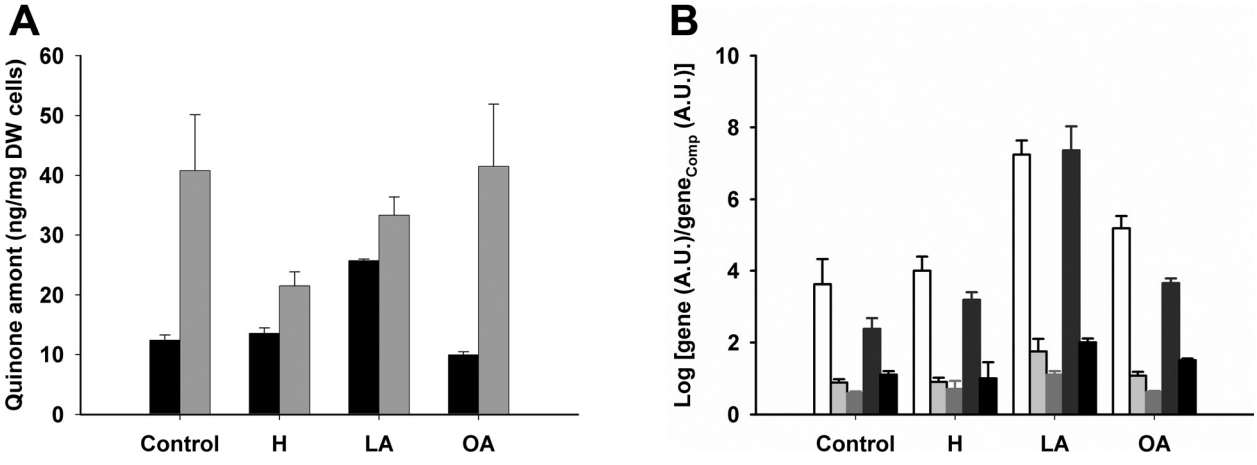

Figure 5. *COQ* RNA levels and content of Q_6 and DMQ_6 in response to oxidative stress. CEN.PK2–1C cells were subjected to different oxidative stress conditions (1mM H_2O_2, or 750 μM linolenic acid) or to control treatments (no addition, or 750 μM oleic acid) for four hours. Cells (900 x 10^6) were collected and analyzed for quinone content as described (*A*; Q_6, black bars; DMQ_6, gray bars). About 200 x 10^6 cells were collected to determine *COQ* RNA content (Fig. S1C), and densitometry data were plotted to calculate gene expression (*B*). Bars correspond (from white to black) to *COQ3*, *COQ4*, *COQ5*, *COQ7* and *ABC1/COQ8* genes. Control, cells without oxidative stress treatment; H, 1mM H_2O_2 incubation; LA, 750 μM linolenic acid; OA, 750 μM oleic acid. RNA levels presented are from the average of two independent PCR reactions ± SD derived from the same total RNA sample. Data are representative of two similar experiments.

concentration as linolenic acid, oleic acid treatment does not produce significant changes in the Q_6 and DMQ_6 levels.

To investigate whether the oxidative stress treatments affected *COQ* gene expression, we analyzed the steady state *COQ* RNA levels. Linolenic acid, but neither H_2O_2 nor oleic acid, increased both *COQ7* and *COQ3* RNA levels following a 4 h treatment relative to control (Fig. 5B). Other *COQ* RNA levels examined did not change significantly, including *COQ5*, in response to oleic acid treatment (Fig. 5B). These results suggest that treatment with linolenic acid acts to increase *COQ7* and *COQ3* RNA levels, and acts to enhance the conversion of DMQ_6 to Q_6 as a mechanism to provide antioxidant protection.

DMQ_6 can be synthesized in yeast in the absence of Coq7p. Yeast strains harboring null mutations in one of the *COQ* genes (*COQ3* to *COQ9)* accumulate HHB (3-hexaprenyl-4-hydroxybenzoic acid), an early Q-intermediate that is the product of the reaction carried out by Coq2p [13, 26, 41, 42]. Several lines of physical evidence now indicate the existence of a biosynthetic Coq-polypeptide complex inside mitochondria [22]. Supplementation of growth media with exogenous Q_6 has been shown to rescue mitochondrial respiration in the yeast *coq* mutants [43] and to preserve steady state levels of Coq3 and Coq4 polypeptides [27] of a *coq7* null mutant. In addition, it has been shown that Q_6 and DMQ_6 are associated with a polypeptide complex defined by Coq3p and Coq4p products [22]. This latter observation suggests that interaction between Q_6 and *COQ* genes products could be important for the biosynthetic complex stabilization and regulation of Q_6 biosynthesis. To determine whether the functional interaction among Coq polypeptides depends on the presence of Q_6, we analyzed the effect of addition of exogenous Q_6 on *coq* null mutants growing in YPD. The analysis of lipid extracts from each of the *coq* mutants (*coq3-abc1/coq8*) indicated that a product co-eluting with DMQ_6 was produced only in the *coq7* mutant strain cultured in the presence of exogenous Q_6 (Fig. S3).

To identify the material co-eluting with DMQ_6, we performed RP-HPLC/MS-MS analysis of the lipid extracts prepared from the wild-type parental strain and the *coq7* null mutant cultured in the presence and absence of exogenously added Q_6. As described in experimental procedures, quantitative data was collected in the multiple reaction monitoring (MRM) mode. This method separates lipid extracts, producing specific retention times for the compounds of interest; the detection of the lipids involves selection of a specific precursor (parent) molecule, which is fragmented for quantification of a specific product (daughter) molecule. By these criteria the Q_6 and DMQ_6 content in the CEN.PK2–1C wild-type yeast were 62.73 ± 15.13 and 4.22 ± 1.04 pmol/gm wet weight, respectively. Neither Q_6 nor DMQ_6 were present at measurable amounts in the *coq7* null yeast strain. However, when cultured in the presence of exogenous Q_6, the *coq7* null mutant contained quantities of both Q_6 (8.05 ± 3.16 pmol/gm wet weight) as well as DMQ_6 (0.24 ± 0.10 pmol/ gm wet weight).

To determine whether the DMQ_6 produced by the *coq7* mutant represented *de novo* synthesis, several of the *coq* null mutant strains were grown in the presence of [^{14}C]-*p*HB, the aromatic ring precursor, and in the presence or absence of exogenously added Q_6. Supplementation of the growth media with exogenously added Q_6 did not affect the incorporation of [^{14}C]-*p*HB into either DMQ_6 or Q_6 in the wild-type strain (Fig. S4). Similar incubations of the *coq3* and *abc1* null mutant strains showed that neither produced detectable DMQ_6 or Q_6, and each produced only a more polar radiolabeled peak eluting at about 6 min, as predicted for HHB (Fig. 6A and C). However, a significant radiolabeled peak corresponding to [^{14}C]-DMQ_6 was observed in lipid extracts prepared from the *coq7* null mutant strain following incubation with [^{14}C]-*p*HB plus exogenous Q_6 (Fig. 6B). Because growth of the *coq7* null mutant in the presence of exogenous Q_6 leads to uptake of Q_6 [44], these results indicate that Q_6 itself supports interaction of the Coq proteins and enables *de novo* synthesis of DMQ_6. Thus, Q_6 is a crucial lipid component of the Coq polypeptide biosynthetic complex, and its presence may be required to facilitate formation of, and/or stabilize, the Coq polypeptide biosynthetic complex. COQ RNA levels were quantified in the CEN MP3.1A (*coq7* null) strain in response to culture in the presence or absence of exogenous Q_6 (Fig. 7). Addition of exogenous Q_6 did not produce a change in *COQ3*, *COQ4*, *COQ5*, or *ABC1/COQ8* RNA levels, indicating that the *de novo* synthesis of DMQ_6 is not driven by changes in RNA levels. Recent studies show that exogenously added Q_6 to *coq7* null mutant cultures enhanced the steady state levels of the Coq3 and Coq4 polypeptides [27]. Hence, it is likely that the addition of Q_6 acts to stabilize the Coq polypeptides.

Abc1p and Coq4p regulate DMQ_6 synthesis. To further understand whether there are more factors involved in the stabilization of Coq polypeptides, we analyzed whether an alteration of the expression of certain Coq proteins would lead to complex stabilization and DMQ_6 synthesis in a *coq7* null mutant strain. Of the *COQ* genes participating in the synthesis of DMQ_6, Coq4p and Abc1/Coq8p do not have an assigned function, although Abc1/Coq8p has recognized sequence motifs that identify it as a potential Hank's Ser/Thr protein kinase [17, 45]. To study the possible functions of Coq4p and Abc1/Coq8 in DMQ_6 biosynthesis, the yeast *coq7* null mutant strain was transformed with a multi-copy plasmid harboring either *COQ4* or *ABC1* coding sequence including the natural promoter. The over-expression of *COQ4* or *ABC1* in the *coq7* null mutant does not produce significant changes in the expression of other *COQ* genes (Fig. 8A). Lipid extracts of these cells were analyzed for content of Q_6 and DMQ_6 by HPLC-ECD. Multicopy expression of *COQ4* produces the synthesis of a small amount of DMQ_6 as compared with the *coq7* null mutant strain; however, the multicopy expression of *ABC1/COQ8* increases dramatically the amount of DMQ_6, to levels much higher than detected in wild-

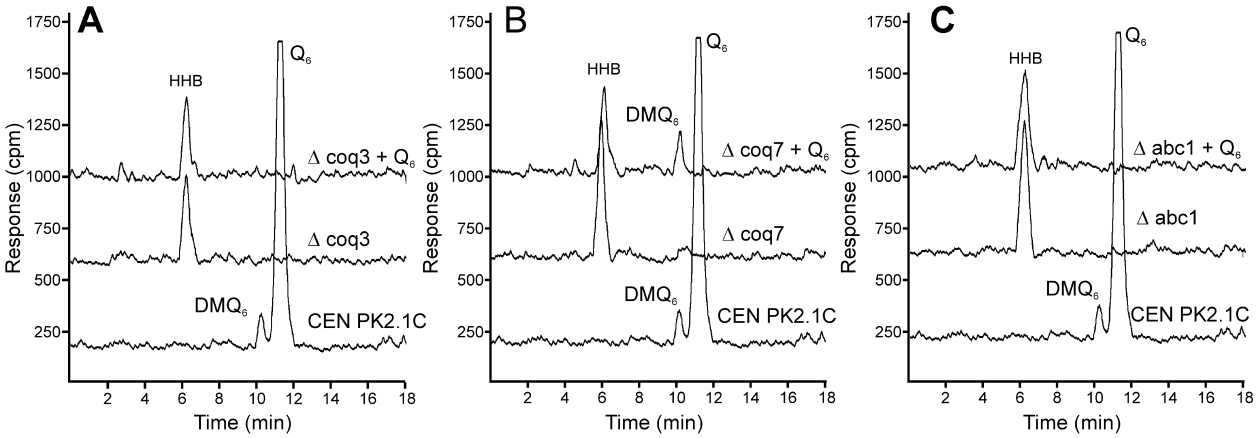

Figure 6. Unique *de novo* synthesis of DMQ_6 in *coq7* null yeast mutants cultured in the presence of exogenously added Q_6. (*A*) Yeast CEN.PK2–1C (wild-type), *coq3*, *coq7,* or *abc1/coq8* null mutants were cultured in YPD in the presence of [*U*-^{14}C]*p*-hydroxybenzoic acid. Where indicated, media was also supplemented with 2 μM Q_6. Cells were collected and subjected to lipid extraction and separation by RP-HPLC coupled to a radiometric detector in order to detect ^{14}C-Q_6 and ^{14}C-Q_6-intermediates. The profile of ^{14}C-labeled material detected in lipid extracts of CEN.PK2–1C is shown as the bottom trace in each panel. (*A*) *coq3*; (*B*), CEN.MP-1A (*coq7)*; and (*C*) *abc1*/*coq8.* Data shown correspond to a representative experiment of a set of two.

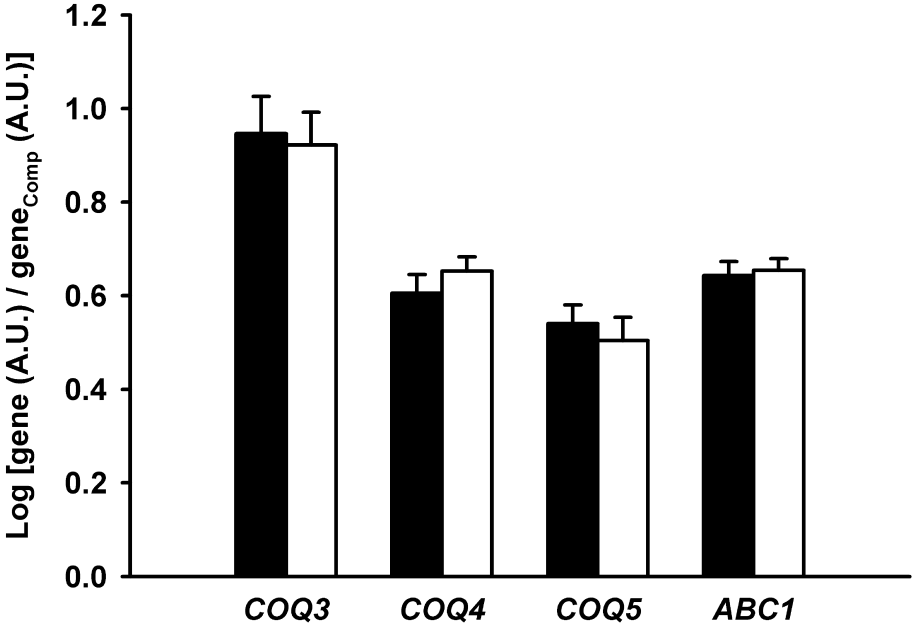

Figure 7. Q_6 supplementation does not affect *COQ* RNA content in the *coq7* null mutant. The CEN.MP3–1A strain (*coq7*) was cultured in YPD in the presence ($+Q_6$) or absence ($-Q_6$) of exogenous Q_6 (2 μM). Total RNA was isolated and the content of *COQ* RNA was determined as previously described (Fig. S2A). Densitometry data were plotted to calculate RNA levels in the absence (black bars) or presence (white bars) of Q_6 supplementation.

type cells (Fig. 8B). These results support a regulatory function for Abc1/Coq8p; and indicate that overexpression of Abc1/Coq8p can facilitate the formation of a Coq7p-independent complex for DMQ_6 biosynthesis in *S. cerevisiae.*

Discussion

Several lines of evidence presented here suggest that hydroxylation of DMQ_6 may serve as a control point in the biosynthesis of Q_6. DMQ_6 is a predominant intermediate in log phase yeast cells. As cells transition from log to stationary phase growth, the DMQ_6 content decreases and the Q_6 content increases. This regulatory point of coenzyme Q biosynthesis is not the only in yeast, another regulatory point may be related for the protein Coq2p. Previous studies [46] have shown that, in yeast cultured with increased concentrations of glucose, a decrease of Q_6 and DMQ_6 levels resulted, while HHB increased. This supports the idea of an initial and strong regulatory point based in a phosphorylation cycle, linked to the fermentative growth phase in yeast. To investigate whether the control of the content of DMQ_6 and Q_6 is due to changes in gene expression, *COQ* RNA levels were

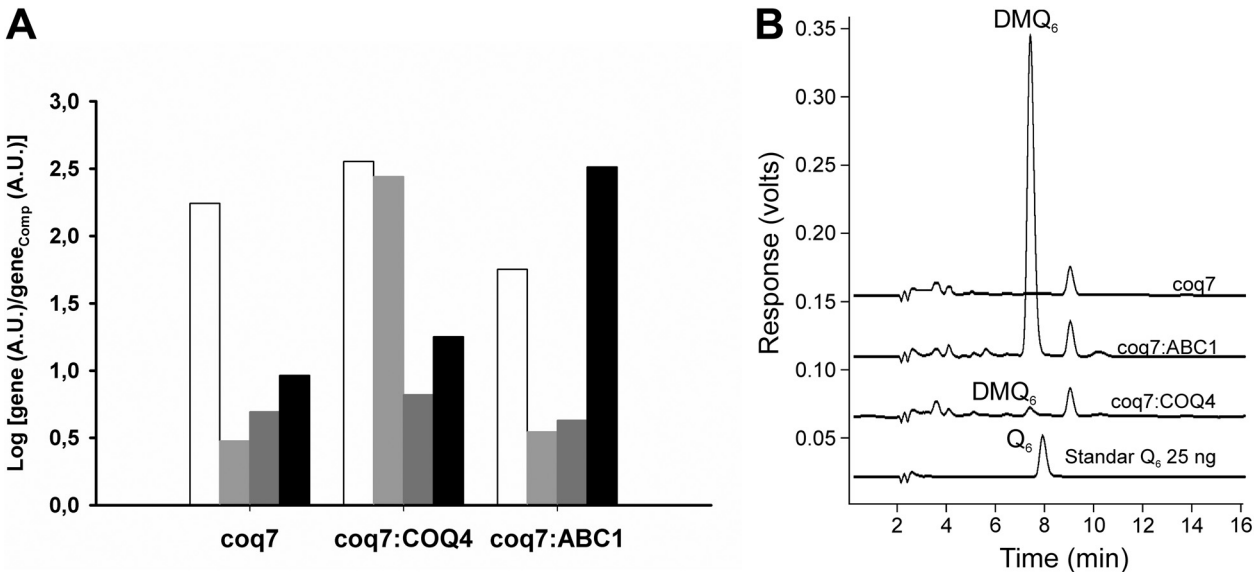

Figure 8. Expression of yeast *ABC1/COQ8* in multicopy facilitates production of DMQ_6 in the *coq7* null mutant. CEN.MP3–1A (*coq7*) null mutant yeast harbouring the *ABC1/COQ8, COQ4,* or empty vector on multicopy plasmids were cultured in SDc–Ura +glucose media for 16 hours. Total RNA was isolated from 200 x 10^6 cells and the content of *COQ* RNA was determined as previously described (Fig. S2B). Band intensities were quantified by densitometry and plotted to calculate *COQ* RNA levels (*A*). Bars correspond to RNA content of *COQ3*, *COQ4*, *COQ5*, and *ABC1/COQ8* (from white to black). The same cells (1 g wet weight) were collected and subjected to lipid extraction and separation by HPLC-ECD to detect lipid quinones (*B*). CEN.MP3–1A with the empty vector pRS426 (coq7); with multicopy *ABC1/COQ8* gene (coq7::ABC1); CEN.MP3–1A with multicopy *COQ4* (coq7::COQ4); Q_6 standard (25 ng).

quantified. *COQ* RNA levels were generally higher in cells grown in media containing glycerol (YPG) as compared to dextrose (YPD). This has been observed previously [15, 47, 48]. In YPG media *COQ7*, *ABC1/COQ8*, *COQ5* and *COQ3* RNA levels increased (from 2- to 7-fold) as cells transitioned from log to stationary phase. In contrast, *COQ* RNA levels in YPD media stay fairly constant, except for *COQ7* RNA levels, which increase about 5-fold in stationary phase cells as compared to log phase. The data suggest that Q_6 synthesis may take place in two phases; the first phase produces DMQ_6 as a predominant intermediate, and the second phase is driven by the induced expression of the *COQ7* gene, which is a member of the di-iron carboxylate family of proteins and catalyzes the hydroxylation of DMQ_6 [49], enabling the final *O*-methylation step to produce Q_6.

We show that treatment with linolenic acid, but not oleic acid, increased Q_6 content, decreased DMQ_6, and increased *COQ7* and *COQ3* RNA levels (Fig. 5). Linolenic acid, a polyunsaturated fatty acid, is prone to autoxidation, and products of linolenic acid autoxidation are toxic to Q-deficient yeast, which can be rescued by restoration of Q_6 biosynthesis, or by the addition of exogenous Q_6 [32, 39]. These studies indicate that reduced Q (QH_2) serves as an essential lipid soluble antioxidant [50]. Because the hypersensitivity is observed in response to linolenic acid, but not oleic acid, (a monounsaturated fatty acid that is much more resistant to autoxidation), we propose that the increase in *COQ7* and *COQ3* RNA levels represents a stress response. Transcription of the yeast *COQ5* gene is induced by growth in media containing oleic acid as sole carbon source [51]. However, in our studies treatment with oleic and linolenic acid were performed at lower concentrations of fatty acid and for only a short time (four hours). Our interpretation is that the linolenic acid-induced oxidative stress acts to degrade Q_6, and the increase in DMQ_6 content and in *COQ3* and *COQ7* RNA levels represents a cellular stress-response to replenish Q_6 and provide antioxidant protection. Up-regulation of Q biosynthesis in response to vitamin E deficiency in rats has been previously demonstrated [24]. Notably, *COQ7* gene expression was induced in small lung cancer cells following treatment with camptothecin [52]. The induction of *COQ7*, and resulting increase in cellular Q content was postulated to protect these cells from the stress caused by this topoisomerase I inhibitor.

The yeast *coq7* null mutant accumulates HHB (hydroxyl hexaprenyl benzoate) [26]. However, we show here that accumulation of newly synthesized DMQ_6 was induced in yeast *coq7* null mutants by either addition of exogenous Q_6 or over-expression of the *ABC1/COQ8* gene. The identity of DMQ_6 was verified by RP-HPLC/MS-MS analyses and identification of the parent ion and its fragmentation to a

specific daughter ion. Furthermore, the production of DMQ_6 represents *de novo* synthesis because incubation of the *coq7* null mutant in the presence of [^{14}C]-4-HB, the aromatic ring precursor of Q biosynthesis, led to the production ^{14}C-DMQ_6, but only if the growth media was supplemented with exogenous Q_6. This was a unique feature of the *coq7* null mutant, as DMQ_6 was not detected when any of the other *coq* null mutants were cultured in the presence of exogenous Q_6. Previous studies have shown that *coq* null mutants take up exogenously supplied Q_6, with a restoration of respiratory electron transport. Supplemental Q_6 also acts to enhance steady state levels of the Coq3 and Coq4 polypeptides in the *coq7* null mutant [27], but does not affect *COQ* RNA levels (Fig. 7). The results presented here indicate that Q_6 itself serves as a crucial lipid component and acts to facilitate formation of, and/or stabilize the Coq polypeptide Q_6-biosynthetic complex. Our results also show that the Abc1/Coq8 polypeptide may serve a similar function, as its overexpression in the *coq7* null mutant also allowed DMQ_6 to be produced.

Aside from its function as a Q biosynthetic intermediate, what is the role of DMQ_6? Although postulated to function in respiratory electron transport or as an antioxidant [53], this seems unlikely because yeast mutants that lack Q_6 and produce only DMQ_6 are respiratory defective and hypersensitive to oxidative stress [32, 44]. Several lines of evidence support the idea that DMQ_6 functions as a lipid component of the Coq polypeptide biosynthetic complex. Genetic evidence argues for the involvement of Q or a Q-intermediate in stabilizing the Coq3, Coq4, and Coq6 polypeptides [21]. DMQ_6 co-elutes with Coq3, Coq4 and Coq6 polypeptides as a high molecular weight complex when digitonin extracts of mitochondria are subjected to size exclusion chromatography [22]. Certain yeast *coq7* point mutants (e.g. *coq7E194K*) have higher steady state levels of the Coq3 and Coq4 polypeptides and produce DMQ_6. Although the enhanced Coq3 and Coq4 polypeptide levels may be due to the presence of the Coq7-E194K polypeptide, it is also possible some of the effects are due to the presence of the DMQ_6 intermediate itself [27]. Finally, we note that as an unsubstituted quinone, DMQ_6 is a potentially potent electrophile that may participate in Michael addition reactions and modify a variety of nucleophilic groups [54]. In fact, H_2O_2 incubation does not affect the expression of COQ genes (Fig. 5B) but increases the Q_6/DMQ_6 ratio (Fig. 5A) decreasing the DMQ_6 level. However, linolenic acid increases the expression of *COQ7* and *COQ3* genes together with an increased Q_6/DMQ_6 ratio. This data point to a possible DMQ_6 degradation but only produced by H_2O_2. This differential behavior of both oxidative stress agents supports the idea that DMQ_6 may be protected by its accumulation in Q_6 biosynthetic complexes or in mitochondrial respiratory complexes containing endogenous Q_6 such the bc_1 complex. Thus, previous studies [32] have shown that in yeasts that only produce DMQ_6, the incubation with linolenic acid increases the superoxide anion generation. This effect was not seen in wild type strains that produce both Q_6 and DMQ_6. The protective effect can be deduced by the fact that DMQ_6-producing strains cannot stabilize the bc_1 complex [32, 44]

These results support a model where DMQ_6 biosynthesis in *S. cerevisiae* may take place in the complete absence of the Coq7 polypeptide, provided either the lipid product Q_6 is present, or Abc1/Coq8 is overexpressed. The role of Abc1/Coq8 polypeptide in Q biosynthesis is not known, but based on sequence motifs it has been identified as Hank's protein kinase [45]. Thus, Abc1/Coq8p is a candidate for the post-translational regulation of Q biosynthesis. Overexpression of *ABC1/COQ8* suppresses the effect of *coq9–1* mutation in *S. cerevisiae* [13]. Here we show *ABC1/COQ8* overexpression allows *coq7* null mutants to produce DMQ_6.

These results support the hypothesis that Coq7p regulates the rate of Q biosynthesis in yeast. A complex of Coq polypeptides serves to generate DMQ_6, a prevalent Q-intermediate in log phase cells. The second phase of Q biosynthesis entails the hydroxylation of DMQ_6 by Coq7p, and subsequent *O*-methylation by Coq3p to form Q_6. This idea was strongly supported by classical studies on coenzyme Q biosynthesis in rat liver, where DMQ_9 was accumulated during Q_9 biosynthesis analysis performed in liver slices [55]. Therefore, the final Q_6 synthesis requires the interaction of the Coq7 polypeptide with the first organized complex. The transitions between these two complexes may serve as a control point for Q_6 biosynthesis, and the accumulation of DMQ_6 itself may provide a reserve for the rapid supply of Q_6.

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1 Trumpower, B. L. (1981). New concepts on the role of ubiquinone in the mitochondrial respiratory chain. J. Bioenerg. Biomembr. 13, 1–24.

2 Villalba, J. M., Crane, F. L., and Navas, P. (1998). Plasma Membrane Redox System and their role in Biological Stress and Disease. H. Asard, A. Berczi, and R.J. Caubergs, eds. (Dordrecht: Kluwer).

3 Fernandez-Ayala, D. J., Brea-Calvo, G., Lopez-Lluch, G., and Navas, P. (2005). Coenzyme Q distribution in HL-60 human cells depends on the endomembrane system. Biochim. Biophys. Acta. 1713, 129–137.
4 Tran, U. C., and Clarke, C. F. (2007). Endogenous synthesis of coenzyme Q in eukaryotes. Mitochondrion. 7 Suppl, S62–71.
5 Teclebrhan, H., Olsson, J., Swiezewska, E., and Dallner, G. (1993). Biosynthesis of the side chain of ubiquinone:transprenyltransferase in rat liver microsomes. J. Biol. Chem. 268, 23081–23086.
6 Kalen, A., Norling, B., Appelkvist, E. L., and Dallner, G. (1987). Ubiquinone biosynthesis by the microsomal fraction from rat liver. Biochim. Biophys. Acta. 926, 70–78.
7 Kaneshiro, E. S., Basselin, M., Merali, S., and Kayser, O. (2006). Ubiquinone synthesis and its regulation in *Pneumocystis carinii*. J. Eukaryot. Microbiol. 53, 435–444.
8 Basselin, M., Hunt, S. M., Abdala-Valencia, H., and Kaneshiro, E. S. (2005). Ubiquinone synthesis in mitochondrial and microsomal subcellular fractions of *Pneumocystis* spp.: differential sensitivities to atovaquone. Eukaryot. Cell. 4, 1483–1492.
9 Asencio, C., Rodriguez-Aguilera, J. C., Ruiz-Ferrer, M., Vela, J., and Navas, P. (2003). Silencing of ubiquinone biosynthesis genes extends life span in *Caenorhabditis elegans*. FASEB J. 17, 1135–1137.
10 Larsen, P. L., and Clarke, C. F. (2002). Extension of life-span in *Caenorhabditis elegans* by a diet lacking coenzyme Q. Science. 295, 120–123.
11 Ishii, N., Senoo-Matsuda, N., Miyake, K., Yasuda, K., Ishii, T., Hartman, P. S., and Furukawa, S. (2004). Coenzyme Q_{10} can prolong C. elegans lifespan by lowering oxidative stress. Mech. Ageing Dev. 125, 41–46.
12 Turunen, M., Olsson, J., and Dallner, G. (2004). Metabolism and function of coenzyme Q. Biochim Biophys Acta. 1660, 171–199.
13 Johnson, A., Gin, P., Marbois, B. N., Hsieh, E. J., Wu, M., Barros, M. H., Clarke, C. F., and Tzagoloff, A. (2005). *COQ9*, a New Gene Required for the Biosynthesis of Coenzyme Q in *Saccharomyces cerevisiae*. J. Biol. Chem. 280, 31397–31404.
14 Tzagoloff, A., and Dieckmann, C. L. (1990). PET genes of *Saccharomyces cerevisiae*. Microbiol. Rev. 54, 211–225.
15 Belogrudov, G. I., Lee, P. T., Jonassen, T., Hsu, A. Y., Gin, P., and Clarke, C. F. (2001). Yeast *COQ4* encodes a mitochondrial protein required for coenzyme Q synthesis. Arch. Biochem. Biophys. 392, 48–58.
16 Do, T. Q., Hsu, A. Y., Jonassen, T., Lee, P. T., and Clarke, C. F. (2001). A Defect in Coenzyme Q Biosynthesis Is Responsible for the Respiratory Deficiency in *Saccharomyces cerevisiae abc1* Mutants. J. Biol. Chem. 276, 18161–18168.
17 Turunen, M., Peters, J. M., Gonzalez, F. J., Schedin, S., and Dallner, G. (2000). Influence of peroxisome proliferator-activated receptor alpha on ubiquinone biosynthesis. J. Mol. Biol. 297, 607–614.
18 Gin, P., Hsu, A. Y., Rothman, S. C., Jonassen, T., Lee, P. T., Tzagoloff, A., and Clarke, C. F. (2003). The *Saccharomyces cerevisiae COQ6* gene encodes a mitochondrial flavin-dependent monooxygenase required for coenzyme Q biosynthesis. J. Biol. Chem. 278, 25308–25316.
19 Poon, W. W., Marbois, B. N., Faul, K. F., and Clarke, C. F. (1995). 3-Hexaprenyl-4-hydrobenzoic acid forms a predominant intermediate pool in ubiquinone biosynthesis in *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. 320, 305–314.
20 Baba, S. W., Belogrudov, G. I., Lee, J. C., Lee, P. T., Strahan, J., Shepherd, J. N., and Clarke, C. F. (2004). Yeast Coq5 C-methyltransferase is required for stability of other polypeptides involved in coenzyme Q biosynthesis. J. Biol. Chem. 279, 10052–10059.
21 Gin, P., and Clarke, C. F. (2005). Genetic evidence for a multisubunit complex in coenzyme Q biosynthesis in yeast and the role of the Coq1 hexaprenyl diphosphate synthase. J. Biol. Chem. 280, 2676–2681.
22 Marbois, B., Gin, P., Faull, K. F., Poon, W. W., Lee, P. T., Strahan, J., Shepherd, J. N., and Clarke, C. F. (2005). Coq3 and Coq4 Define a Polypeptide Complex in Yeast Mitochondria for the Biosynthesis of Coenzyme Q. J. Biol. Chem. 280, 20231–20238.
23 Hsu, A., Do, T., Lee, P., and Clarke, C. (2000). Genetic evidence for a multi-subunit complex in the O-methyltransferase steps of coenzyme Q biosynthesis. Biochem. Biophys. Acta. 1484, 287–297.
24 Navarro, F., Navas, P., Burgess, J. R., Bello, R. I., De Cabo, R., Arroyo, A., and Villalba, J. M. (1998). Vitamin E and selenium deficiency induces expression of the ubiquinone- dependent antioxidant system at the plasma membrane. FASEB J. 12, 1665–1673.
25 De Cabo, R., Cabello, R., Rios, M., Lopez-Lluch, G., Ingram, D. K., Lane, M. A., and Navas, P. (2004). Calorie restriction attenuates age-related alterations in the plasma membrane antioxidant system in rat liver. Exp. Gerontol. 39, 297–304.
26 Marbois, B. N., and Clarke, C. F. (1996). The *COQ7* gene encodes a protein in *Saccharomyces cerevisiae* necessary for ubiquinone biosynthesis. J. Biol. Chem. 271, 2995–3004.
27 Tran, U. C., Marbois, B., Gin, P., Gulmezian, M., Jonassen, T., and Clarke, C. F. (2006). Complementation of *Saccharomyces cerevisiae coq7* mutants by mitochondrial targeting of the Escherichia coli UbiF polypeptide: two functions of yeast Coq7 polypeptide in coenzyme Q biosynthesis. J. Biol. Chem. 281, 16401–16409.
28 Miyadera, H., Amino, H., Hiraishi, A., Taka, H., Murayama, K., Miyoshi, H., Sakamoto, K., Ishii, N., Hekimi, S., and Kita, K. (2001). Altered quinone biosynthesis in the long-lived clk-1 mutants of *Caenorhabditis elegans*. J. Biol. Chem. 276, 7713–7716.
29 Nakai, D., Yuasa, S., Takahashi, M., Shimizu, T., Asaumi, S., Isono, K., Takao, T., Suzuki, Y., Kuroyanagi, H., Hirokawa, K., Koseki, H., and Shirsawa, T. (2001). Mouse homologue of coq7/clk-1, longevity gene in Caenorhabditis elegans, is essential for coenzyme Q synthesis, maintenance of mitochondrial integrity, and neurogenesis. Biochem. Biophys. Res. Commun. 289, 463–471.
30 Jonassen, T., Marbois, B. N., Faull, K. F., Clarke, C. F., and Larsen, P. L. (2002). Development and fertility in *Caenorhabditis elegans* clk-1 mutants depend upon transport of dietary coenzyme Q_8 to mitochondria. J. Biol. Chem. 277, 45020–45027.
31 Hihi, A. K., Kebir, H., and Hekimi, S. (2003). Sensitivity of *Caenorhabditis elegans* clk-1 mutants to ubiquinone side-chain length reveals multiple ubiquinone-dependent processes. J. Biol. Chem. 278, 41013–41018.
32 Padilla, S., Jonassen, T., Jimenez-Hidalgo, M. A., Fernandez-Ayala, D. J. M., Lopez-Lluch, G., Marbois, B., Navas, P., Clarke, C. F., and Santos-Ocana, C. (2004). Demethoxy-Q, An Intermediate of Coenzyme Q Biosynthesis, Fails to Support Respiration in *Saccharomyces cerevisiae* and Lacks Antioxidant Activity. J. Biol. Chem. 279, 25995–26004.
33 Lundblad, V., and Struhl, K. (2003). *Saccharomyces cerevisiae*. In Current Protocols in Molecular Biology. F. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl, eds. (New York: John Wiley & Sons, Inc.).
34 Gietz, R.D. and Schiestl, R.H. (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Protoc. 2007, 35–7.
35 Jonassen, T., and Clarke, C. F. (2000). Isolation and functional expression of human *COQ3*, a gene encoding a methyltransferase required for ubiquinone biosynthesis. J. Biol. Chem. 275, 12381–12387.
36 Radin, N. S. (1981). Extraction of tissue lipids with a solvent of low toxicity. Methods. Enzymol. 72, 5–7.
37 Gattei, V., Degan, M., De Iuliis, A., Rossi, F. M., Aldinucci, D., and Pinto, A. (1997). Competitive reverse-transcriptase PCR: a useful alternative to northern blotting for quantitative estimation of relative abundances of specific mRNAs in precious samples. Biochem. J. 325, 565–567.

38 Kohler, T., Rost, A. K., and Remke, H. (1997). Calibration and storage of DNA competitors used for contamination-protected competitive PCR. Biotechniques. 23, 722–726.
39 Do, T. Q., Schultz, J. R., and Clarke, C. F. (1996). Enhanced sensitivity of ubiquinone-deficient mutants of *Saccharomyces cerevisiae* to products of autoxidized polyunsaturated fatty acids. Proc. Natl. Acad. Sci. USA. 93, 7534–7539.
40 Hagerman, R. A., Trotter, P. J., and Willis, R. A. (2002). The regulation of *COQ5* gene expression by energy source. Free Radic. Res. 36, 485–490.
41 Forsgren, M., Attersand, A., Lake, S., Grunler, J., Swiezewska, E., Dallner, G., and Climent, I. (2004). Isolation and functional expression of human COQ2, a gene encoding a polyprenyl transferase involved in the synthesis of CoQ. Biochem. J. 382, 519–526.
42 Poon, W. W., Do, T. Q., Marbois, B. N., and Clarke, C. F. (1997). Sensitivity to treatment with polyunsaturated fatty acids is a general characteristic of the ubiquinone-deficient yeast coq mutants. J. Mol. Asp. Med., 121–127.
43 Clarke, C. F. (2001). New advances in Coenzyme Q biosynthesis. Protoplasma. 213, 134–147.
44 Santos-Ocana, C., Do, T. Q., Padilla, S., Navas, P., and Clarke, C. F. (2002). Uptake of Exogenous Coenzyme Q and Transport to Mitochondria Is Required for bc_1 Complex Stability in Yeast *coq* Mutants. J. Biol. Chem. 277, 10973–10981.
45 Leonard, C. J., Aravind, L., and Koonin, E. V. (1998). Novel Families of Putative Protein Kinases in Bacteria and Archaea: Evolution of the "Eukaryotic" Protein Kinase Superfamily. Genome Res. 8, 1038–1047.
46 Sippel, C. J., Goewert, R. R., Slachman, F. N., and Olson, R. E. (1983). The regulation of ubiquinone-6 biosynthesis by *Saccharomyces cerevisiae.* J. Biol. Chem. 258, 1057–1061.
47 Clarke, C. F., Williams, W., and Teruya, J. H. (1991). Ubiquinone biosynthesis in *Saccharomyces cerevisiae.* Isolation and sequence of *COQ3*, the 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase gene. J. Biol. Chem. 266, 16636–16644.
48 Trotter, P. J. (2001). The genetics of fatty acid metabolism in *Saccharomyces cerevisiae.* Annu. Rev. Nutr. 21, 97–119.
49 Stenmark, P., Grunler, J., Mattsson, J., Sindelar, P. J., Nordlund, P., and Berthold, D. A. (2001). A new member of the family of di-iron carboxylate proteins: Coq7 (clk-1), a membrane-bound hydroxylase involved in ubiquinone biosynthesis. J. Biol. Chem. 276, 33297.
50 Kagan, V. E., Nohl, H., and Quinn, P. J. (1996). Coenzyme Q: Its role in scavenging and generation of radicals in membranes. E. Cadenas, and L. Packer, eds. (New York: Marcel Decker Inc.), pp. 157–201.
51 Hagerman, R. A., and Willis, R. A. (2002). The yeast gene *COQ5* is differentially regulated by Mig1p, Rtg3p and Hap2p. Biochim. Biophys. Acta. 1578, 51–58.
52 Brea-Calvo, G., Rodriguez-Hernandez, A., Fernandez-Ayala, D. J., Navas, P., and Sanchez-Alcazar, J. A. (2006). Chemotherapy induces an increase in coenzyme Q_{10} levels in cancer cell lines. Free Radic. Biol. Med. 40, 1293–1302.
53 Miyadera, H., Kano, K., Miyoshi, H., Ishii, N., Hekimi, S., and Kita, K. (2002). Quinones in long-lived clk-1 mutants of *Caenorhabditis elegans.* FEBS Lett. 512, 33–37.
54 MacDonald, M. J., Husain, R. D., Hoffmann-Benning, S., and Baker, T. R. (2004). Immunochemical identification of coenzyme Q_0-dihydrolipoamide adducts in the E2 components of the alpha-ketoglutarate and pyruvate dehydrogenase complexes partially explains the cellular toxicity of coenzyme Q_0. J. Biol. Chem. 279, 27278–27285.
55 Trumpower, B. L., Aiyar, A. S., Opliger, C. E., and Olson, R. E. (1972). Studies on Ubiquinone. The isolation and identification of 5-demethoxyubiquinone-9 as an intermediate in biosynthesis of ubiquinone-9 in the rat. J. Biol. Chem. 247, 2499–2511.
56 Proft, M., Kötter, P., Hedges, D., Bojunga, N., and Entian, K. D. (1995). *CAT5*, a new gene necessary for derepression of gluconeogenic enzymes in *Saccharomyces cerevisiae.* EMBO J. 14, 6116–6126.