Coenzyme Q (Qn) is a vital lipid component of the electron transport chain that functions in cellular energy metabolism and as a membrane antioxidant. In the yeast Saccharomyces cerevisiae, coq1–coq9 deletion mutants are respiratory-imcompetent, sensitive to lipid peroxidation stress, and unable to synthesize Q6. The yeast coq10 deletion mutant is also respiratory-deficient and sensitive to lipid peroxidation, yet it continues to produce Q6 at an impaired rate. Thus, Coq10 is required for the function of Q6 in respiration and as an antioxidant and is believed to chaperone Q6 from its site of synthesis to the respiratory complexes. In several fungi, Coq10 is encoded as a fusion polypeptide with Coq11, a recently identified protein of unknown function required for efficient Q6 biosynthesis. Because “fused” proteins are often involved in similar biochemical pathways, here we examined the putative functional relationship between Coq10 and Coq11 in yeast. We used plate growth and Seahorse assays and LC-MS/MS analysis to show that COQ11 deletion rescues respiratory deficiency, sensitivity to lipid peroxidation, and decreased Q6 biosynthesis of the coq10Δ mutant. Additionally, immunoblotting indicated that yeast coq11Δ mutants accumulate increased amounts of certain Coq polypeptides and display a stabilized CoQ synthome. These effects suggest that Coq11 modulates Q6 biosynthesis and that its absence increases mitochondrial Q6 content in the coq10Δcoq11Δ double mutant. This augmented mitochondrial Q6 content counteracts the respiratory deficiency and lipid peroxidation sensitivity phenotypes of the coq10Δ mutant. This study further clarifies the intricate connection between Q6 biosynthesis, trafficking, and function in mitochondrial metabolism.

Coenzyme Q (ubiquinone or Q)2 is a benzoquinone lipid that functions as an essential electron carrier within the electron transport chain (1). Because of its redox activities, Q is a versatile electron acceptor in biological pathways such as cellular respiration, oxidation of proline and sulfide, fatty acid β-oxidation, and pyrimidine biosynthesis (1–3). The reduced hydroquinone form of Q (ubiquinol or QH2) also serves as an important chain-breaking antioxidant shown to alleviate lipid peroxidative damage in cellular membranes (4).

For proper functional localization, Q relies on its polyisoprenoid tail to remain anchored at the mid-plane of phospholipid bilayers. The number of isoprene units (n) that comprise the polyisoprenoid tail of Qn depends on a species-specific polypropenyl diphosphate synthase (5), with Q10 representing the major isomer in humans (6). Patients unable to produce adequate levels of Q10 display a wide variety of health issues that stem from mitochondrial dysfunction across tissues (7). Attempts to ameliorate the consequences of primary Q10 deficiency by early Q10 supplementation have been partially successful in some cases (8); however, many patients fail to demonstrate full recovery, which is related to inefficient uptake of orally-supplied Q10. Because of the striking homology between human COQ genes and those of Saccharomyces cerevisiae (7,9), studies of Q6 biosynthesis in S. cerevisiae may provide insight

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This article contains Figs. S1–S3, Tables S1 and S2, and supporting Refs. 1–8.

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Coq10 knockout phenotypes are rescued by deletion of COQ11

into human Q_{10} biosynthesis, leading to the discovery of potential therapeutic targets.

In *S. cerevisiae*, at least 14 nuclear-encoded mitochondrial proteins (Coq1–Coq11, Yah1, Arh1, and Hfd1) drive Q_{6} biosynthesis (7, 9). Many Coq polypeptides (Coq3–Coq9, and Coq11) are localized to the matrix side of the mitochondrial inner membrane, where they organize into a high-molecular-weight multisubunit complex known as the “CoQ synthome” (7, 9). Several lines of evidence suggest that correct assembly of the CoQ synthome is necessary for efficient Q_{6} biosynthesis (9–12). In fact, deletion of certain COQ genes results in decreased levels of other Coq polypeptides and contributes to a destabilized CoQ synthome in these mutants (12, 13). Recently, a protein of unknown function encoded by the ORF YLR290C was identified to associate with the CoQ synthome, via proteomic analysis of tandem affinity-purified tagged Coq proteins (14). YLR290C copurified with Coq5, Coq7, and Coq9, in addition to Q_{6} and late-stage Q_{6}-intermediates (14). Furthermore, the ylr290cΔ mutant exhibited impaired de novo Q_{6} biosynthesis, despite preserving growth on a nonfermentable carbon source (14). Given its effects on Q_{6} biosynthesis and involvement with the CoQ synthome, YLR290C was renamed Coq11 (14).

In several fungi, Coq11 and Coq10 have evolved as fusion proteins (14), suggesting that Coq11 may have a functional relationship with Coq10 (15). High-throughput genetic analyses found COQ11 to correlate with both COQ2 and COQ10 (16). Whereas the coq mutants generally lack Q_{6}, the coq10Δ mutant is different because it produces near WT amounts of Q_{6} in stationary phase and only has decreased de novo Q_{6} biosynthesis in log phase (17, 18). Although Q_{6} biosynthesis is only minimally decreased in the absence of COQ10, the coq10Δ mutant has decreased NADH and succinate oxidase activity and displayed sickly growth on respiratory medium (18). The coq10Δ mutant is sensitive to lipid peroxidation initiated by exogenously supplemented polyunsaturated fatty acids (PUFAs), indicating that the Coq10 polypeptide is also required for antioxidant protection by Q_{6} (17, 19).

The NMR structure of a Coq10 ortholog in *Caulobacter crescentus* was shown to possess a steroidogenic acute regulatory protein–related lipid transfer (START) domain (20) that can directly bind Q and late-stage Q-intermediates (17). Purified Coq10 from either *S. cerevisiae* or *Schizosaccharomyces pombe* eluted with the respective species’ Q isoform (18, 21). This observation has prompted speculation that Coq10 acts as a Q_{6} chaperone protein required for delivery of Q_{6} from its synthesis site to sites where Q_{6} functions as an antioxidant and to the respiratory complexes, thereby bridging efficient de novo Q_{6} biosynthesis with respiration (17). Recent studies have shown a spatial compartmentalization of the mitochondrial inner membrane with the identification of different sites, such as the inner boundary membrane, the cristae membrane, and the ER-mitochondrial contact sites (22–25). Thus, for optimal respiratory competence, newly-synthesized Q_{6} must move from its site of synthesis (i.e. the ER-mitochondrial contact sites (23, 24)) to the cristae membrane where the respiratory complexes are concentrated (22). The presence of Coq10–Coq11 fusions in fungal species indicates that Coq11 may have a functional association with the Coq10 chaperone to facilitate or regulate Q_{6} transport for respiration in yeast.

In this work, the functional relationship between Coq10 and Coq11 was investigated using a series of single- and double-knockout mutants. Deletion of COQ11 alleviated the coq10Δ respiratory defect, increased Coq polypeptides and CoQ synthome stability, and partially rescued Q_{6} production. Based on this evidence, we propose a novel function for Coq11 as a negative modulator of Q_{6} biosynthesis in the mitochondria.

Results

Coq10 and Coq11 reside in similar compartments within the mitochondria

Previous phylogenetic analyses of numerous fungi revealed that Coq11-like proteins are fused to Coq10 (14). Protein fusions often indicate a functional relationship between corresponding homologs in other organisms, such as direct protein–protein interaction or operation within the same biological pathway (15). Although Coq10 and Coq11 are not physically localized to the same site, there is a functional link between the two proteins. Because protein localization is often associated with function, we first performed mitochondrial fractionation to localize both Coq10 and Coq11. Coq10 has been localized previously (18), but fractionation was re-performed here in the context of Coq11.

*S. cerevisiae* mitochondria were fractionated as described under “Experimental procedures.” Purified mitochondria were incubated in hypotonic buffer to disrupt the outer membrane and release soluble components of the intermembrane space (IMS). The inner membrane was kept intact following hypotonic buffer treatment, protecting inner membrane and matrix proteins. Analysis of the fractions via immunoblot suggested that both Coq10 and Coq11 remained associated with the mitoplast fraction as opposed to colocalizing with the IMS marker cytochrome b_{562}. Mitoplasts were further fractionated after sonication to separate soluble matrix components (supernatant, S) from membrane components (pellet, P). The soluble matrix marker Hsp60 was partially released into the supernatant by sonication as demonstrated in earlier work (26). Although Coq11 remained associated with the membrane fraction, Coq10 was partially dissociated in a similar manner to Hsp60 (Fig. 1B). Previous Coq10 colocalization following sonication demonstrated that Coq10 was solely associated with the membrane fraction (18). The detection of Coq10 in the supernatant shown in Fig. 1B may be due to increased sensitivity of the polyclonal antisera used in this study.

Alternatively, mitoplasts were subjected to alkaline carbonate extraction to separate peripheral membrane components (supernatant, S) from integral membrane and matrix components (pellet, P) (27). Coq10 and Coq11 were released into the supernatant following alkaline treatment (Fig. 1B), matching the peripheral inner membrane marker Atp2 (28). There was no colocalization with the pellet fraction, marked by the integral membrane protein Cyt_b (29). These results indicate that Coq10 and Coq11 are both peripheral inner membrane proteins, and Coq10 has additional localization to the matrix. The localization of Coq10 to the inner membrane is consistent with
its putative role as a START domain protein possessing a hydrophobic cavity to bind and chaperone Q₆ from its site of synthesis to complex III for respiration (17, 18). We hypothesize that dual mitochondrial matrix localization occurs when Coq10 is tightly bound to protein partners to decrease its hydrophobicity.

For better insight into the membrane association of Coq10 and Coq11, intact mitochondria or mitoplasts were treated with proteinase K in the absence or presence of two individual detergents (1% Triton X-100 or 0.5% SDS). Coq10 and Coq11 were both protected from protease treatment in purified mitochondria and mitoplasts, as was the matrix marker Hsp60 (Fig. 1C). When protease was used in the presence of either detergent in mitochondria or mitoplasts, all proteins became sensitive to the protease and were degraded. Expanding on the subfractionation results, these data indicate that Coq10 and Coq11 polypeptides are peripherally associated with the inner membrane facing the matrix side in yeast mitochondria, and Coq10 is also found in the mitochondrial matrix itself. The mitochondrial peripheral membrane association of these two proteins is also in agreement with their submitochondrial localization previously identified in a study of the yeast mitochondrial proteome (30).

Figure 1. Coq11 and Coq10 are peripherally associated with the mitochondrial inner membrane facing the matrix, and Coq10 is additionally found in the mitochondrial matrix. A, Coq10 and Coq11 are fused in multiple fungi, suggesting an evolutionarily functional relationship between these proteins, although they are not found fused in S. cerevisiae. B, S. cerevisiae mitochondria purified from yeast strains cultured on YPGal medium were subjected to hypotonic swelling and centrifugation to separate the IMS proteins from mitoplasts. The mitoplasts were alkaline-treated (Na₂CO₃; pH 11.5) or sonicated and then separated by centrifugation (100,000 × g for 1 h) into supernatant (S) or pellet (P) fractions. C, intact mitochondria or mitoplasts were treated with 100 µg/ml proteinase K for 30 min on ice, with or without detergent. Mitochondrial polypeptide markers are as follows: Atp2, peripheral inner membrane protein; Cyb₂, intermembrane space protein; Cyt₁, integral inner membrane protein; and Hsp60, soluble matrix protein. Results are representative of two experiments.

Coq10 knockout phenotypes are rescued by deletion of COQ11

**U. maydis**: Coq11-Coq10 fusion

**S. cerevisiae**: No fusion, functional relationship?

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**Figure 1.** Coq11 and Coq10 are peripherally associated with the mitochondrial inner membrane facing the matrix, and Coq10 is additionally found in the mitochondrial matrix. A, Coq10 and Coq11 are fused in multiple fungi, suggesting an evolutionarily functional relationship between these proteins, although they are not found fused in *S. cerevisiae*. *B*, *S. cerevisiae* mitochondria purified from yeast strains cultured on YPGal medium were subjected to hypotonic swelling and centrifugation to separate the IMS proteins from mitoplasts. The mitoplasts were alkaline-treated (Na₂CO₃; pH 11.5) or sonicated and then separated by centrifugation (100,000 × g for 1 h) into supernatant (S) or pellet (P) fractions. *C*, intact mitochondria or mitoplasts were treated with 100 µg/ml proteinase K for 30 min on ice, with or without detergent. Mitochondrial polypeptide markers are as follows: Atp2, peripheral inner membrane protein; Cyb₂, intermembrane space protein; Cyt₁, integral inner membrane protein; and Hsp60, soluble matrix protein. Results are representative of two experiments.
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Table 1

| Strain            | Genotype                                    | Source          |
|-------------------|---------------------------------------------|-----------------|
| W303-1B           | MAT a ade2-1 his3-1,15 leu2-112trp1-1 ura3-1 | R. Rothstein*   |
| BY4742            | MAT a his3Δ leu2Δ met15Δ ura3Δ            | 53              |
| JM6               | MAT a his-4Δ                            | 68              |
| JM8               | MAT a his-1Δ                            | 68              |
| BY4742 coq1Δ      | MAT a his3Δ leu2Δ met15Δ ura3Δ coq1::KanMX4 | 69              |
| BY4742 coq2Δ      | MAT a his3Δ leu2Δ met15Δ ura3Δ coq2::KanMX4 | 69              |
| BY4742 coq4Δ      | MAT a his3Δ leu2Δ met15Δ ura3Δ coq4::KanMX4 | 69              |
| BY4742 coq5Δ      | MAT a his3Δ leu2Δ met15Δ ura3Δ coq5::KanMX4 | 69              |
| BY4742 coq6Δ      | MAT a his3Δ leu2Δ met15Δ ura3Δ coq6::KanMX4 | 69              |
| BY4742 coq8Δ      | MAT a his3Δ leu2Δ met15Δ ura3Δ coq8::KanMX4 | 69              |
| BY4742 coq9Δ      | MAT a his3Δ leu2Δ met15Δ ura3Δ coq9::KanMX4 | 69              |
| BY4742 coq10Δ     | MAT a his3Δ leu2Δ met15Δ ura3Δ coq10::KanMX4 | 69              |
| BY4742 coq11Δ     | MAT a his3Δ leu2Δ met15Δ ura3Δ coq11::LEU2  | This work       |
| BY4742 coq10Δcoq11Δ | MAT a his3Δ leu2Δ met15Δ ura3Δ coq10::His3 coq11::LEU2 | This work       |
| W303 coq1Δ        | MAT a ade2-1 his3-1,15 leu2-3,112trp1-1 ura3-1 coq1::His3 | 18              |
| W303 coq10Δ       | MAT a ade2-1 his3-1,15 leu2-3,112trp1-1 ura3-1 coq10::His3 sup | 18              |
| MB-10             | Diploid produced from W303 a coq10Δ × W303 a coq10Δrev | This work       |
| W303 coq1Δ        | MAT a ade2-1 his3-1,15 leu2-3,112trp1-1 ura3-1 coq1::His3 | This work       |
| W303 coq10Δcoq11Δ | MAT a ade2-1 his3-1,15 leu2-3,112trp1-1 ura3-1 coq10::His3 coq11::LEU2 | This work       |
| BY4741 cor1Δ      | MAT a his3Δ leu2Δ met15Δ ura3Δ coq1::KanMX4 | 69              |

* Gift from Dr. Rodney Rothstein Department of Human Genetics, Columbia University.

coq10Δ respiratory defect is alleviated by deletion of COQ11

Based on similar mitochondria localization and genetic evolutionary evidence, a putative functional relationship between Coq10 and Coq11 was further probed using a series of coq10 and coq11 single- and double-knockout mutants. Strain descriptions are listed in Table 1. The Coq10 polypeptide is required for respiration in yeast, and mutants lacking coq10 have poor growth on nonfermentable carbon sources, including YPGlycerol, hereafter referred to as “YPG” (18). Unlike deletion of COQ10, coq11Δ mutants are respiratory-capable and have comparable growth to WT on nonfermentable carbon sources (14). When COQ11 was deleted in a coq10Δ mutant in two different yeast genetic backgrounds, the sickly growth of coq10Δ on nonfermentable YPG was rescued (Fig. 2A).

Quantitative respiratory capacity of each mutant was evaluated with an XF96 Extracellular Flux Analyzer (Fig. 2, B and C). Representative and normalized traces of oxygen consumption rates (OCR) of four independent experiments performed in nonrepulsive medium (YPGal) are shown in Fig. 2B. Basal rates of OCR were measured prior to the addition of any small molecule inhibitors. Consistent with its slow growth on nonfermentable medium, the coq10Δ mutant had a low rate of basal oxygen consumption compared with WT (p = 0.052) (Fig. 2C). Basal OCR was rescued in the coq10Δ coq11Δ double mutant (Fig. 2C). Following the addition of two sequential injections of FCCP, a mitochondrial oxidative phosphorylation uncoupler, maximal respiration was also quantified. The maximal respiration of coq10Δ coq11Δ was rescued to that of WT (Fig. 2C). These results show that the deletion of COQ11 in a coq10Δ mutant confers a beneficial effect, such that both growth on respiratory medium and OCR are rescued to WT.

Deletion of COQ11 rescues PUFA sensitivity of the coq10Δ mutant

PUFA autoxidation is initiated by the radical-mediated abstraction of vulnerable hydrogen atoms at bis-allylic positions (31). The ensuing carbon-centered radical adds to molecular oxygen to form a lipid peroxyl radical that propagates lipid peroxidation, with the resulting lipid hydroperoxides ultimately driving cellular toxicity (32). The coq10Δ mutant is sensitive to treatment with exogenous PUFAs (Fig. 2D) (17, 19), likely because the Q6 chaperone function of Coq10 is required for the antioxidant function of Q6. Attenuated respiration in coq10Δ is rescued in the coq10Δ coq11Δ double knockout (Fig. 2, A–C), presumably through regained function of Q6 in the electron transport chain. To test whether the antioxidant capability of Q6 is also restored in the coq10Δ coq11Δ mutant, yeast strains were evaluated for sensitivity to added PUFAs (Fig. 2D). As anticipated, all strains were resistant to treatment with the monounsaturated oleic acid (Fig. 2D). Q6-less coq9Δ was sensitive to α-linolenic acid due to the lack of Q6 antioxidant protection (Fig. 2D). Conversely, the Q6-replete yet respiratory-deficient cor1Δ remained resistant to α-linolenic acid (Fig. 2D). Deletion of COQ11 rescued the α-linolenic acid sensitivity of the coq10Δ mutant, suggesting that the double knockout has restored Q6 antioxidant protection (Fig. 2D) despite the absence of Coq10 as a Q6 chaperone.

Independent coq10 revertant with rescued growth on respiratory medium harbors a mutation within COQ11

Although the coq10Δ mutant is unable to grow robustly on nonfermentable medium, an earlier study identified a spontaneous coq10 revertant (coq10rev) that arose when coq10Δ yeast was cultured for several weeks on nonfermentable medium containing ethanol and glycerol as carbon sources (18). Characterization of this revertant revealed a suppressor mutation within the COQ11 ORF, resulting in a truncated Coq11 protein that is predicted to be nonfunctional (Fig. 3A). This mutation was further assessed for dominance to determine whether it was sufficient to explain the respiratory competence of coq10rev. A haploid coq10Δ mutant crossed with haploid coq10 rev produced diploid MB-10 (Table 1), which was capable of growth on respiratory medium (Fig. 3B). Illustrated growth
patterns suggest that the coq11 truncated allele present in coq10rev is a dominant-negative mutation. Because the dominant mutation in coq10rev restores growth on respiratory medium via a functionally suppressive Coq11 truncation mutation, this mutant effectively validates the coq10/H9004 coq11/H9004 phe notype in an independent system.

Deletion of COQ11 fails to fully restore coq10Δ Q₆ biosynthesis in whole cells

When a coq mutant displays anemic growth on respiratory medium, it is often indicative of inefficient Q₆ biosynthesis (7, 9); yeast lacking COQ10 exhibit both poor growth on respiratory medium and decreased Q₆ biosynthesis in log phase whole cells (17, 19). Although the coq11Δ mutant retains the ability to grow on nonfermentable medium, it is also characterized by impaired Q₆ biosynthesis (14). Only a small amount of Q₆ is required for growth on respiratory medium, ~0.2–3% of the total Q₆ found in WT (9, 33, 34). Because the coq10Δcoq11Δ double mutant has rescued respiration, we wanted to assess whether recovered growth was accompanied by increased Q₆ biosynthesis. Whole-cell de novo-synthesized [13C₆]Q₆ and [12C]Q₆ were measured in yeast by feeding the quinone ring-labeled precursor, [13C₆]4HB, or EtOH vehicle control (Fig. 4).

Consistent with previous results (14, 17), coq10Δ and coq11Δ had significantly decreased de novo-synthesized [13C₆]Q₆ and [12C]Q₆ compared with WT (Fig. 4A). The coq10Δ mutant had a lower total Q₆ content ([13C₆]Q₆ + [12C]Q₆) than WT and also a lower total Q₆ than coq11Δ (Fig. 4B). Deletion of COQ11 in coq10Δ yeast led to a slight increase in de novo-synthesized [13C₆]Q₆ and unchanged [12C]Q₆ compared with coq10Δ (Fig. 4A). Therefore, the coq10Δcoq11Δ double mutant presented total Q₆ contents that were significantly lower than either WT or coq11Δ (Fig. 4B). Given the robust growth of the coq10Δcoq11Δ double mutant on YPG, restored respiration, and resistance to PUFA treatment, the low Q₆ concentrations observed are surprising.

Next, we quantified the concentrations of key Q₆-intermediates in the same whole-cell yeast pellets. As shown previously...
Coq10 knockout phenotypes are rescued by deletion of COQ11

(17, 19), the coq10Δ mutant contained lower amounts of the late-stage intermediate [13C6]DMQ6 and [12C]DMQ6 (Fig. 4C) than WT, and it accumulated the early-stage intermediate [13C6]HHB and [12C]HHB (Fig. 4D). In contrast to coq10/H9004, the coq11/H9004 mutant mirrored WT production of both de novo-synthesized and unlabeled early- and late-stage intermediates (Fig. 4, C and D), as shown previously (14). Q6-intermediate trends in coq10/H9004 coq11/H9004 matched those of the coq10/H9004 mutant rather than coq11/H9004 (Fig. 4, C and D). The low Q6 content and accumulation of early-stage Q6-intermediates in the coq10Δcoq11Δ double knockout suggest the absence of COQ10 still produces a notable effect on Q6 biosynthesis, although respiratory capacity is rescued.

cboq10Δcoq11Δ double mutant has increased mitochondrial Q6 compared with the coq10Δ single mutant

Although Q6 biosynthesis solely occurs within mitochondria, it is found in all cellular membranes (9). Therefore, Q6 was quantified in both whole cells and purified mitochondria from mutant and WT cells cultured under the same conditions (36). Whole-cell Q6 determined under mitochondrial purification conditions matched those determined in Figs. 4 and 5A. The coq10Δcoq11Δ double mutant made slightly more Q6 than the coq10Δ single mutant, but overall less Q6 compared with the coq11Δ single mutant. All mutants had lower whole-cell Q6 amounts than WT (Fig. 5A).

Similarly, mitochondrial Q6 content per microgram of mitochondrial protein was lower in coq11/H9004 than WT (Fig. 5B). However, deletion of COQ11 in the coq10Δ mutant increased the mitochondrial Q6 5-fold (Fig. 5B). Despite these profound differences in mitochondrial Q6 content, mitochondrial mass was consistent between strains as determined by three distinct assays (Fig. 5, C–E). Increased mitochondrial Q6 in the coq10Δcoq11Δ double mutant compared with coq10Δ indicates that the absence of COQ11 in part rescues defective Q6 synthesis in the coq10Δ mutant.

Low Coq protein content and destabilized CoQ synthome of the coq10Δ mutant are restored in the coq10Δcoq11Δ double mutant

Proper formation of the CoQ synthome from component Coq polypeptides is required for efficient Q6 biosynthesis in yeast (9, 12, 13). Deletion of COQ10 causes a decrease in several other Coq polypeptides, including Coq3–Coq7, Coq9, as well as overall CoQ synthome destabilization (12, 13, 19). These results were confirmed when purified mitochondria from coq10Δ yeast were analyzed for each Coq polypeptide (Fig. 6A). The coq10Δ mutant had significantly decreased amounts of Coq3,
Figure 4. Low amounts of de novo [13C6]Q6 in whole-cell lipid extracts of the coq10Δ mutant are only partially restored by deletion of COQ11. Triplicates of 6-ml cultures in YPGal were labeled at A600 = 1 with 5 μg/ml [13C6]4HB or EtOH vehicle control, and 5 ml of each culture were collected after 4 h, lipid-extracted, and analyzed by LC-MS/MS. A, unlabeled [12C]Q6 and de novo–synthesized [13C6]Q6 (blue); B, total amount of Q6 determined from the sum of [13C6]Q6 and [12C]Q6 (red); C, [12C]DMQ6 and [13C6]DMQ6 (red); and D, [12C]HHB and [13C6]HHB (purple) were measured from the whole-cell lipid extracts of WT and the coq10Δ, coq11Δ, and coq10Δcoq11Δ mutants. Values are the mean of three replicates. The data show mean ± S.D., and the statistical significance as compared with WT is represented by *, p < 0.05; **, p < 0.001; and ****, p < 0.0001.

Coq4, Coq7, and Coq9 compared to and plotted as a percentage of WT (Fig. 6B).

In contrast to coq10Δ, the coq11Δ single mutant had elevated Coq4, Coq6, Coq7, and Coq9 (Fig. 6A), with protein quantification shown in Fig. 6B. Furthermore, the coq10Δcoq11Δ double mutant also had raised amounts of Coq4, Coq7, and Coq9 polypeptides compared with WT (Fig. 6A and B). This increase in Coq proteins could not be explained by enhanced COQ transcription as there was no corresponding change in the concentration of the respective mRNAs, (Fig. 6C), although COQ4 mRNA was not detected.

CoQ synthome formation was probed using two-dimensional blue native/SDS-PAGE (2D-BN/SDS-PAGE) with Coq4 and Coq9 serving as sensitive indicators of a high-molecular-weight complex (13). As expected, the CoQ synthome in WT yeast presented as a heterogeneous high-molecular-weight complex, spanning a range of ~140 kDa to >1 MDa for Coq4 (Fig. 7A) and from ~100 kDa to >1 MDa for Coq9 (Fig. 7B). Consistent with prior results (13, 19), the coq10Δ mutant displayed a highly-destabilized CoQ synthome, with a disappearance of large complexes that were replaced by lower-molecular-weight subcomplexes less than ~440 kDa for Coq4 (Fig. 7A) and less than ~232 kDa for Coq9 (Fig. 7B). The coq11Δ mutant had a stabilized CoQ synthome compared with WT, with high-molecular-weight complexes shifting to the left and collapsing into a more homogeneous complex spanning ~900 kDa to >1 MDa for both Coq4 (Fig. 7A) and Coq9 (Fig. 7B). When COQ11 was deleted in combination with COQ10, there was a substantial rescue of high-molecular-weight complex formation compared with the coq10Δ single mutant (Fig. 7, A and B). The CoQ
Coq10 knockout phenotypes are rescued by deletion of COQ11

The Coq8 polypeptide is a member of an ancient atypical kinase family (37), with several conserved kinase motifs that are essential for Q biosynthesis (13, 38). Prior studies have demonstrated that overexpression of Coq8 in a coq10Δ mutant increased the otherwise low amounts of several key Coq polypeptides and stabilized CoQ synthome formation (13). This is similar to the phenotype observed when COQ11 was deleted in the coq10Δ mutant. Furthermore, Coq8 overexpression has also been shown to influence Q₆ biosynthesis, including the restoration of late-stage Q₆-intermediates in coq5–coq9 null mutants (39). Although the CoQ synthome of the coq10Δ mutant was stabilized by deletion of COQ11, Q₆ and late-stage Q₆-intermediates remained lower compared with WT and the coq11Δ single mutant (Fig. 4C). We hypothesized that the overexpression of Coq8 in the coq10Δ and coq10Δcoq11Δ mutant may restore Q₆ content in both mutants.

WT, coq10Δ, coq11Δ, and coq10Δcoq11Δ were analyzed for growth on nonfermentable medium and Q₆ biosynthesis upon transformation with high-copy COQ8 (hcCOQ8, Table 2) or empty vector control (Fig. 8). Similar to previous observations in a different yeast genetic background (18), coq10Δ-expressing hcCOQ8 regained the ability to grow on respiratory medium (Fig. 8A). This growth phenotype may be explained by a stabilized CoQ synthome in the coq10Δ mutant harboring hcCOQ8 (13). However, hcCOQ8 had no material effect on the growth properties of WT, coq11Δ, or coq10Δcoq11Δ strains on YPG (Fig. 8A).

Each strain was grown in minimal selection medium to maintain plasmid expression and was analyzed for Q₆ biosynthesis following metabolic labeling with the ring-labeled Q₆ precursor, [¹³C₆]HB (Fig. 8, B and C). Changing the growth medium from rich (i.e. YPGal) to minimal synthetic (i.e. SD and dropout dextrose media (DOD)) changed the relative amounts of Q₆ content among the mutants (Figs. 4B versus 8C). Although WT [¹³C₆]Q₆ and total Q₆ content is similar in Figs. 4B and 8C, the values for coq11Δ and the double mutant are quite different. When grown in YPGal, coq10Δ had the lowest Q₆ content, followed by the double mutant coq10Δcoq11Δ, with coq11Δ hav-
Coq10 knockout phenotypes are rescued by deletion of COQ11

Figure 6. Several Coq polypeptides have increased abundance in coq11Δ and coq10Δcoq11Δ mutants compared with WT. A, aliquots of purified mitochondria (25 μg) from WT, coq10Δ, coq11Δ, and coq10Δcoq11Δ yeasts were subjected to 10 or 12% Tris-glycine SDS-PAGE. Mitochondrial malate dehydrogenase (Mdh1) was included as a loading control, with a representative blot shown. The Coq5 protein also serves as a qualitative loading control, because the Coq5 polypeptide amounts remain unchanged across the panel of coq1–coq4 and coq6–coq10 deletion mutants (12). Aliquots of purified coqΔ mitochondria (coq1A–coq7A) were included as negative controls for immunoblotting with antisera to each of the Coq polypeptides. Black arrows highlight the location of each protein on the membrane. B, ImageStudioLite was used to quantify triplicates of each Coq protein band’s intensity by hand, which were normalized to Mdh1 and plotted as a percentage of WT. The data show mean ± S.D., and the statistical significance is as compared with WT is represented by *, p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001. C, qPCR was used to determine COQ gene expression from whole-cell cultures of WT, coq10Δ, coq11Δ, or coq10Δcoq11Δ, and data were normalized to actin. COQ RNA levels remain unchanged in the coq10Δ, coq11Δ, or coq10Δcoq11Δ mutants as compared with WT.

had no effect on de novo or unlabeled Q6 content in either the coq11Δ single mutant or the coq10Δcoq11Δ double mutant (Fig. 8B). Total Q6 contents of both coq11Δ and coq10Δcoq11Δ remained significantly decreased compared with WT (Fig. 8C). Together, these results show that the rescue of the coq10Δ mutant mediated by Coq8 overexpression requires Coq11.

Expression of low-copy COQ11 rescues only some of the phenotypes of the coq10Δcoq11Δ mutant

The functional complementation of coq11Δ single and coq10Δcoq11Δ double mutants with low-copy COQ11 was assessed (lcCOQ11, Table 2). As expected, the coq10Δ mutant, coq10Δ with empty vector, and coq10Δ complemented with lcCOQ11 showed slow growth on the nonfermentable carbon
Coq10 knockout phenotypes are rescued by deletion of COQ11

Source YPGlycerol (Fig. 9A). Because yeast lacking COQ11 retain respiratory capacity (Fig. 2) (14), coq11Δ complemented with lcCOQ11 had no detectable change in growth phenotype compared with either the coq11Δ mutant or coq11Δ with empty vector (Fig. 9A). Intriguingly, when lcCOQ11 was expressed in the coq10Δcoq11Δ double mutant, there was no repression of growth on YPG compared with that of the coq10Δ mutant (Fig. 9A).

This observation suggests that Q6 biosynthesis in coq10Δcoq11Δ may not be affected by lcCOQ11. To determine the effect of lcCOQ11 expression on mutant Q6 biosynthesis, yeast was grown in selection medium to maintain plasmid expression. We tested whether lcCOQ11 expression rescued Q6 content in the coq11Δ mutant. Expression of lcCOQ11 in coq11Δ efficiently rescued total Q6 ([13C6]Q6 + [12C]Q6) to WT amounts (Fig. 9B).

Table 2

| Plasmid | Relevant genes/markers | Source |
|---------|------------------------|--------|
| pRS305  | Yeast vector with LEU2 marker | 70     |
| pRS313  | Yeast vector with HIS3 marker | 70     |
| pRS316  | Yeast shuttle vector; low-copy | 70     |
| lcCOQ11 | pRS316 with yeast COQ11; low-copy | This work |
| pRS426  | Yeast shuttle vector; multi-copy | 71     |
| p4HN4   | pRS426 with yeast COQ8; multi-copy | 12     |

Figure 7. Deletion of COQ11 in the coq10Δ mutant restores the CoQ synthome. Aliquots (100 μg) of purified mitochondria from WT, coq10Δ, coq11Δ, and coq10Δcoq11Δ yeasts cultured in YPGal were solubilized with digitonin and separated with two-dimensional BN/SDS-PAGE. Following transfer of proteins to membranes, the CoQ synthome was visualized as a heterogeneous signal from ~66 to ~669 kDa in WT control with antibodies to A, Coq4, or B, Coq9. Intact mitochondria (25 μg) from each designated strain was included as a loading control (M). Aliquots of coq4Δ or coq9Δ-purified mitochondria (25 μg) were included as a negative control for the antisera to Coq4 and Coq9, as the Coq9 polypeptide is absent from the coq4Δ mutant. Red arrowheads and brackets indicate distinct complexes.
Finally, whole-cell steady-state Q₆ concentrations were evaluated in all mutants. Even though lcCOQ11 complementation did not suppress coq10Δcoq11Δ growth on YPGlycerol (Fig. 9A), Q₆ concentrations were increased in coq10Δcoq11Δ to a level comparable with that of coq10ΔH9004 (Fig. 9C). This implies that Coq11’s role in Q₆ biosynthesis is not effective when the COQ11 ORF is expressed on a single-copy plasmid in the absence of COQ10. Perhaps Coq11 expression from a plasmid does not account for multiple levels of regulation that occur through endogenous expression. Alternatively, the coq10Δcoq11Δ double mutant may have slightly lower amounts of the Coq11 polypeptide compared with coq11Δ when both are complemented by lcCOQ11 (Fig. 9D), and these lower levels may not be sufficient to suppress respiration (Fig. 9A).

**Discussion**

This work investigated a putative functional relationship between Coq10 and Coq11 within the S. cerevisiae Q₆ biosynthetic pathway. The presence of Coq10–Coq11 fusions in several Ustilaginaceae species suggests that these proteins may directly interact or participate in the same biological pathway in yeast (Fig. 1A) (14). Yeast Coq10 and its orthologs were previously shown to be required for efficient de novo Q biosynthesis and respiration (17, 18). We were surprised to discover that the yeast coq10Δ growth defect on nonfermentable medium and oxygen consumption rates were rescued upon deletion of COQ11 (Fig. 2). Moreover, spontaneous revertants isolated from coq10Δ yeast were previously found to exhibit growth on
We have shown that this reversion is due to a dominant base pair deletion within the COQ11 gene, likely resulting in a nonfunctional, truncated Coq11 protein (Fig. 3). Mutants lacking both COQ10 and COQ11 when cultured on YPGal have increased de novo Q6 production (Fig. 4A) in addition to a 5-fold increase in mitochondrial Q6 content compared with the coq10/H9004 single knockout (Fig. 5B). Therefore, we have demonstrated that deletion of the Coq11 polypeptide in a coq10/H9004 mutant confers a beneficial effect on both respiration and Q6 biosynthesis (Fig. 10).

Enhanced Q6 content in the coq10/H9004 coq11/H9004 double mutant compared with the coq10/H9004 single mutant may be partially due to increased amounts of several key Coq polypeptides (Fig. 6) and CoQ synthome stabilization (Fig. 7). The ring-modifying enzymes within the Q6 biosynthetic pathway colocalize to numerous distinct “CoQ domains” in vivo, and proper assembly of the CoQ synthome components is required for the presence of these CoQ domains (23). Two recent studies demonstrated that mitochondria isolated from yeast lacking COQ10 have a reduced number of CoQ domain puncta (23, 24). This is likely due to lower levels of certain Coq polypeptides and partial CoQ synthome destabilization in the coq10/H9004 mutant (17, 19), which was confirmed in this work (Figs. 6 and 7). In contrast, coq11/H9004 yeast displayed significantly higher amounts of Coq4, Coq6, Coq7, and Coq9 polypeptides (Fig. 6). The CoQ synthome was likewise shifted to a higher molecular weight in coq11/H9004 mitochondria compared with WT (Fig. 7). When Coq9–yEGFP was used as a marker for CoQ domains, coq11/H9004 had increased CoQ
domain intensity that stemmed from amplified expression of Coq9–yEGFP, although the number of domains was similar to WT. Mutants lacking essential Coq polypeptides coq1–coq9, or coq10, displayed significantly less Coq9-labeled domains (24). The CoQ synthome stabilization seen in coq11Δ via 2D-BN/SDS-PAGE analyses performed in this work (Fig. 7) agrees with the observation of increased CoQ domains and argues that the CoQ synthome is truly stabilized upon deletion of COQ11, as opposed to inducing a greater number of domains.

These observations are consistent with the biochemical data that led to the notion of a CoQ synthome whose formation relies on the presence of prenylated Q-intermediates (13, 40, 41). The coq10Δ mutant produced more early-stage intermediates (HHB) and had less late-stage intermediates (DMQ6) compared with WT (Fig. 4, C and D), resulting in less CoQ synthome formation (Fig. 7). Because coq11Δ yeast displayed comparable amounts of early- and late-stage Q6-intermediates to WT (Fig. 4, C and D), this mutant retained the ability to fully form the CoQ synthome (Fig. 7). The double knockout synthesized varying amounts of early- and late-stage Q6-intermediates that were largely in-between those of the single knockouts (Fig. 4, C and D). The CoQ synthome is thus able to form in coq10Δ coq11Δ double mutant, coq10Δ + hcCOQ8 has rescued Q6 content pointing to an additional role of Coq11 in Q6 biosynthesis, redox regulation, or transportation.
Coq10 knockout phenotypes are rescued by deletion of COQ11

A currently unidentified Q₆ chaperone with lower efficiency than Coq10 that is able to rescue respiration only in the absence of both Coq10 and Coq11.

Overexpression of the Q₆-biosynthetic protein Coq8 also rescued coq10Δ mutant growth on nonfermentable medium (Fig. 8A) and de novo Q₆ biosynthesis (Fig. 8, B and C) (17). Coq8 has been implicated in the partial extraction of Q₆ intermediates out of the mitochondrial inner membrane for enzymatic modification by other Coq proteins, allowing for appropriate Q₆ biosynthesis (25). Prior investigations also revealed that Coq8 overexpression in coq10Δ yeast increased Coq4 and Coq9 polypeptides and stabilized the CoQ synthome (13, 24). Despite the substantial benefit of Coq8 overexpression in a coq10Δ single mutant, Coq8 overexpression failed to enhance Q₆ biosynthesis in the coq10Δcoq11Δ double knockout (Figs. 8, B and C, and 10). The absence of COQ11 in the double mutant is sufficient to restore Coq polypeptides and CoQ synthome formation (Figs. 6, A and B, 7, and 10). Coq11 and Coq8 may therefore work by different mechanisms to serve opposing functions for CoQ synthome and Coq polypeptide stabilization. Furthermore, it is clear that Coq11 is required to perform an additional function to Coq8, as hccCOQ8 requires the presence of Coq11 to restore Q₆ biosynthesis (Figs. 8, B and C, and 10).

Proper CoQ synthome formation is not only required for efficient Q₆ biosynthesis, but it is also vital for the establishment of ER-mitochondrial contact sites mediated by the ER-mitochondrial encounter structure (ERMES) complex (23, 24). The ERMES complex is essential for lipid exchange between the ER and mitochondria (42). Specifically, ERMES null mutants have irregular Q₆ cellular distribution and a destabilized CoQ synthome (23). When COQ10 was deleted in yeast expressing Coq6–GFP, there was a significant decrease in Coq6–GFP puncta colocalization with Mdm34–mCherry, a component of the ERMES complex (23). These results indicate that Coq synthome positioning next to the ERMES complex, and subsequent Q₆ distribution from the mitochondria, depends on Coq10. Because COQ11 deletion stabilizes the CoQ synthome (Figs. 6, A and B, and 7), it is possible that coq11Δ mutants have more ER-mitochondrial contacts through the ERMES complex and improved transfer of lipids between these organelles. Therefore, one possible role of Coq11 may be an auxiliary protein mediating lipid transport between the ER and mitochondria.

In a recent study, Coq11 was named Mrx2, as part of the mitochondrial organization of gene expression (MIOREX) complex involved in the mitochondrial genetic expression system (43). Considering the proposed regulatory function of Coq11 in Coq synthome assembly, it is tempting to speculate that Coq11 offers a mechanism to couple Q₆ synthesis with the assembly of the respiratory complexes. When the synthesis of the respiratory complexes is more active, Coq11 is associated to the MIOREX complex, and Q₆ synthesis at ER junctions is stimulated. Coq11 dual localization in the mitochondrial inner membrane, to the MIOREX complex and the CoQ synthome, would also explain the sensitivity of yeast cells to the number of COQ11 copies, as well as present another example of a loop system control for balanced expression of mitochondrial products (44).

Another potential explanation for the phenotypes induced by the knockout of Coq11 relates to its structural connection with the short-chain dehydrogenase/reductase (SDR) superfamily of NAD(P)(H)-dependent oxidoreductases (14, 45). These enzymes catalyze an assortment of reactions, including isomerization, decarboxylation, epimerization, imine reduction, and carbonyl–alcohol oxidoreduction (45). SDR superfamily proteins contain a conserved protein structural motif known as a Rossmann fold, a feature used in the binding of nucleotide cofactors such as FAD, FMN, and NAD(P) (46). The crystal structure of the Pseudomonas aeruginosa gene UbiIX, which catalyzes the decarboxylation step in Q₆ biosynthesis, revealed a Rossmann fold with a bound FMN (47). Thus, Coq11 may use its Rossmann fold in conjunction with a nucleotide cofactor to perform similar redox chemistry in S. cerevisiae Q₆ biosynthesis. The ratio of QH₂/Q serves as a metabolic sensor for electron transport chain efficiency (48). High QH₂/Q ratios induce respiratory complex I-mediated reverse electron transport (RET) under physiological conditions in both Drosophila and mammalian cell lines (48, 49). Superoxide and secondary reactive species produced specifically through complex I RET extended Drosophila lifespan and improved mitochondrial function in a model of Parkinson’s disease (49). RET induced by over-reduction of the Q pool presumably generates a superoxide-dependent signal essential for homeostasis, such that manipulation of the Q redox state is beneficial for mitochondrial function (48, 49). Mitochondrial phenotypes in the absence of COQ11, including restored respiration in coq10Δ coq11Δ and up-regulated Q₆ machinery (Figs. 2, A–C, 6, and 7), seem to correlate well with the aforementioned effects of Q₆H₂ accumulation. Yeast coq11Δ and coq10Δcoq11Δ mutants retain antioxidant protection by Q₆H₂, demonstrated by their resistance to treatment with exogenously-added PUFAs (Fig. 2D). Because cells lacking the Coq11 polypeptide maintain Q₆H₂ as an antioxidant, it follows that Coq11 could be involved in the oxidation of Q₆H₂ to Q₆.

The phenotypes of Coq10 and Coq11 seen in this work are similar to those in both fungi and mammalian hosts. Several fungi use Coq11 or Coq11-like proteins as NAD-dependent epimerases/dehydratases, NADH-ubiquinone oxidoreductases, and NADH dehydrogenase subunits (14). Coq11 orthologs are commonly found in plant and algae genomes, including the chloroplast-localized flavin reductase protein At1g32220 from the land plant Arabidopsis thaliana, which is thought to be involved in plastoquine biosynthesis and storage (14, 50). The closest but distinct higher eukaryotic Coq11-like protein is the SDR subfamily protein NDUFA9 (14), an auxiliary subunit of complex I in humans (51). Patients with decreased NDUFA9 expression are unable to properly assemble complex I and may develop a degenerative infancy respiratory disorder known as Leigh syndrome (52). Although yeast cells do not possess complex I, this evidence indicates that Coq11 may play a crucial role in respiratory regulation or function, supporting the observations of this study.

The function of Coq10 is widely conserved across different organisms. Expression of the Coq10 homolog from C. crescentus (CC1736) rescues the impaired respiration and antioxidant function of Q₆ in coq10 yeast mutants (17). The NMR structure
Coq10 knockout phenotypes are rescued by deletion of COQ11

of CC1736 reveals a START domain, which is known to bind lipids via a hydrophobic tunnel (20). Studies of S. pombe Coq10 demonstrate that it is able to bind Q₁₀ (21). One proposed function of Q binding by CC1736 and Coq10 from S. pombe may be to regulate Q delivery to its proper sites in the respiratory complexes. Humans have two distinct homologs of yeast Coq10: COQ10A and COQ10B. Expression of either human protein rescues the coq₁₀Δ respiratory deficiency and sensitivity to oxidative stress, and it restores the amounts of Coq polypeptides to WT (19). The conserved function of yeast Coq10 with human COQ10A and COQ10B suggests that the findings of this work will shed light on the role of Coq10 as a chaperone in humans, leading to a better understanding of the pathobiology of Q₁₀ diseases.

In summary, this work reveals that Coq11 plays a regulatory role to maintain Q₆ homeostasis in concert with Coq10 in S. cerevisiae (Fig. 10). The absence of COQ11 caused an augmentation of Q₆ production and respiration in the coq₁₀Δ mutant, indicating that Coq11 confers a negative effect on the CoQ synthome. Coq11 may be crucial for Q₆ function in addition to Q₆ biosynthesis, as total whole-cell and mitochondrial Q₆ content remained lower than WT in coq₁₁Δ and coq₁₀Δcoq₁₁Δ mutants.

Experimental procedures

All reagents were obtained commercially from Thermo Fisher Scientific unless otherwise specified.

Yeast strains and growth medium

S. cerevisiae strains used in this study are described in Table 1. Yeast strains were derived from S288C (BY4742 (53)) or W303 (54). Growth media were prepared as described previously (55), and plate medium contained 2% bacto-agar. Growth media included the following:YPD (2% glucose, 1% yeast extract, and 2% peptone), YPGal (2% galactose, 1% yeast extract, 2% peptone, and 0.1% dextrose), YPG (3% glycerol, 1% yeast extract, and 2% peptone), and YPEG (3% glycerol, 2% ethanol, 1% yeast extract, and 2% peptone). Synthetic dextrose/selection media (SD–Complete, SD–Ura, SD–Leu, SD–His, SD–His–Leu, SD–Ura–Leu) were prepared as described previously (55) and consisted of all components minus uracil, leucine, histidine, or both uracil and leucine. Drop-out dextrose (DOD) medium was prepared as described previously (14).

COQ11 was disrupted by the one-step gene replacement method (56). The LEU2 gene from pRS305 was amplified by polymerase chain reaction (PCR), with COQ11 upstream and downstream flanking sequences 5'–GGGAAATATGTTACGTATAACAAAATACAGCTAAGCTGACCTG and 3'–GCTCTTTACTATACTACAGCTTGGTGTTAATTTTTAAA TGTTAATAAC. Transformations of PCR products into yeast cells were performed using the Li-acetate method (57). The double coq₁₀Δcoq₁₁Δ mutant was constructed via disruption of COQ10 within the coq₁₁Δ strain. The HIS3 gene from pRS313 was amplified by PCR with COQ10 upstream and downstream flanking sequences 5'–GGATAAGGAGCACAACATTTAACGGCTAAGAGTACCCGGG and 3'–CAGGATACAAAGATCATGCCATCCAGATAAGCTGAGCCCTG, and transformation was performed as for the COQ11 disruption.

Priming were designed using SnapGene (GSL Biotech, LLC, Chicago, IL).

Mitochondria isolation from BY4742 WT and mutant yeast

Yeast cultures of BY4742, coq₁₀Δ, coq₁₁Δ, and coq₁₀Δcoq₁₁Δ were grown overnight in 5 ml of YPD. Yeast-containing plasmids were grown overnight in 5 ml of selection medium (SD–Ura). All pre-cultures were back-diluted with YPGal and grown overnight with shaking (30 °C, 250 rpm) until cell density reached an A₆₀₀ ~ 4. Spheroplasts were prepared with Zymolyase-20T (MP Biomedicals) and fractionated as described previously (36), in the presence of Completem™ EDTA-free protease inhibitor mixture tablets (Roche Applied Science), phosphatase inhibitor mixture set I (Sigma-Aldrich), phosphatase inhibitor mixture set II (Sigma-Aldrich), and phenylmethylsulfonyl fluoride (Thermo Fisher Scientific). Nycodenz (Sigma-Aldrich) density gradient purified mitochondria were frozen in liquid nitrogen, aliquoted, and stored at −80 °C until further use. Protein concentration of mitochondria was measured by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific).

Submitochondrial localization of Coq10 and Coq11 polypeptides

Purified mitochondria from BY4742 yeast (3 mg of protein, 150 μl) were subfractionated, as described previously (13). Proteinase K treatment of purified BY4742 mitochondria was also performed as described previously (13). Proteinase K-treated mitoplasts and control samples were resuspended in SDS sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromphenol blue, and 1.33% β-mercaptoethanol); equal aliquots were separated by SDS-gel electrophoresis on 10 or 12% Tris-glycine polyacrylamide gels as detailed below. Several mitochondrial compartment markers and proteins of interest, Coq10 and Coq11, were detected with rabbit polyclonal antibodies prepared in blocking buffer at dilutions listed in Table S1.

Oxygen consumption evaluation by Seahorse

Mitochondrial function was assessed using the XF96 extracellular flux analyzer (Seahorse Bioscience, Agilent Technologies). Seahorse plates were coated with 50 μg/ml poly-D-lysine (Sigma-Aldrich), diluted 1:1 in UltraPure distilled water (Thermo Fisher Scientific). Volumes of 25 μl were added to each well for 30 min at room temperature and then aspirated before plates were dried overnight at room temperature. The Seahorse XF96 sensor cartridge was hydrated with Seahorse XF calibrant solution (Agilent) and was incubated overnight at room temperature.

Yeast cultures of BY4742, coq₁₀Δ, coq₁₁Δ, and coq₁₀Δcoq₁₁Δ were grown overnight in 25 ml of YPGal medium. On the day of measurement, all cultures were diluted to seed an A₆₀₀ = 0.1 cells/well of BY4742, coq₁₀Δ, coq₁₁Δ, and coq₁₀Δcoq₁₁Δ into a Seahorse XF96 microplate in a total volume of 175 μl YPGal. Four wells containing only medium were included for background measurement. The loaded plate was centrifuged at 500 × g for 3 min at room temperature (with no brakes). Following centrifugation, the loaded plate was incubated for 30
Coq10 knockout phenotypes are rescued by deletion of COQ11

min at 37 °C with no CO₂ to aid in the transitioning of the plate into the Seahorse machine’s temperature. Cells were stimulated sequentially with two injections of 4 μM FCCP in ports A and B (optimized for maximum oxygen consumption rate) (Enzo Life Sciences) and 2.5 μM antimycin A in port C (Enzo Life Sciences), delivered in YPGal. Mix, wait, and measure times were 2 min, 30 s, and 2 min, respectively. Basal respiration included four measurements, and then following each injection three measurements were made. All OCR were subtracted for nonmitochondrial respiration and normalized to A₀. Basal respiration was calculated as an average of OCR prior to the first FCCP addition. Maximal respiration was calculated as an average of OCR following the second FCCP addition. Nonmitochondrial respiration was measured as average OCR following antimycin A addition.

**Fatty acid sensitivity assay**

Sensitivity of yeast cells to PUFA-induced oxidative stress was performed as described previously (19, 58, 59), with some modifications. Briefly, BY4742 WT, coq10 Δ, coq9 Δ, coq10A Δcoq11 Δ, and coq10 A Δcoq11 Δ were inoculated in 5 ml of YPD medium and incubated overnight at 30 °C, 250 rpm. Cultures were subinoculated to an A₀ = 0.25 in 15 ml of fresh YPD medium and incubated at 30 °C, 250 rpm until they reached an A₀ = 1. Cells were harvested, washed twice with 10 ml of sterile H₂O, and diluted in 0.1 M phosphate buffer with 0.2% dextrose, pH 6.2, to an A₀ = 0.2. This cell suspension was divided into 5-ml aliquots and treated with an ethanol vehicle control (final concentration 0.1% v/v), ethanol-diluted oleic acid (Nu-Chek Prep), or α-linolenic acid (Nu-Chek Prep) to a final concentration of 200 μM. Fatty acid-treated cultures were incubated for 4 h at 30 °C, 250 rpm, after which cell viability was assessed via plate dilutions. Cell viability prior to the addition of fatty acids was determined via plate dilutions, represented in the 0-h plate.

**Analysis of Q₆ and Q₆-intermediates**

Standards of Q₆ were obtained from Avanti Polar Lipids, and Q₄ was from Sigma-Aldrich. Yeast cultures were grown overnight in 30 ml of YPGal, or selection medium (SD-complete or SD-Ura) for strains harboring plasmids. Cultures were diluted into triplicates of 6 ml of fresh medium to A₀ = 0.5, and 5 ml of medium was harvested by centrifugation once they reached A₀ = 4. Cell pellets were stored at −20 °C. Following collection, frozen cell pellets were lipid-extracted in the presence of internal standard Q₆ and analyzed for Q₆ and Q₆-intermediates by LC-MS/MS as described previously (19).

**Stable isotope labeling for determination of de novo Q₆ and Q₆-intermediates**

Yeast cultures were grown overnight in 30 ml of YPGal and diluted in triplicates of 6 ml of fresh medium to an A₀ = 0.1. Cultures were incubated until they reached an A₀ ~ 1, at which point ethanol vehicle control (0.1% v/v) or 5 μg/ml of the stable isotope [¹³C₆]4HB (Cambridge Isotope Laboratories, Inc.) was added. Cultures were allowed to grow for an additional 4 h when 5 ml of each culture was harvested by centrifugation and stored at −20 °C. Cell pellets were lipid extracted and analyzed by LC-MS/MS as described previously (19).

**Mitochondrial DNA determination by qPCR analysis**

DNA was extracted from yeast cells as follows. Yeast pellets (10 ml) grown in YPGal were collected at an A₀ ~ 4 by centrifugation at 3000 × g for 5 min, washed with 5 ml of H₂O, and transferred to 2-ml screw-cap tubes. Pellets were frozen at −80 °C until DNA extraction was carried out. Cell pellets were resuspended in 200 μl of lysis buffer (10 mM Tris-Cl, pH 8.0, 2% (v/v) Triton X-100, 1 mM EDTA, 100 mM NaCl, 1% SDS), and 200 μl of acid-washed glass beads with 200 μl of phenol/chloroform/isoamyl alcohol (25:24:1) were added to the cell suspension. Cells were lysed using a bead-beater (Precellys 24; Bertin Technologies) three times for 10 s at 6500 rpm with a 45-s break between rounds at 4 °C. Triton-EDTA (TE, 200 μl) was added, and the cell suspension was centrifuged at 13,000 × g for 5 min at room temperature. The aqueous layer was removed to a new tube containing 200 μl of chloroform, mixed by inversion, and centrifuged at 13,000 × g for 5 min at room temperature. This was repeated once more. The aqueous layer was then transferred to a 2-ml screw-cap tube containing 1 ml of 95% EtOH, mixed by inversion, and centrifuged at 13,000 × g for 2 min at room temperature. The resulting pellet was resuspended in 400 μl of TE containing 30 μl of RNase A and incubated at 37 °C for 30 min. Then, 10 μl of 3 M sodium acetate and 1 ml of 95% EtOH was added, mixed by inversion, and incubated at −20 °C for 1 h. After 1 h at −20 °C, the suspension was centrifuged at 13,000 × g for 5 min, and the pellet was washed twice with 70% EtOH and air-dried. The dried pellet was resuspended in 25 μl of TE; concentration was measured by Nanodrop (Thermo Fisher Scientific), and the pellet was stored at −20 °C until use.

qPCR was performed on a CFX384 instrument (Bio-Rad) using the SensiFAST™SYBR® NO ROX kit (Bioline) as per the manufacturer’s instructions. Each sample was run in duplicate with 150 ng of total DNA used per reaction using the following thermocycling protocol (95 °C for 2 min, 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 20 s, plate read and cycle repeated ×40, melt curve 40–92 °C with plate read and 40 °C for 10 s). Melting-curve analysis confirmed that all PCRs produced a single product. mtDNA-specific primers (forward (13,999), 5′-GTG CGT ATA TTT CGT TGA TGC GT-3′; reverse (14,297), 5′-TTC ACA CTG CCT GTG CTA TCT AA-3′) and actin-specific primers (forward, 5′-GAA TTG AGA GTT GCC CCA GA-3′; reverse, 5′-ATC ACC GGA ATC CAA AAC AA-3′) were used. The relative level of gene expression of mitochondrial DNA was normalized to the expression level of actin as described previously (61).

**Citrate synthase activity**

The measurement of citrate synthase activity in cells was carried out as described previously (62). Briefly, yeast pellets (10 ml) grown in YPGal were collected at an A₀ ~ 4 by centrifugation at 3000 × g for 5 min, washed with 5 ml of H₂O, and transferred to 2-ml screw-cap tubes. Pellets were resuspended in 200 μl of lysis buffer (100 mM Tris-Cl, pH 7.4, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1× Complete™ Protease Inhibitor Mixture (Roche...
Coq10 knockout phenotypes are rescued by deletion of COQ11

Applied Science), and then 200 μl of acid-washed glass beads were added. Cells were lysed using a bead-beater (Precellys 24; Bertin Technologies) three times for 10 s at 6500 rpm with a 45-s break between rounds at 4°C. The clarified cell lysate was collected after centrifugation at 16,000 × g for 10 min at 4°C. The concentration of protein was determined with the BCA assay (Thermo Fisher Scientific). Cell lysates were normalized to 0.05 μg/μl protein. The colorimetric citrate synthase assay was carried out using a VersaMax plate reader (Molecular Devices) and a flat-bottom 96-well plate. First, 40 μl of 500 mM Tris-Cl, pH 7.4, 2 μl of 30 mM acetyl-CoA, 8 μl of 2.5 mM 5,5’-dithiobis(2-nitrobenzoic acid), 90 μl of H2O2, and 50 μl cell lysate (2.5 μg total protein) were added into each well. Then, 10 μl of 10 mM oxaloacetic acid were added per well and mixed by pipetting up and down. A412 was measured every 30 s at 25°C. The initial slope was calculated by using data from the first 10 min and used to determine the enzyme reaction rate using the extinction coefficient for 2-nitro-5-thiobenzoate of 14.15 mM−1 cm−1 (63).

Porin quantification

Porin content was quantified via immunoblot of yeast WT and mutant whole cells. Protein extraction from whole cells was performed (64), and 25 μg of each sample was separated by SDS-gel electrophoresis as described below. Three replicates of the immunoblots were performed and quantified by hand using ImageStudioLite software normalized to Ponceau total protein staining.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from yeast cells using TRIzol reagent (Invitrogen). DNA contamination from the resulting RNA was removed using the DNase TURBO kit as per the manufacturer’s instructions (Invitrogen). RNA concentration was measured by Nanodrop (Thermo Fisher Scientific), and RNA was stored at −20°C. Reverse transcription was carried out using the SuperScript III first strand synthesis system using random hexamer primers (Invitrogen). cDNA was stored at −20°C until qPCR analyses were carried out. Quantitative real-time PCR was performed on a CFX384 instrument (Bio-Rad) using the SensiFAST™SYBR® NO-ROX kit (Bioline) in duplicate. The relative levels of gene expression were normalized to the expression level of actin. Melting curve analysis confirmed that all PCRs produced a single product. The primers (forward/reverse) used in real-time PCR were designed using Primer3 on line (RRID: SCR_003139). Primers used are given in Table S2.

SDS-PAGE and immunoblot analysis

Purified mitochondria (25 μg) were resuspended in SDS sample buffer and separated by SDS-gel electrophoresis on 10 or 12% Tris-glycine polyacrylamide gels. Proteins were transferred to a 0.45-μm polyvinylidene difluoride membrane (Millipore) and blocked with blocking buffer (0.5% BSA, 0.1% Tween 20, 0.02% SDS in PBS). Representative Coq polypeptides and loading control mitochondrial malate dehydrogenase (Mdh1) were probed with rabbit polyclonal antibodies prepared in blocking buffer at dilutions listed in Table S1. IRDye 680LT goat anti-rabbit IgG secondary antibody (LiCOR) was used at a dilution of 1:10,000. Proteins were visualized using a LiCOR Odyssey IR Scanner (LiCOR). Immunoblots are representative of three replicates and were quantified by hand using ImageStudioLite software normalized to Mdh1.

Two-dimensional Blue Native/SDS-PAGE immunoblot analysis of high-molecular-weight complexes

2D-BN/SDS-PAGE was performed as described previously (13, 65, 66). Briefly, 200 μg of purified mitochondria were solubilized at 4 mg/ml for 1 h on ice with 16 mg/ml digitonin (Biosynth) in the presence of the protease and phosphatase inhibitors used during mitochondrial isolation. Protein concentration of solubilized mitochondria was determined by BCA assay. NativePAGE 5% G-250 sample additive (Thermo Fisher Scientific) was added to a final concentration of 0.25%. Solubilized mitochondria (100 μg) were separated on NativePAGE 4–16% BisTris gels (Thermo Fisher Scientific) in the first dimension, and native gel slices were further separated on 12% Tris-glycine polyacrylamide gel in the second dimension. Following the second-dimension separation, immunoblot analyses were performed as described above, using antibodies against Coq4 and Coq9 at the dilutions indicated in Table S1. Molecular weight standards for BN gel electrophoresis and SDS gel electrophoresis were obtained from GE Healthcare (Sigma-Aldrich) and Bio-Rad, respectively.

Construction of low-copy COQ11 yeast expression vectors

Plasmids used in this study are described in Table 2. A low-copy COQ11-containing plasmid was constructed using the pRS316 low-copy empty vector. The COQ11 ORF and regions corresponding to 842 bp upstream and 256 bp downstream were cloned into pRS316 using Gibson Assembly (New England Biolabs). Clones were sequenced by Laragen, and successful clones were transformed into WT and mutant yeast, along with the corresponding empty vector (pRS316) control as described above.

Revertant isolation

As reported previously, coq10 mutant growth deficiency on nonfermentable carbon sources (YPEG) spontaneously revert due to nuclear suppression mutations (18). Here, W303 coq10Δ yeast was grown on glucose to stationary phase, and ~10 million cells were plated on YPEG. After several weeks, a colony began to appear on this medium. The colony was purified, and its genome was sequenced.

Genome sequencing

The Wizard® genomic purification kit (Promega) was used to extract total DNA from the parental respiratory-deficient mutant W303 coq10Δ and from the spontaneous revertant W303 coq10rev. The DNA was quantified using the QUBIT DNA™ high-sensitivity assay, and 1 ng of the normalized DNA was tagged by the Nextera XTMM (Illumina) protocol. The libraries were amplified and pooled as described (67). The pooled libraries were subjected to sequencing with the NextSeq™ (Illumina) equipment in the Genome Investigation and Analysis Laboratory of the Institute of Biomedical Sciences at the University of Sao Paulo. The BWA Aligner tool,
version 1.1.4 (Base Space Labs-Illumina), was used to align ~23,000,000 reads obtained from each strain with the reference genomes of *S. cerevisiae*. The alignments were compared using the Integrative Genomics Viewer (Base Space Labs Illumina).

**Statistical analyses**

All data sets were tested for normality using GraphPad Prism 7 with the Shapiro-Wilk normality test. Because a majority of sets passed the normality test (α = 0.5), statistical analyses were performed using GraphPad Prism 7 with parametric one-way analysis of variance correcting for multiple comparisons using Tukey’s test, comparing the mean of each sample to the mean of its corresponding WT or empty vector control. The data show the means ± S.D., and the statistical significance as compared with WT or empty vector control is represented by the following: *, p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001. The denotation ns indicates values with “not significant” differences from the corresponding control.

**Data availability**

The MS source data for determination of Qₒ and Qₐ intermediates will be shared upon request. Please contact Catherine Clarke at cath@chem.ucla.edu. All remaining data are contained within the article.

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