UbiB proteins regulate cellular CoQ distribution in Saccharomyces cerevisiae

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Beyond its role in mitochondrial bioenergetics, Coenzyme Q (CoQ, ubiquinone) serves as a key membrane-embedded antioxidant throughout the cell. However, how CoQ is mobilized from its site of synthesis on the inner mitochondrial membrane to other sites of action remains a longstanding mystery. Here, using a combination of *Saccharomyces cerevisiae* genetics, biochemical fractionation, and lipid profiling, we identify two highly conserved but poorly characterized mitochondrial proteins, Ypl109c (Cqd1) and Ylr253w (Cqd2), that reciprocally affect this process. Loss of Cqd1 skews cellular CoQ distribution away from mitochondria, resulting in markedly enhanced resistance to oxidative stress caused by exogenous polyunsaturated fatty acids, whereas loss of Cqd2 promotes the opposite effects. The activities of both proteins rely on their atypical kinase/ATPase domains, which they share with Coq8—an essential auxiliary protein for CoQ biosynthesis. Overall, our results reveal protein machinery central to CoQ trafficking in yeast and lend insights into the broader interplay between mitochondria and the rest of the cell.
CoQ is synthesized in mitochondria, where it functions as an essential cofactor in multiple processes including oxidative phosphorylation, fatty acid oxidation, and nucleotide biosynthesis. CoQ is also present in membranes throughout the cell, suggesting that it has a more widespread cellular importance than is currently appreciated. Recently, one such role for extramitochondrial CoQ in mammalian cells was identified with the discovery that plasma membrane-localized FSP1 exhibits CoQ-dependent activity in mitigating ferroptosis, a form of regulated cell death caused by aberrant iron-dependent lipid peroxidation. To our knowledge, no proteins have yet been directly associated with cellular CoQ trafficking from mitochondria, but the extreme hydrophobicity of CoQ suggests that this process likely requires dedicated machinery.

Here, we demonstrate that two members of the poorly characterized UbiB family of atypical kinases/ATPases influence the cellular distribution of mitochondria-derived CoQ in the budding yeast *Saccharomyces cerevisiae*. We show that disruption of *CQD1* and *CQD2* diminishes and enhances the levels of mitochondrial CoQ, respectively, without altering total cellular CoQ abundance. Our findings help to define the functions of two mitochondrial proteins and advance our still nascent understanding of how CoQ is distributed throughout the cell.

**Results**

**Extramitochondrial CoQ combats oxidative stress.** We sought to identify proteins related to CoQ trafficking by exploiting the extramitochondrial antioxidant role of CoQ₀—the major form of CoQ in *S. cerevisiae* (hereafter referred to as CoQ). *S. cerevisiae* lacking CoQ₀ or phospholipid hydroperoxide glutathione peroxidases (PHGPx) are sensitive to the oxidative stress conferred by exogenous polyunsaturated fatty acids (PUFAs), such as α-linolenic acid (18:3). PUFAs undergo uncontrolled autooxidation reactions in the absence of these antioxidant factors, leading to the toxic accumulation of lipid peroxides and peroxyl radicals. To force cells into relying more heavily on the antioxidant properties of CoQ₀, we deleted all three PHGPx genes in W303 *S. cerevisiae Δgpx1/2/3* (hereafter referred to as Δgpx1/2/3). We validated that this strain is sensitized to 18:3 treatment and demonstrated that this sensitivity is dampened when cellular CoQ₀ levels are augmented through supplementation with the soluble CoQ precursor 4-hydroxybenzoate (4-HB) (Fig. 1a, b). Importantly, the CoQ analog decylubiquinone was markedly more effective at protecting against PUFA stress than its mitochondria-targeted counterpart, mitoquinone, suggesting that extramitochondrial CoQ₀ is the predominant mediator of PUFA resistance (Fig. 1c). This is consistent with previous data showing that exogenous PUFAs are incorporated into endogenous membranes slowly and, therefore, likely populate non-mitochondrial membranes first. Thus, we established a strain whose survival in the presence of PUFAs is especially dependent on extramitochondrial CoQ₀.

**Loss of Cqdl confers PUFA resistance.** We reasoned that suppressor mutations that increase extramitochondrial CoQ₀ levels would enhance PUFA resistance in the *Δgpx1/2/3* strain, so we performed a forward-genetic suppressor screen (Fig. 2a). We randomly mutagenized this strain with ethyl methanesulfonate (EMS) and isolated colonies tolerant of 18:3 treatment. From ~20,000 unique mutant colonies, we obtained four hit strains with substantial PUFA resistance (Fig. 2b). We then performed whole-genome sequencing that revealed non-synonymous mutations in 442 unique genes across these four strains (Supplementary Data 1). These mutants were ranked using PROVEAN (Protein Variation Effect Analyzer), a software tool for predicting deleterious protein changes. PROVEAN assigns a disruption score (D-Score) that reflects the likelihood that a given mutation is deleterious. In our collective dataset, 99 genes achieved a D-Score below the strict threshold of −4.1 (Fig. 2c; Supplementary Data 1). Given the overall limited overlap in hits between mutant strains, it is likely that our dataset includes multiple genes that contribute to an enhanced PUFA resistance phenotype.

We chose to focus on mitochondrial proteins for further examination since, to our knowledge, trafficking machinery at the site of CoQ synthesis in mitochondria has yet to be identified. Of the nine mitochondrial proteins harboring likely deleterious mutations, one, Ypl109c (renamed here as Cqdl, see below), is an uncharacterized protein that resides on the inner mitochondrial membrane (IMM), making it an attractive candidate for further study (Fig. 2c; Supplementary Fig. 1a). Moreover, Cqdl possesses the same UbiB family atypical kinase/ATPase domain as Coq8, an essential protein for CoQ₀ synthesis that resides on the matrix face of the IMM. Our recent work suggests that Coq8 ATPase activity may be coupled to the extraction of hydrophobic CoQ₀ precursors from the IMM for subsequent processing by membrane-associated matrix enzymes. Cqdl resides on the opposite side of the IMM, facing the intermembrane space, physically separated from the other CoQ-related enzymes but still positioned for direct access to membrane-embedded CoQ₀ precursors and mature CoQ₀. Furthermore, a recent study reported that haploinsufficiency of human CQD1 ortholog ADCK2 led to aberrant mitochondrial lipid oxidation and myopathy associated with CoQ₀ deficiency.

In our screen, each resistant strain (mutA-D) possesses more than 100 protein-coding mutations, a combination of which likely contributes to the PUFA resistance phenotype. Mutant C (mutC) contains an early stop codon in CQD1 (Fig. 2c, Supplementary Fig. 1c). To test whether this CQD1 mutation is important for mutC’s phenotype, we reintroduced WT CQD1 into this strain under its endogenous promoter. Indeed, this reintroduction re-
confers PUFA sensitivity (Fig. 2d). Furthermore, deletion of CQD1 in the parent Δgpx1/2/3 strain, which lacks all other mutC mutations, was sufficient to enhance PUFA resistance (Fig. 2e, f). We also confirmed that deletion of CQD1 had no effect in background strains lacking CoQ (Δcoq2 and Δgpx1/2/3Δcoq2), establishing that this PUFA-resistant phenotype is CoQ-dependent (Supplementary Fig. 1d, e). Collectively, these data demonstrate that disruption of CQD1 is at least partially causative for mutC’s PUFA-resistant phenotype.

Cqdl affects CoQ distribution. Our results above suggest that loss of CQD1 confers cellular resistance to PUFA-mediated oxidative stress by increasing extramitochondrial CoQ. We reasoned that this was likely rooted either in a general increase in CoQ production or in its redistribution. To test these models, we first measured total levels of CoQ and its early mitochondrial precursor polypropenyl-4-hydroxybenzoate (PPHB) in cells lacking CQD1 or control genes (Fig. 3a–c). As expected, disruption of HFD1, which encodes the enzyme that produces the soluble CoQ precursor 4-HB17,18, led to a loss of CoQ and PPHB, while disruption of COQ8 caused complete loss of CoQ with the expected buildup of the PPHB precursor. However, we found no significant change in CoQ or PPHB levels in the Δcqd1 strain, demonstrating that Cqdl is essential neither for CoQ biosynthesis nor the import of CoQ precursors under the conditions of our analyses.

To next examine CoQ distribution, we fractionated yeast and measured CoQ levels (Fig. 3d; Supplementary Fig. 2a). We measured measured total levels of CoQ and its early mitochondrial precursor polypropenyl-4-hydroxybenzoate (PPHB) in cells lacking CQD1 or control genes (Fig. 3a–c). As expected, disruption of HFD1, which encodes the enzyme that produces the soluble CoQ precursor 4-HB17,18, led to a loss of CoQ and PPHB, while disruption of COQ8 caused complete loss of CoQ with the expected buildup of the PPHB precursor. However, we found no significant change in CoQ or PPHB levels in the Δcqd1 strain, demonstrating that Cqdl is essential neither for CoQ biosynthesis nor the import of CoQ precursors under the conditions of our analyses.

To next examine CoQ distribution, we fractionated yeast and measured CoQ levels (Fig. 3d; Supplementary Fig. 2a). We observed that Δcqd1 yeast had a significant increase in CoQ from the non-mitochondrial (NM) fraction, consisting of organelles and membranes that do not pellet with mitochondria, and a corresponding significant decrease in mitochondrial (M) CoQ. Deletion of the tricarboxylic acid (TCA) cycle enzyme Kgd1 had no effect on relative CoQ levels (Fig. 3d) despite causing a deficiency in respiratory growth (Fig. 3e), indicating that general mitochondrial dysfunction does not perturb CoQ distribution. The increased extramitochondrial CoQ in Δcqd1 yeast is consistent with the observation that deleting CQD1 increases PUFA resistance (Fig. 2e, f).
To our knowledge, this is the first example of a genetic disruption leading to altered cellular distribution of endogenous CoQ, hence our renaming of this gene CoQ Distribution 1 (CQD1). To further validate this finding, we examined growth in glycerol, a non-fermentable carbon source, which requires an intact mitochondrial electron transport chain. We reasoned that a decrease in mitochondrial CoQ would disrupt respiratory growth in media depleted of CoQ precursors. Indeed, deletion of CQD1 reduced respiratory growth rate in this medium appreciably (~4 h in this growth condition). Growth rate of WT, ∆cqd1, and ∆kgd1 yeast assayed under conditions described in (d) (mean ± SD; none n = 6 independent samples). Yeast enter the respiratory phase of growth after ~4 h in this growth condition. f Growth rate of WT, ∆cqd1, and ∆kgd1 yeast assayed under conditions described in (d) (mean ± SD; none n = 6 independent samples, all others n = 3). Yeast were grown in the presence and absence of 100 µM CoQ analogs (CoQ2, CoQ4) and 1 µM CoQ precursors (pABA, 4-HB). g Growth rate of WT and ∆cqd1 yeast transformed with the indicated plasmids (EV, CQD1 or CQD1 point mutants) and grown in Ura− media containing 0.1% (w/v) glucose and 3% (w/v) glycerol (mean ± SD, n = 3 independent samples). Yeast were treated with 0 (colored bars) or 1 µM 4-HB (white bars, superimposed) to determine rescue of respiratory growth. h Model for Cqd1’s putative role in cellular CoQ distribution. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; EMM, extramitochondrial membranes. b-d Significance calculated by an unpaired, two-tailed Student’s t-test.

Cqd2 counteracts Cqd1 function. Beyond Coq8 and Cqd1, the S. cerevisiae genome encodes just one other member of the UbiB family—Ylr253w (aka Mcp2, and renamed here Cqd2). Cqd2 is also poorly characterized and resides in the same location as Cqd1, on the outer face of the IMM11,15,21 (Supplementary Fig. 1b). Previous studies have identified genetic and physical interactions connecting Cqd2 to mitochondrial lipid homeostasis, but not to a specific pathway.21–23 Given the similarity between these three proteins (Supplementary Fig. 2d, e), we anticipated that Cqd2 might also be connected to CoQ biology.

To test this hypothesis, we disrupted CQD2 in ∆gpx1/2/3 yeast and subjected this strain to PUFA-mediated stress. Surprisingly, ∆gpx1/2/3∆cqd2 yeast exhibited an enhanced sensitivity to PUFA treatment—the opposite phenotype to that of ∆gpx1/2/3∆cqd1
This phenotype is also CoQ-dependent, as deletion of \textit{CQD2} likewise had no effect in background strains lacking CoQ (Supplementary Fig. 1d, e). Furthermore, \textit{Δgpx1/2/3 Δcqd1 Δcqd2} yeast phenocopied the parental (\textit{Δgpx1/2/3}) strain (Fig. 4a; Supplementary Fig. 3a).

Under respiratory conditions, \textit{Δcqd2} yeast exhibited no detectable change in growth. However, deleting \textit{CQD2} from \textit{Δcqd1} yeast (\textit{Δcqd1 Δcqd2}) restored this strain’s impaired respiratory growth rate to WT levels (Fig. 4b, c). Conversely, reintroduction of \textit{CQD2} into the \textit{Δcqd1 Δcqd2} strain recapitulated the respiratory growth deficiency of \textit{Δcqd1} (Fig. 4d). Total cellular CoQ levels remained unchanged (Supplementary Fig. 3b), again suggesting these CoQ-related phenotypes are unrelated to CoQ biosynthesis. Similar to Cqd1 (Fig. 3g), Cqd2 function was dependent on intact canonical PKL and UbiB-specific residues (Fig. 4d, Supplementary Fig. 3c–e), suggesting that all three UbiB family proteins in yeast are active phosphoryl transfer enzymes. Consistent with these results, subcellular fractionation revealed significantly increased CoQ levels in the pure mitochondrial fraction from \textit{Δcqd2} yeast (Fig. 4e; Supplementary Fig. 3f). Furthermore, the \textit{Δcqd1 Δcqd2} strain possessed mitochondrial and non-mitochondrial CoQ levels between those of the \textit{Δcqd1} and \textit{Δcqd2} strains (Fig. 4e). However, our fractionation approach, which prioritizes high purity over yield, only detected very low levels of CoQ in the WT and \textit{Δcqd2} non-mitochondrial samples; therefore, a quantifiable loss of CoQ in this fraction for the \textit{Δcqd2} was not detectable (Fig. 4e).
Fig. 4 Cqd2 function opposes Cqd1 control of CoQ distribution. a Growth rate of Δgpx1/2/3 and the described yeast strains in pABA− media containing 2% (w/v) glucose and the indicated additives (mean ± SD, n = 3 independent samples). b Growth assay of WT, Δcqd1, Δcqd2, and Δcqd1Δcqd2 yeast in pABA− media containing 0.1% (w/v) glucose and 3% (w/v) glycerol (mean ± SD, n = 6 independent samples). c Growth rate of yeast strains in b treated with 0 (colored bars) or 1 mM 4-HB (white bars, superimposed) (mean ± SD; 0 μM 4-HB n = 6 independent samples, 1 mM 4-HB n = 3). d Growth rate of WT and Δcqd1Δcqd2 yeast transformed with the indicated plasmids (EV, CQD2, or CQD2 point mutants) and grown in Ura−, pABA− media containing 0.1% (w/v) glucose and 3% (w/v) glycerol (mean ± SD, n = 3 independent samples). Yeast were treated with 0 (colored bars) or 1 mM 4-HB (white bars, superimposed) to determine recapitulation of respiratory growth defect. e CoQ from subcellular fractions derived from WT, Δcqd1, Δcqd2, and Δcqd1Δcqd2 yeast (p = 0.0392 WT CM vs Δcqd2 CM, **p = 0.0081 WT NM vs Δcqd1 NM, ***p = 0.0075 WT NM vs Δcqd1Δcqd2 NM, p = 0.0105 WT M vs Δcqd1 M, p = 0.0112 WT M vs Δcqd2 M; mean ± SD, n = 3 independent samples). SP, spheroplast; CM, crude mitochondria; NM, non-mitochondrial fraction; M, enriched mitochondria. f Schematic of Sdh4-GFP styrene-maleic acid (SMA) lipid particle (SMALP) isolation. g Western blot to assess the purity of SMALP isolation samples from endogenously tagged Sdh4-GFP yeast. SP, spheroplast; CM, crude mitochondria; S, soluble; E, elution (or IMM patch). Kar2, endoplasmic reticulum; Tom70, outer mitochondrial membrane (OMM); Vdac, OMM; Sdh4-GFP, SMALP target/IMM; Sdh2, IMM; Cht1, mitochondrial matrix. A representative western blot from three independent experiments. h CoQ from subcellular fractions derived from SMALP isolation described in (f) for the indicated strains (p = 0.0026 WT IMM patch vs Δcqd1 IMM patch, p = 0.0114 WT IM patch vs Δcqd2 IMM patch; mean ± SD, n = 3). i Summary model depicting opposing roles for yeast UbiB family proteins in the cellular distribution of CoQ. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; EMM, extramitochondrial membranes. e, h Significance calculated by an unpaired, two-tailed Student’s t-test.

Discussion

Our work demonstrates that two previously uncharacterized UbiB family proteins influence the cellular distribution of mitochondria-derived CoQ. To our knowledge, Cqd1 and Cqd2 are the first proteins implicated in this process, which is essential for providing membranes throughout the cell with the CoQ necessary for enzymatic reactions and antioxidant defense. Further efforts are needed to establish how these proteins support CoQ distribution mechanistically; however, their similarity to Coq8 and the requirement for canonical PKL residues in their active sites suggests that Cqd1 and Cqd2 may couple ATPase activity to the selective extraction/deposition of CoQ from/to the IMM.

Our investigations here focused on CoQ; however, it is possible that Cqd1 and Cqd2 (aka Mcp2) influence lipid transport and homeostasis more broadly. Previous work has identified an array of genetic interactions for Cqd1 and Cqd2 with lipid biosynthesis and homeostasis genes. Moreover, Cqd2 was previously identified as a high-copy suppressor of a growth defect caused by loss of the ERMES subunit Mdm10. More recently, conserved Cqd2 active site residues were shown to mitigate rescue of Δmdm10 yeast growth, results that we confirmed (Cqd2 K210R) and expand upon with six additional residue mutations.

Interestingly, mitochondrial CoQ export still occurs in the absence of Cqd1/2, indicating that additional factors can participate in this process. This observation is consistent with multiple other recent studies demonstrating that various aspects of phospholipid transport are highly redundant in yeast. For example, ERMES and vCLAMP appear to have overlapping functions while normally operating under different growth conditions. In the absence of Cqd1 and Cqd2, CoQ transport between the OMM and IMM might be achieved by a combination of MICOS and other lipid-binding proteins. Although our data demonstrate that...
MICOS disruption is insufficient to thwart the PUFA resistance mediated by disruption of COQ1. MICOS alone is often not sufficient to facilitate lipid movement between these membranes, which instead relies on dedicated phospholipid trafficking proteins39. COQ9 is a lipid-binding protein that likely delivers which instead relies on dedicated phospholipid trafficking proteins41,42, they have been localized to mitochondria11,15,43. Of note, although Gpx1-3 are primarily cytosolic41,42, they have been localized to mitochondria11,15,43. Thus, our screen may also be equipped to identify genes that protect against loss of mitochondrial-based Gpx defenses.

Finally, UbIB family proteins are found across all domains of life44. UbIB homologs in plants (termed ABC1K proteins) are abundant, with 17 found in Arabidopsis45. Many of these ABC1K proteins are localized to plastoglobules—plastid-localized lipid-protein particles that contain various lipid-derived metabolites—and recent work suggests that ABC1K1 and ABC1K3 may affect the mobility and exchange of their subcellular plastoquinone-9 pools46, suggesting UbIB proteins might function in quinone distribution across species. In humans, five UbIB proteins have been identified, ADCK1-5. While COQ8A (ADCK3) and COQ8B (ADCK4) have established roles in CoQ biosynthesis and human disease13,47,48, the biological roles of other ADCK proteins remain elusive. Genome-wide knockdown studies have implicated these uncharacterized ADCK genes in several cancer disease states49–52. As novel targets for human disease intervention, it will be important to determine if functional conservation exists between Cqd1 and Cqd2 and their putative human orthologs, ADCK2 and ADCK1/5, respectively. Recently, a crucial new role for extramitochondrial CoQ was identified in mitigating ferropotosis, a type of cell death stemming from a buildup of toxic lipid peroxides, suggesting that manipulating CoQ distribution could provide therapeutic benefits53,54. Notably, we have developed small-molecule modulators for Coq814 and COQ8A33, indicating that UbIB proteins are promising druggable targets.

Collectively, our work to de-orphanize these poorly characterized mitochondrial proteins represents the first step in addressing enduring questions regarding endogenous cellular CoQ distribution and unlocking the therapeutic potential of manipulating this pathway.

**Methods**

**Yeast strains and cultures.** Unless otherwise described, Saccharomyces cerevisiae haploid W303 (MATa his3 leu2 met15 trp1 ura3) yeast were used. For SMA-mediated homologous recombination or CRISPR-mediated methods (all primers used in this study are detailed in Supplementary Data 2), for homologous recombination, open reading frames were replaced with the KanMX6, HygMX6, or NatMX6 cassette as previously described55. Cassette insertion was confirmed by a PCR assay and DNA sequencing. CRISPR-mediated deletions were performed as described in20. 20-mer guide sequences were designed with the ATUM CRISPR gRNA design tool (https://www.atum.bio/eCommerce/cas9/input) and cloned into pRCC-K, and 500 ng of the guide-inserted pRCC-K was used per yeast transformation. Donor DNA was 300 nM of an 80-nM Ultramer consisting of 40 bp upstream and 40 bp downstream of the ORF (for scarless deletions) or ~8 μg of PCR-amplified Longqite and Hisqite with flanking homology 40 bp upstream and 40 bp downstream of the ORF (for cassette-replacement deletions).

Synthetic complete (and dropout) media contained drop-out mix (US Biological), yeast nitrogen base (with ammonium sulfate and without amino acids) (US Biological), and the indicated carbon source. pABA (or dropout) media contained Complete Supplement Mixture (Formedium). Yeast Nitrogen Base without amino acids and without pABA (Formedium), and the indicated carbon source. All media were sterilized by filtration (0.22 μm pore size).

**Yeast growth assay and drop assay**

**PUFA growth assays.** To assay yeast growth in liquid media, individual colonies were used to inoculate synthetic complete (or synthetic complete dropout) media (2% glucose, w/v) starter cultures, which were incubated overnight (30 °C, 230 rpm). Yeast were diluted to 1.1 × 10^6 cells/mL in pABA− (or pABA+ dropout) media (2% glucose, w/v) and incubated until early log phase (30 °C, 7–8 h, 230 rpm). Yeast were swamped into fresh pABA+ media (2% glucose, w/v) at an initial density of 5 × 10^6 cells/mL with indicated additives. The cultures were incubated (30 °C, 1440 rpm) in an Epitope plate reader (BioTek®) in a sterile 96 well polystyrene round bottom microwell plate (Thermo) with a Breathe-Easy cover seal (Diversified Biotech). Optical density readings (A_600) were obtained every 10 min, and growth rates were calculated with Gen5 v3.0.2.2 software (BioTek®), excluding time points before the diauxic shift and during stationary phase growth.

**Respiratory growth assays.** Individual colonies of S. cerevisiae were used to inoculate synthetic complete media (2% glucose, w/v) starter cultures, which were incubated overnight (30 °C, 230 rpm). For transformed yeast strains, the corresponding Ura+ media was used. Yeast were diluted to 1 × 10^6–3.3 × 10^6 cells/mL in pABA− media (2% glucose, w/v) and incubated until early log phase (30 °C, 7–8 h, 230 rpm). Yeast were swamped into pABA+ media with glucose (0.1%, w/v) and glyceral (3%, w/v) at an initial density of 5 × 10^6 cells/mL with indicated additives. The cultures were incubated (30 °C, 1440 rpm) in an Epitope plate reader (BioTek®) in a sterile 96 well polystyrene round bottom microwell plate (Thermo) with a Breathe-Easy cover seal (Diversified Biotech). Optical density readings (A_600) were obtained every 10 min, and growth rates were calculated with Gen5 v3.0.2.2 software (BioTek®), excluding time points before the diauxic shift and during stationary phase growth.

**Forward-genetic screen.** Individual colonies of Δgpx1/2/3 yeast were used to inoculate YEPD starter cultures, which were incubated overnight. 1.0 × 10^6 cells were pelleted, washed once with sterile water, and resuspended in 2.5 mL of 100 mM sodium phosphate buffer, pH 7.0. Ethyl methanesulfonate (EMS) (80 μL) was added, and cells were incubated (90 min, 30 °C, 230 rpm). Cells were washed thrice with sodium thiosulfate (5%, w/v) to inactivate EMS. Cells were resuspended in water. 1.0 × 10^6 cells were plated on pABA− limited (2% w/v glucose, 100 mM pABA) agar plates. After 3 days, cells were replica-plated onto pABA− (2% glucose, w/v) plates with 0 μM or 25 μM α-linolenic acid (C18:3, Sigma). Colonies that grew on 25 μM linolenic acid were picked into YEPD overnight cultures and struck on YEPD plates, and PUFA resistance phenotypes were confirmed with plate reader (BioTek®). For mutant strain growth, yeast were grown in the presence of α-linolenic acid, genomic DNA was isolated with the MasterPure Yeast DNA Purification Kit (Lucigen) and submitted to GENEWIZ for whole-genome sequencing. S. cerevisiae genome assembly and variation calling were performed with SeqMan NGen 14 and ArrayStar 14 (DNASTAR Lasergene suite). Variant D-Score predictions were obtained using the PROVEAN v1.1.3 web server (http://provean.jcvi.org/seq_submit.php).

**Plasmid cloning.** Expression plasmids were cloned with standard restriction enzyme cloning methods. ORF-specific primers (Supplementary Data 2) were used to amplify Cqd1 (Ypl109c) and Cqd2 (Ylr253w) from W303 yeast genomic DNA. Amplicons were treated with DpnI to degrade genomic DNA and ligated into the digested p146 GPD plasmid (Addgene). Cloning products were then transformed into E. coli TOP10 chemically competent cells (Lucigen). Plasmids were isolated from transformants and Sanger sequencing was used to identify those containing the correct insert.

Constrains constructing Cqd1 and Cqd2 were digested with SalI and BamHI or HindIII to liberate the GPD promoter. Digested backbones were then combined with a modified endogenous promoter regions (1000 bases upstream for Cqd1, 500 bases upstream for Cqd2) and ligated to generate endogenous promoter vectors for Cqd1 and Cqd2.

**Site-directed mutagenesis.** Point mutants were constructed as described in the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) and were confirmed via Sanger sequencing. Yeast were transformed as previously described57 with plasmids encoding Cqd1 and Cqd2 variants with their endogenous promoters and grown on uracil drop-out (Ura−) synthetic media plates containing glucose (2%, w/v).

**Homology model generation.** Amino acid sequences of Cqd1 and Cqd2 were threaded through COQ8A apo crystal structure (PDB:4PED) via the online I-TASSER webserver58. Superimposed homology models were visualized in the PyMOL Molecular Graphics System (Version 2.0, Schrodinger, LLC). Color
schemes depicting protein domain organization were chosen according to the previous work. Subcellular fractionation. Individual colonies of S. cerevisiae were used to inoculate synthetic complete media (2% glucose, w/v) starter cultures and were incubated for 14–16 h (30 °C, 230 rpm). Yeast were diluted to 5 × 10⁶ cells/mL in 50 mL pAβA® media (2% glucose, w/v) and incubated until mid-log phase (30 °C, 16 h, 230 rpm). Yeast were swelled into 2 L of pAβA® media with glucose (0.1%, w/v) and glycerol (3%, w/v) at an initial density of 2.5 × 10⁸ cells/mL and incubated until early log phase (30 °C, 16 h, 230 rpm). 1 × 10⁸ cells were collected for whole-cell (WC) analyses. The remaining culture was pelleted by centrifugation (4,500 × g, 7 min) and weighed (2–3 g). Pellets were then fractionated using previously described methods. For preparative scale affinity purification, crude mitochondria were resuspended in 50 mL BB7.4 (0.6 M sorbitol, 20 mM HEPES-KOH pH 7.4), dialyzed in 930 mL ice-cold BB7.5 (20 mM HEPES-KOH pH 7.4), vortexed for 10 s (medium setting, Rotor, Vertex Genie) and incubated on ice for 30 min. Swollen mitochondria were then sonicated briefly (1/8 tip, 20 amplitude) for 2–5 s pulses with 60 between pulses. Mitoplasts with osmotically ruptured outer membranes were recovered by centrifugation (20,000 × g, 10 min, 4 °C). Pellet from removing the supernatant fraction was washed once with 1 mL of Buffer B (20 mM HEPES-KOH pH 8.0, 200 mM NaCl) containing 2% (w/v) styrene-maleic acid copolymer (SMA, Polyacrylo Smal® 25010) by repeat pipetting and rotated end-over-end (4 h, 4 °C). Soluble SMA extracts were separated from non-extracted material by centrifugation at 21,000 × g for 10 min at 4 °C. Soluble material was then added to NTA nickel resin (400 µL slurry, Qiagen), which was pre-charged (overnight at 4 °C, end-over-end) with radiolabeled His-tagged GFPb (12.5 µL, 20 mM imidazole). This mixture of soluble SMA extracts and charged nickel resin was rotated end-over-end (24 h, 4 °C). Nickel resin was pelleted by centrifugation (700 × g, 2 min, 4 °C) and the supernatant fraction was carefully collected. Nickel resin was washed twice with Buffer B and twice with 500 µL Wash Buffer [Buffer B containing 20 mM imidazole]. Native nanodisc isolation. Individual colonies of BY4741 were used to inoculate synthetic complete media (2% glucose, w/v) starter cultures, which were incubated for 14–16 h (30 °C, 230 rpm). Yeast were diluted to 5 × 10⁶ cells/mL in 50 mL pAβA® media (2% glucose, w/v) and incubated until mid-log phase (30 °C, 16 h, 230 rpm). Yeast were swelled into 2 L of pAβA® media with glucose (0.1%, w/v) and glycerol (3%, w/v) at an initial density of 2.5 × 10⁸ cells/mL and incubated until early log phase (30 °C, 16 h, 230 rpm). 1 × 10⁸ cells were collected for whole-cell (WC) analyses. The remaining culture was pelleted by centrifugation (4,500 × g, 7 min) and weighed (2–3 g). Pellets were then fractionated using previously described methods. For preparative scale affinity purification, crude mitochondria were resuspended in 50 mL BB7.4 (0.6 M sorbitol, 20 mM HEPES-KOH pH 7.4), dialyzed in 930 mL ice-cold BB7.5 (20 mM HEPES-KOH pH 7.4), vortexed for 10 s (medium setting, Rotor, Vertex Genie) and incubated on ice for 30 min. Swollen mitochondria were then sonicated briefly (1/8 tip, 20 amplitude) for 2–5 s pulses with 60 between pulses. Mitoplasts with osmotically ruptured outer membranes were recovered by centrifugation (20,000 × g, 10 min, 4 °C). Pellet from removing the supernatant fraction was washed once with 1 mL of Buffer B (20 mM HEPES-KOH pH 8.0, 200 mM NaCl) containing 2% (w/v) styrene-maleic acid copolymer (SMA, Polyacrylo Smal® 25010) by repeat pipetting and rotated end-over-end (4 h, 4 °C). Soluble SMA extracts were separated from non-extracted material by centrifugation at 21,000 × g for 10 min at 4 °C. Soluble material was then added to NTA nickel resin (400 µL slurry, Qiagen), which was pre-charged (overnight at 4 °C, end-over-end) with radiolabeled His-tagged GFPb (12.5 µL, 20 mM imidazole). This mixture of soluble SMA extracts and charged nickel resin was rotated end-over-end (24 h, 4 °C).
MS2 spectra (Top2) within the same injection. Acquisition parameters for all MS scans in both modes were 17,500 resolution, 1 × 105 automatic gain control (AGC) target, 100 ms ion accumulation time (max IT), and 200–1600 m/z scan range. MS2 scans in both modes were then performed at 17,500 resolution, 1 × 105 AGC target, 50 ms max IT, 1.0 m/z isolation window, stepped normalized collision energy (NCE) at 20, 30, 40, and a 10.0 s dynamic exclusion.

Parallel reaction monitoring (PRM) of Coenzyme Q10, Q8, and Q9 was utilized to monitor the four microwells, [M–H]–, and [M–NH4]+, of each CoQ species. For CoQ9, we targeted the mass to charge ratio of 592.449 and 609.475; for CoQ10, 728.574 and 745.601; and for CoQ11, 864.67 and 881.727. PRM MS settings were: automatic gain control (AGC) target at 5 × 105; Maximum IT at 100 ms, resolving power of 50,000 at 2 m/z isolation window at 3.0 m/z, and pseudo selected reaction at 35. Another experiment performed in tandem with PRM used targeted single ion monitoring (t-SIM) in negative mode to determine the primary adduct, [M–H]–, of CoQ intermediates. For PPRHs, we targeted the mass to charge ratio of 544.908 and used the following t-SIM MS settings: AGC target at 5 × 105, Maximum IT at 100 ms, and resolving power at 140,000 with an isolation window of 4.0 m/z.

Data analysis. The resulting LC-MS data were manually processed using a custom TraceFinder 4.1 (Thermo Scientific) method using a mass precision of 4 and mass tolerance of 10 ppm to detect and identify the different species and adducts of CoQ9 and CoQ10 and intermediates.

Targeted HPLC-ECD for yeast CoQ9. For yeast whole-cell measurements, 5 × 108 cells were collected by centrifugation at 4,000 × g, 5 min and layered with 100 µL of glass beads (0.5 mm; RPI). Lipids from whole-cell samples and other fractions were extracted according to the “Petroleum Ether:MeOH Extraction” section above. Samples were analyzed by reverse-phase high-pressure liquid chromatography with electrochemical detection (HPLC-ECD) using a C18 column (Thermo Scientific; Betasil C18, 100 × 2.1 mm, particle size 3 µm) at a flow rate of 0.3 mL/min with a mobile phase of 75% methanol, 20% 2-propanol, and 5% ammonium acetate (1 M, pH 4.4). After separation on the column, the NaBH4-reduced quinones were quantified on an ECD detector (Thermo Scientific ECD3000-RS) equipped with 6020RS Dual Column Guard Cells. Reference peaks for ECD were 601185 ultra Analizol Cell “E2” and “E3”. To prevent premature quinone oxidation, the E1 guard electrode was set to −200 mV. Measurements were made using the analytical E2 electrode operating at 600 mV after complete oxidation of the quinone sample and E3 electrode (600 mV) was used to ensure that the total signal was recorded on the E2 cell. For each experiment, a CoQ9 standard in 2-propanol was also prepared with sodium borohydride and methanol treatment, and different volumes were injected to make a standard curve. Quinones were quantified by integrating respective peaks using the Chromelon 7.210 software (Thermo) and normalized to CoQ9 internal standard.

Antibodies and western blots. Antibodies. Primary antibodies used in this study include anti-Kar2 (SCBT sc-33630, 15000; RRID: AB_627118), anti-Cit1 (custom made at Biomatik, 14000), anti-Tom70 (1:1000, a gift from Nora Vogtle, University of Freiburg), anti-Vdac (Abcam ab110326, 1:2000; RRID: AB_10865182); anti-GFP (SCBT sc-9996, 1:1000; RRID: AB_621842) and goat anti-rabbit (LI-COR 926-32210, 1:15000; RRID: AB_621842) and goat anti-mouse (LI-COR 926-32211, 1:15000; RRID: AB_621843).

SMALP fractionation western blot. Fractions described above in “Native Nanodisc Isolation” and “SMALP solubility western blot” were collected and used for western blot analysis. Merges of 200 micrograms of spheroplasts (SP) and crude mitochondria (CM) were loaded, along with equal volumes of extracted soluble (S) and final elution (E) samples. Western blots were performed as described above.

Statistical analysis. All experiments were performed in at least biological triplicate, unless stated otherwise. In all cases, ‘mean’ refers to the arithmetic mean, and “sd” refers to standard deviation. Statistical analyses were performed using Microsoft Excel. p-values were calculated using an unpaired, two-tailed, Student’s t-test. In all cases, n represents independent replicates of an experiment. For all western blot, Coomassie gel, and drop assay data, a representative blot from three independent experiments is displayed.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The next generation sequencing data generated in this study (Fig. 2c, Supplementary Fig. 1c) have been deposited to NCBI SRA (BioProject: accession PRJNA679831). Source data for Figs 1–4 and Supplementary Figs 1–4 are provided in the Source Data file. All other data supporting the findings of this study are available from the corresponding authors on reasonable request. Source data are provided with this paper.

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