UbiB proteins regulate cellular CoQ distribution in \textit{Saccharomyces cerevisiae}

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 \textit{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\(^\text{1,3}\). CoQ is also present in membranes throughout the cell\(^\text{14}\), 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\(^\text{5,6}\), 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 CoQ\(_{\text{a}}\) — the major form of CoQ in S. cerevisiae (hereafter referred to as CoQ). S. cerevisiae lacking CoQ or phospholipid hydroperoxide glutathione peroxidases (PHGPx) are sensitive to the oxidative stress conferred by exogenous polyunsaturated fatty acids (PUFAs), such as \(\alpha\)-linolenic acid (18:3)\(^\text{7,8}\). PUFAs undergo uncontrolled autooxidation reactions in the absence of these antioxidant factors, leading to the toxic accumulation of lipid peroxides and peroxyl radicals\(^\text{7,9}\). To force cells into relying more heavily on the antioxidant properties of CoQ, we deleted all three PHGPx genes in W303 S. cerevisiae \(\Delta\)gpx1/2/3 (hereafter referred to as \(\Delta\)gpx1/2/3). We validated that this strain is sensitized to 18:3 treatment and demonstrated that this sensitivity is dampened when cellular CoQ 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 CoQ (Fig. 1a, b). Importantly, the CoQ analog decylubiquinone was markedly more effective at protecting against PUFA stress than CoQ (Fig. 1a, b). Importantly, the CoQ analog decylubiquinone was markedly more effective at protecting against PUFA stress than CoQ (Fig. 1a, b).

Loss of Cqd1 confers PUFA resistance. We reasoned that suppressor mutations that increase extramitochondrial CoQ levels would enhance PUFA resistance in the \(\Delta\)gpx1/2/3 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 \(\sim\)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\(^\text{9}\). 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 Cqd1, 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, Cqd1 possesses the same UbIB family atypical kinase/ATPase domain as Coq8, an essential protein for CoQ synthesis that resides on the matrix face of the IMM\(^\text{10-13}\). Our recent work suggests that Coq8 ATPase activity may be coupled to the extraction of hydrophobic CoQ precursors from the IMM for subsequent processing by membrane-associated matrix enzymes\(^\text{14}\). Cqd1 resides on the opposite side of the IMM, facing the intermembrane space\(^\text{11,15}\) (Supplementary Fig. 1b), physically separated from the other CoQ-related enzymes but still positioned for direct access to membrane-embedded CoQ precursors and mature CoQ. Furthermore, a recent study reported that haploinsufficiency of human CQD1 ortholog ADCK2 led to aberrant mitochondrial lipid oxidation and myopathy associated with CoQ\(_{10}\) deficiency\(^\text{16}\).

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-
Cqd1 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 polyprenyl-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-HB, 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 Cqd1 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 of WT and Δcqd1 yeast. Similar to Coq813,14,19, the ability of Cqd1 to rescue the respiratory growth defect of Δcqd1 yeast. Similar to Coq8, Cqd1 possesses an atypical protein kinase-like (PKL) fold that endows ATPase activity but occludes larger proteinaceous substrates from entering the active site13,19 (Supplementary Fig. 2c–e). Unlike Coq8, Cqd1 is recalcitrant to recombinant protein purification; therefore, in lieu of direct in vitro activity assays, we examined the ability of Cqd1 point mutants to rescue the respiratory growth defect of Δcqd1 yeast. Similar to Coq8, the ability of Cqd1 to rescue the Δcqd1 respiratory growth deficiency depended on core protein kinase-like (PKL) family residues20 required for phosphorl transfer (Fig. 3g) and on quintessential UbiB motif residues (Supplementary Fig. 2e–h). Further biochemical work is required to prove Cqd1’s enzymatic activity; however, these data support a model whereby Cqd1’s ability to promote CoQ distribution relies on atypical kinase/ATPase activity (Fig. 3h).

**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 pathway21–23. 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 Δgpx1/2/3 yeast and subjected this strain to PUFA-mediated stress. Surprisingly, Δgpx1/2/3Δcqd2 yeast exhibited an enhanced sensitivity to PUFA treatment—the opposite phenotype to that of Δgpx1/2/3Δcqd1.
This phenotype is also CoQ-dependent, as deletion of CQD2 likewise had no effect in background strains lacking CoQ (Supplementary Fig. 1d, e). Furthermore, Δgpx1/2/3Δcqd1Δcqd2 yeast phenocopied the parental (Δgpx1/2/3) strain (Fig. 4a; Supplementary Fig. 3a). Under respiratory conditions, Δcqd2 yeast exhibited no detectable change in growth. However, deleting CQD2 from Δcqd1 yeast (Δcqd1Δcqd2) restored this strain’s impaired respiratory growth rate to WT levels (Fig. 4b, c). Conversely, reintroduction of CQD2 into the Δcqd1Δcqd2 strain recapitulated the respiratory growth deficiency of 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 phosphorl transfer enzymes. Consistent with these results, subcellular fractionation revealed significantly increased CoQ levels in the pure mitochondrial fraction from Δcqd2 yeast (Fig. 4e; Supplementary Fig. 3f). Furthermore, the Δcqd1Δcqd2 strain possessed mitochondrial and non-mitochondrial CoQ levels between those of the Δcqd1 and 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 Δcqd2 non-mitochondrial samples; therefore, a quantifiable loss of CoQ in this fraction for the Δcqd2 was not detectable (Fig. 4e). The analyses above, coupled with the submitochondrial location of Cqdl and Cqd2, suggest a model whereby these enzymes may reciprocally regulate the amount of CoQ within the IMM. To test this directly, we used the amphipathic polymer "F01"; Supplementary Fig. 3a).
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 µM 4-HB (white bars, superimposed) (mean ± SD; 0 µM 4-HB n = 6 independent samples, 1 µM 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 µM 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 mitochondrion. 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; NM, non-mitochondrial fraction; SP, spheroplast; CM, crude mitochondria; M, enriched mitochondrion. h 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. i, 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 confirm (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 CQD1, 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 (background). Of note, although Gpx1-3 are primarily manipulated this pathway. First step in addressing enduring questions regarding endogenous cellular pathways (e.g., by performing similar screens in a Δcqd1Δcqd2 background). Of note, although Gpx1-3 are primarily

Methods

Yeast strains and cultures. Unless otherwise described, Saccharomyces cerevisiae haploid W303 (MATa his3 leu2 met15 trp1 ura3) yeast were used. For SMA-derived lipid nanodisc work, endogenous GFP-tagged BY4741 (MATa his3Δ1 leu2Δ2 met15Δ130 ura3Δ10) yeast strains46 were used. Yeast deletion strains were generated using standard 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 described35. Cassette insertion was confirmed by a PCR assay and DNA sequencing. CRISPR-mediated deletions were performed as described45. 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 pmol of an 80-nt Ulitimer consisting of 40 bp upstream and 40 bp downstream of the ORF (for scarless deletions) or ~6 µg of PCR-amplified Longtine cassette 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− (and 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

Yeast 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 × 10^5 cells/mL in PABA− (or pABA− dropout) media (2% glucose, 1% w/v) with or without added indicated additives. Yeast were incubated until early log phase (30 °C, 7–8, 230 rpm). Yeast were swayed into fresh pABA− media (2% glucose, w/v) at an initial density of 5 × 10^5 cells/mL with indicated additives. The cultures were incubated (30 °C, 1140 rpm) in an Eppendorf 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 × 10^6–1.33 × 10^6 cells/mL in pABA− media (2% glucose, w/v) and incubated until early log phase (30 °C, 7–8, 230 rpm). Yeast were swayed into pABA− media with glucose (0.1%, w/v) and glycerol (3%, w/v) at an initial density of 5 × 10^5 cells/mL with indicated additives. The cultures were incubated (30 °C, 1140 rpm) in an Eppendorf 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 (Bio-Tek), excluding time points before the diauxic shift and during stationary phase growth.

Drop assays. Individual colonies of yeast were used to inoculate pABA-limited media (2% w/v glucose, 100 nm pABA) starter cultures, which were incubated overnight (30 °C, 230 rpm). Cells were spun down (21,000 × g, 2 min) and resuspended in water. Serial dilutions of yeast (10^5, 10^4, 10^3, 10^2, or 10^1 cells) were dropped onto pABA− media (2% glucose and 1% EOH, w/v) agar plates with indicated additives and incubated (30 °C, 2–3 d).

Forward-genetic screen. Individual colonies of Δgpx1/2/3 yeast were used to inoculate YEPD starter cultures, which were incubated overnight. 1.0 × 10^7 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 thioulate (5% w/v) to inactivate EMS. Cells were resuspended in water, and 1.0 × 10^7 cells were plated on pABA− (2% glucose, w/v) plates with 0 µM or 25 µM α-linolenic acid (C18:3, Sigma). Colonies that grew on 25 µM α-linolenic acid were picked into YEPD overnight cultures and struck on the pABA− plate. For mutant strains that grew 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/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 DpnII to degrade genomic DNA and ligated into the digested pGPD plasmid (Addgene). Cloning products were then transformed into E. coli TOP10 chemically competent cells (Lucigen). Plasmids were isolated from transformants. Amplification and sequencing was used to identify those containing the correct insertion.

Constructions containing CqD1 and CqD2 were digested with Sall and BamHI or HindIII to liberate the GPD promoter. Digested backbone was then combined with a P0.5 (GPD) endogenous promoter region (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 described37 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, Schrödinger, LLC). Color
Subcellular fractionation. Individual colonies of S. cerevisiae were used to inoculate synthetic complete medium (2% glucose, w/v) starter cultures and were incubated for 14–16 h (30 °C, 230 rpm). Yeast were diluted to 5 x 10^6 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 x 10^6 cells/mL and incubated until early log phase (30 °C, 16 h, 230 rpm). 1 x 10^8 cells were collected for whole-cell (WC) analyses. The remaining culture was pelleted by centrifugation (4,500 x, 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), diluted in 930 µL ice-cold BB7.5 (20 mM HEPES-KOH pH 7.4), vortexed for 10 s (medium setting), 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 µw between pulses. Mitoplasts with osmotically ruptured outer membranes were recovered by centrifugation (20,000 x, 10 min, 4 °C), and resuspended in 500 µL of Buffer B (20 mM HEPES-KOH pH 8.0, 200 mM NaCl) containing 2% (w/v) styrene-maleic acid copolymer (SMA, PolyScience SMALP® 25010 P) by repeat pipetting and rotated end-over-end (4h, 4 °C). Soluble SMA extracts were separated from non-extracted material by centrifugation at 21,000 x 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 remaining His-tagged GFPnb (12.5 µL, 20 mg/mL). 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 x, 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 nanodiscs bound to His-GFPnb were eluted with Buffer B containing 250 mM imidazole by rotating end-over-end for 20 min at 4 °C. Due to the presence of GFP nanobody in the elution samples, relative target abundance was determined by western blot analysis and anti-GFP band quantification. Protein concentrations of all other samples were quantified by Pierce® BCA Protein Assay Kit (Thermo).

Lipid extraction. 

ChCl₃:MeOH extraction. 1 x 10⁸ yeast cells were harvested by centrifugation (4,000 x, 5 min, 4 °C). The supernatant was removed, and the cell pellet was flash-frozen in N₂ (l) and stored at −80 °C. Frozen yeast pellets were thawed on ice and resuspended in 100 µL cold water. To this, 10 µL of glass beads (0.5 mm; RPI) and CoQ₁₀ internal standard (10 µL) were added and bead beating (2 min, 4 °C, 900 x, 2 × 10⁶ rpm) was performed. The final concentration of 4 µM was determined by western blot analysis and anti-GFP band quantification. Protein concentrations of all other samples were quantified by Pierce® BCA Protein Assay Kit (Thermo).

Petroleum ether:MeOH extraction. Yeast whole-cell preparations, 1 x 10⁶ cells were collected by centrifugation (4,000 x, 5 min, 4 °C) and layered with 100 µL of glass beads (0.5 mm; RPI). Whole-cell samples and all other fractions were then suspended in ice-cold methanol (500 µL; with 1 µL CoQ₁₀, internal standard) and vortexed (10 min). ~500 µL of petroleum ether was added to extract lipids, and samples were vortexed (3 min, 4 °C) and centrifuged (17,000 x, 1 g, 4 °C) to separate phases. The petroleum ether (upper) layer was collected, and the extract was repeated with another round of petroleum ether (500 µL), vortexing (3 min, 4 °C), and centrifugation (17,000 x, 1 g). The petroleum ether layers were pooled and dried under argon. Lipids were resuspended in 2-propanol (15 µL) and transferred to amber glass vials (Sigma; Q5ertVial™, 12 x 32 mm, 0.3 mL) for LC-MS analysis.

Lipidomic analysis. Targeted LC-MS for yeast CoQ₆ and PPHB₆. LC-MS analysis was performed on an Acquity UPLC C18 column held at 50 °C (100 mm x 1.1 mm x 1.7 μm particle size; Waters) using a Vanquish Binary Pump (400 µL/min flow rate; Thermo Scientific). Mobile phase A consisted of 10 mM ammonium acetate and 250 µL/L acetic acid in ACN:H₂O (70:30, v/v). Mobile phase B consisted of IPA:ACN (90:10, v/v) also used as mobile phase. Gradient was run for 2 min (1% B), 3 min (10% B), 3.5 min (50% B), 4 min (100% B). Data was acquired from 0.5 to 10 min, with a sweeping mass profile from 250 to 1,250 Da. Thermal data were collected with QuantStudio Real-Time PCR v1.2 software and analyzed with Protein Thermal Shift v1.3 software.

Native nanodisc isolation. Individual colonies of S. cerevisiae (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 x 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 x 10⁶ cells/mL and incubated until early log phase (30 °C, 16 h, 230 rpm). Yeast were pelleted by centrifugation (4,500 x, 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), diluted in 930 µL ice-cold BB7.5 (20 mM HEPES-KOH pH 7.4), vortexed for 10 s (medium setting), 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 µw between pulses. Mitoplasts with osmotically ruptured outer membranes were recovered by centrifugation (20,000 x, 10 min, 4 °C) and resuspended in 500 µL of Buffer B (20 mM HEPES-KOH pH 8.0, 200 mM NaCl) containing 2% (w/v) styrene-maleic acid copolymer (SMA, PolyScience SMALP® 25010 P) by repeat pipetting and rotated end-over-end (4h, 4 °C). Soluble SMA extracts were separated from non-extracted material by centrifugation at 21,000 x 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 remaining His-tagged GFPnb (12.5 µL, 20 mg/mL). 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 x, 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 nanodiscs bound to His-GFPnb were eluted with Buffer B containing 250 mM imidazole by rotating end-over-end for 20 min at 4 °C. Due to the presence of GFP nanobody in the elution samples, relative target abundance was determined by western blot analysis and anti-GFP band quantification. Protein concentrations of all other samples were quantified by Pierce® BCA Protein Assay Kit (Thermo).
MS2 spectra (Top2) within the same injection. Acquisition parameters for full MS scans in both modes were 17,500 resolution, 1 × 106 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 × 107 AGC target, 50 ms max IT, 1.0 m/z interval, stepped normalized collision energy (NCE) at 20, 30, 40, and a 10.0s dynamic exclusion.

Parallel reaction monitoring (PRM) used for that stability and was utilized to monitor two primary adducts, [M-H]- and [M-NH2]+, for each CoQ species. For CoQ8, we targeted the mass to charge ratio of 592.449 and 609.475; for CoQ9, 728.574 and 745.601; and for CoQ10, 864.7 and 881.727. PRM MS settings were: automatic gain control (AGC) target at 5 × 105, Maximum IT at 100 ms, resolving power at 2, isolation window 3.0 m/z, and collision energy 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 PPHP8, 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 CoQs and CoQs and intermediates.

Targeted HPLC-ECD for yeast CoQs. For yeast whole-cell measurements, 5 × 108 cells were collected by centrifugation (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 electrophoretical 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 ECD detector (Thermo Scientific ECD3000-RS) equipped with 6020RS omni Coulometric Guarding Cell 1", 6018RS ultra Analytical Cell "E2" and "E3". To prevent premature quinone oxidation, the E1 guard electrode was set to -200 mV. Measurements were made using the analytical electrode E2 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 CoQ 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 7.2.10 software (Thermo) and normalized to CoQx internal standard.

Antibodies and western blots
Antibodies. Primary antibodies used in this study include anti-Kar2 (SCB7 sc-33630, 1:5000; RRID: AB_627118), anti-Cit1 (commercial made at Biomatik, 1:4000), anti-Tom70ff (1:1000, a gift from Nora Vogle, University of Freiburg), anti-Vdac (Abcam ab110326, 1:2000; RRID: AB_10865182); anti-GFP (SCBT sc-9996, 1:1000; RRID: AB_627695), anti-Sdh265 (1:5000, a gift from Oleh Khalimonchuk, University of Nebraska). Secondary antibodies include goat anti-mouse IgG (horseradish peroxidase (HRP)), 1:15,000 in 1% NFDM in TBST (1.5 h, r.t.). The membrane was washed three times in TBST and the secondary antibodies were diluted 1:15,000 in 1% NFDM in TBST (1.5 h, r.t.). The membrane was washed three times in TBST and imaged on a LI-COR Odyssey CLx using Image Studio v5.2 software.

SMALP fractionation western blot. Fractions described above in "Native Nanodisc Isolation" and "SMALP Solubility western Blot" were collected and used for western blot analysis. Aliquots (milligrams 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 sample 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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References
1. Hatfi, Y., Haavik, A. G., Fowler, L. R. & Griffiths, D. E. Studies on the electron transfer system. XII. Reconstitution of the electron transfer system. J. Biol. Chem. 237, 2661–2669 (1962).
2. Freeman, F. E. Acyl-CoA dehydrogenases, electron transfer flavoprotein, and electron transfer flavoprotein dehydrogenase. Biochem Soc. Trans. 16, 416–418 (1988).
3. Jones, M. E. Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. Annu Rev. Biochem. 49, 253–279 (1980).
4. Bentinger, M., Brismar, K. & Dallner, G. The antioxidant role of coenzyme Q. Mitochondrion 7, 541–550 (2007).
5. Bersuker, K. et al. The CoQ oxidoreductase FSP1 acts parallel to GPX4 to inhibit ferroptosis. Nature 575, 688–692 (2019).
6. Doll, S. et al. FSP1 is a glutathione-independent ferroptosis suppressor. Nature 575, 693–698 (2019).
7. Do, T. Q., Schulz, J. R. & Clarke, C. F. Enhanced sensitivity of ubiquinone-deficient mutants of Saccharomyces cerevisiae to products of autoxidized polyunsaturated fatty acids. Proc. Natl Acad. Sci. USA 93, 7534–7539 (1996).
8. Avery, A. M. & Avery, S. V. Saccharomyces cerevisiae expresses three phospholipid hydroperoxide glutathione peroxidases. J. Biol. Chem. 276, 33730–33735 (2001).
9. Choi, Y., Sims, G. E., Murphy, S., Miller, J. R. & Chan, A. P. Predicting the functional effect of amino acid substitutions and indels. PLoS One 7, e46688 (2012).
10. Tauche, A., Krause-Buchholz, U. & Rodel, G. Ubiquinone biosynthesis in the context of the electronic transport chain. J. Biol. Chem. 267, 2253–2259 (1992).
11. Vogle, F. N. et al. Landscape of sub mitochondrial protein distribution. Nat. Commun. 8, 290 (2017).
12. Rhee, H. W. et al. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. Science 339, 1328–1331 (2013).
13. Stefelj, J. A. et al. Cerebellar Ataxia and coenzyme Q deficiency through loss of unorthodox kinase activity. Mol. Cell 63, 680–682 (2016).
14. Reidenbach, A. G. et al. Conserved lipid and small-molecule modulation of COQ8 reveals regulation of the ancient kinase-like Ublt family. Cell Chem. Biol. 25, 154–165 e11 (2018).
15. Morgenstern, M. et al. Domains of a high-confidence mitochondrial proteome at quantitative scale. Cell Rep. 19, 2836–2852 (2017).
16. Vazquez-Fonseca, L. et al. ADCK2 haploinsufficiency reduces mitochondrial lipid oxidation and causes myopathy associated with CoQ deficiency. J. Clin. Med. 8, 1374 (2019).
17. Payet, L. A. et al. Mechanistic details of early steps in coenzyme Q biosynthesis pathway in yeast. Cell Chem. Biol. 23, 1241–1250 (2016).
18. Stefelj, J. A. et al. Mitochondrial protein functions elucidated by multi-omic mass spectrometry profiling. Nat. Biotechnol. 34, 1191–1197 (2016).
19. Stefly, J. A. et al. Mitochondrial ADCK3 employs an atypical protein kinase-like fold to enable coenzyme Q biosynthesis. *Mol. Cell* 57, 83–94 (2015).

20. Kannan, N., Taylor, S. S., Zhai, Y., Venter, J. C. & Manning, G. Structural and functional diversity of the microbial kinome. *PLoS Biol.* 5, e17 (2007).

21. Tan, T., Ozbalci, C., Brugger, B. & Rapaport, D. & Dimmer, K.S. Mcp1 and Mcp2, two novel proteins involved in mitochondrial lipid homeostasis. *J. Cell Biol.* 201, 3563–3572 (2013).

22. Odendall, E. et al. The mitochondrial intermembrane space-facing proteins Mcp2 and Tgl2 are involved in yeast lipid metabolism. *Mol. Biol. Cell.* 30, 2681–2694 (2019).

23. Costanzo, M. et al. A global genetic interaction network maps a wiring diagram of cellular function. *Science* 353, aaf420 (2016).

24. Lee, S. E. et al. A method for detergent-free isolation of membrane proteins in their local lipid environment. *Nat. Protoc.* 11, 1149–1162 (2016).

25. Calzada, E. et al. Phosphatidylethanolamine made in the inner mitochondrial membrane is essential for yeast cytochrome bc1 complex function. *Nat. Commun.* 10, 1432 (2019).

26. Oyedotun, K. S. & Lemire, B. D. The quinone-binding sites of the mitochondrial supercomplex, two novel proteins involved in mitochondrial lipid homeostasis. *MCP2, two novel proteins involved in mitochondrial lipid homeostasis.*

27. Stefely, J. A. et al. Mitochondrial ADCK3 employs an atypical protein kinase-like fold to enable coenzyme Q biosynthesis. *Mol. Cell* 57, 83–94 (2015).

28. Wiedemeyer, W. R. et al. Pattern of retinoblastoma pathway inactivation dictates response to CDK4/6 inhibition in GRM. *Proc. Natl Acad. Sci. USA* 107, 11501–11506 (2010).

29. Brough, R. et al. Functional viability profiles of breast cancer. *Cancer Discov.* 1, S1–3563–3572 (2011).

30. Simpson, K. J. et al. Identification of genes that regulate epithelial cell migration using an siRNA screening approach. *Nat. Cell Biol.* 10, 1027–1038 (2008).

31. Breker, M., Gymrek, M., Moldavski, O. & Schuldiner, M. LoQAtE software for the rapid and accurate assignment of interactome data to protein complexes. *Nat. Methods* 12, 2694–2702 (2015).

32. Lohman, D. C. et al. An isoprene lipid-binding protein promotes eukaryotic gene expression. *Proc. Natl Acad. Sci. USA* 112, 112002 (2020).

33. Asquith, C. R. M., Murray, N. H. & Pagliarini, D. J. ADCK3/COQ8A: the role of mitochondrial ADCK3 in the regulation of mitochondrial gene expression. *J. Biol. Chem.* 285, 15,125–15,133 (2010).

34. Tamura, Y. et al. Role for two conserved intermembrane space proteins, Mcp1 and Mcp2, two novel proteins involved in mitochondrial lipid homeostasis. *J. Cell Biol.* 201, 3563–3572 (2013).

35. Osman, C. et al. The genetic interactome of prohibitins: coordinated control of mitochondrial bioenergetic function and respiratory supercomplex stability. *J. Proteome Res.* 15, 323–330 (2016).

36. Hoppins, S. et al. A mitochondrial-focused genetic interaction map reveals a conserved functional diversity of the microbial kinome. *Sci. Rep.* 7, 16142 (2017).

37. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. & Cullin, C. A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. *Nucleic Acids Res.* 21, 3329–3330 (1993).

38. Generoso, W. C., Gottardi, M., Oreb, M. & Boles, E. Simplified CRISPR-Cas genome editing for Saccharomyces cerevisiae. *J. Microbiol. Methods* 127, 203–205 (2016).

39. Gietz, R. D. & Woods, R. A. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* 350, 87–96 (2002).

40. Yang, J. et al. The I-TASSER Suite: protein structure and function prediction. *Nat. Methods* 12, 7–8 (2015).

41. Meininger, C., Pfanner, N. & Truschnik, S. K. Introduction of yeast mitochondria. *Methods Mol. Biol.* 313, 33–39 (2006).

42. Formar, F., Arriaga, E. A. & Mann, M. Mild protease treatment as a small-scale biochemical method for mitochondria purification and proteomic mapping of cytoplasm-exposed mitochondrial proteins. *J. Proteome Res.* 5, 3277–3287 (2006).

43. Fox, B. G. & Blommel, P. G. Autoinduction of protein expression. *Curr. Protoc. Protein Sci.* https://doi.org/10.1002/0471140864.pd0535s56 (2009).

44. Niesen, F. H., Berglund, H. & Vedadi, M. The use of differential scanning fluorometry to detect ligand interactions that promote protein stability. *Nat. Protoc.* 2, 2212–2221 (2007).

45. Asquith, C. R. M., Murray, N. H. & Pagliarini, D. J. ADCK3/COQ8A: the role of mitochondrial ADCK3 in the regulation of mitochondrial gene expression. *J. Biol. Chem.* 285, 15,125–15,133 (2010).

46. Hoppins, S. et al. A mitochondrial-focused genetic interaction map reveals a conserved functional diversity of the microbial kinome. *Sci. Rep.* 7, 16142 (2017).

47. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. & Cullin, C. A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. *Nucleic Acids Res.* 21, 3329–3330 (1993).

48. Managing the organellar kinase void. *J. Proteome Res.* 5, 3277–3287 (2006).

49. Vogel, N. F. et al. Mutations in PMP12 encoding the catalytic subunit of the mitochondrial bc1 complex cause neurodegeneration in early childhood. *Am. J. Hum. Genet.* 102, 557–573 (2018).

50. Bohorvych, I. et al. Metalloprotease OMA1 fine-tunes mitochondrial biogenesis and respiratory supercomplex stability. *Sci. Rep.* 5, 13989 (2015).

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Author contributions Z.A.K. and K.P.R. conceived of the project and its design. Z.A.K. and K.P.R. conducted experiments and performed data analysis. J.M.S. purified and characterized GFP nanobody. M.M. performed and analyzed HIPLEC-ECD experiments. B.R.P. and P.D.H. performed and analyzed mass spectrometry experiments. J.C.J. oversaw all mass spectrometry-based experiments. A.I. contributed to new reagents (cloning). All authors edited the paper. Z.A.K., K.P.R., and D.J.P. wrote the paper. D.J.P. supervised the project.

Competing interests J.C.J. is a consultant for Thermo Fisher Scientific. The authors declare no other competing interests.
