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Genes and lipids that impact uptake and assimilation of exogenous coenzyme Q in *Saccharomyces cerevisiae*

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Abstract

Coenzyme Q (CoQ) is an essential player in the respiratory electron transport chain and is the only lipid-soluble antioxidant synthesized endogenously in mammalian and yeast cells. In humans, genetic mutations, pathologies, certain medical treatments, and aging, result in CoQ deficiencies, which are linked to mitochondrial, cardiovascular, and neurodegenerative diseases. The only strategy available for these patients is CoQ supplementation. CoQ supplements benefit a small subset of patients, but the poor solubility of CoQ greatly limits treatment efficacy. Consequently, the efficient delivery of CoQ to the mitochondria and restoration of respiratory function remains a major challenge. A better understanding of CoQ uptake and mitochondrial delivery is crucial to make this molecule a more efficient and effective therapeutic tool. In this study, we investigated the mechanism of CoQ uptake and distribution using the yeast *Saccharomyces cerevisiae* as a model organism. The addition of exogenous CoQ was tested for the ability to restore growth on non-fermentable medium in several strains that lack CoQ synthesis (*coq* mutants). Surprisingly, we discovered that the presence of CoQ biosynthetic intermediates impairs assimilation of CoQ into a functional respiratory chain in yeast cells. Moreover, a screen of 40 gene deletions considered to be candidates to prevent exogenous CoQ from rescuing growth of the CoQ-less *coq2Δ* mutant, identified six novel genes (*CDC10, RTS1, RVS161, RVS167, VPS1,* and *NAT3*) as necessary for efficient trafficking of CoQ to mitochondria. The proteins encoded by these genes represent essential steps in the pathways responsible for transport of exogenously supplied CoQ to its functional sites in the cell, and definitively associate CoQ distribution with endocytosis and intracellular vesicular trafficking pathways conserved from yeast to human cells.

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Conflict Of Interests
The authors declare no conflict of interest for any of the work presented in this article.

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Keywords

Coenzyme Q; ubiquinone; transport; uptake; endocytosis; CoQ_6 rescue; *Saccharomyces cerevisiae*

Introduction

Coenzyme Q (CoQ) is essential for energy production in mitochondria. It functions as an electron carrier in the respiratory chain, moving electrons from Complex I (NADH oxidase in *Saccharomyces cerevisiae*), II, and several other enzymes, to Complex III [1, 2]. In this manner, CoQ participates in multiple cellular pathways including β-oxidation and *de novo* synthesis of pyrimidines. In addition, $CoQH_2$ (the reduced or hydroquinone form) has an important role as an antioxidant, protecting DNA, lipids, and proteins from oxidative stress [3, 4]. CoQ is composed of a benzoquinone ring connected to a polyisoprenoid side chain, whose length is species-specific [1, 2]. Six isoprene subunits are present in *Saccharomyces cerevisiae* (CoQ_6), eight in *Escherichia coli* (CoQ_8), and nine and ten (CoQ_9 and CoQ_{10}) in rodents and humans, although CoQ_9 predominates in rodents and CoQ_{10} is most prevalent in humans [1, 2]. Almost all cells have the capacity to produce CoQ, and at least 13 genes are necessary for its endogenous production in yeast (see Fig. 1) [1, 2, 5].

In humans, insufficient CoQ_{10} can result in profound deficits in mitochondrial function [9, 10]. Mutations in genes that participate directly in CoQ_{10} biosynthesis lead to primary CoQ_{10} deficiencies, rare conditions that severely affect multiple organ systems, such as the central and peripheral nervous systems, kidney, skeletal muscle, heart, or sensory systems, in highly variable manners [11]. CoQ_{10} content may also be decreased in other conditions attributed to secondary deficiencies, in which depletion of CoQ_{10} is caused by mutations in genes unrelated to CoQ_{10} biosynthesis including genes involved in mitochondrial myopathies or in the mitochondrial DNA depletion syndrome [12, 13]. Secondary deficiencies can also result from non-genetic conditions such as fibromyalgia, clinical treatments (e.g. hypercholesterolemia treatment with statins) [14, 15], environmental toxins, metabolic disorders, aging [16], and age-related diseases [2, 4]. Secondary deficiencies are more common than primary deficiencies and reflect the diversity of biological functions and metabolic pathways that involve CoQ_{10} [11, 12].

To date, the only treatment to ameliorate the symptoms of human CoQ_{10} deficiencies is CoQ_{10} supplementation. At high doses, CoQ_{10} supplements increase CoQ_{10} levels in all tissues, especially with certain formulations, and are beneficial for various pathophysiological conditions [11, 17]. For example, the Q-SYMBIO study demonstrated that CoQ_{10} supplementation improves heart failure symptoms with a significant reduction in major adverse cardiovascular events and mortality [4, 18]. Other studies reported that CoQ_{10} supplementation could stop the progression of encephalopathy [19] and ameliorate the symptoms of renal disease in patients with defects in *CoQ_2*, *CoQ_6*, or *ADCK4* (the human homolog of yeast Coq8) [20, 21]. However, bioavailability studies show that the response of individuals to CoQ_{10} supplementation is inconsistent, and treatment failure is common [1, 17]. The absorption of CoQ_{10} is slow and limited due to its high molecular weight and negligible aqueous solubility, making delivery by oral supplementation inefficient [11, 17,

22]. Thus, developing therapies for CoQ_{10} deficiencies remains a challenge. Current efforts are focused on new delivery strategies for CoQ [23–25], as well as on alternative approaches using compounds that enhance endogenous CoQ_{10} synthesis in CoQ_{10}-deficient patients [1, 5, 11, 26–29].

In order to improve the therapeutic efficacy of CoQ_{10} supplementation, it is crucial to define the mechanisms responsible for the uptake and distribution of CoQ within cells. However, complete understanding of these mechanisms remains elusive. In mammalian cells and yeast, CoQ is synthesized in the inner membrane and matrix of mitochondria, yet is found in all cellular membranes, indicating the existence of a transport mechanism from the mitochondria to the rest of the cell [30–32]. Conversely, transport to the mitochondria can also occur from the extracellular environment through the plasma membrane as shown by uptake and assimilation of exogenously supplied CoQ in studies with yeast [33, 34] and mice [35], as well as through successful CoQ_{10} supplementation in patients with CoQ_{10} deficiencies [36]. Bidirectionality of CoQ transport, from the extracellular media to the mitochondria and from the mitochondria to other cellular membranes, has been attributed to endomembrane trafficking, yet the pathways and mechanisms remain obscure [32, 33].

In the yeast *S. cerevisiae*, CoQ_6 is not essential for viability as strains that lack CoQ_6 (*coq* mutants) can grow by fermentation on glucose without the involvement of the respiratory chain. However, *coq* mutants are unable to grow on non-fermentable carbon sources unless they are supplemented with exogenous CoQ, thereby restoring mitochondrial oxidative phosphorylation [34, 37, 38]. In this study, we investigated the mechanism of CoQ uptake and intracellular distribution in yeast. To do this, we interrogated a series of *ORFΔcoq2Δ* double mutants for their ability to grow in a non-fermentable glycerol-based medium (designated YPG) in the presence of exogenous CoQ_6. These mutants harbor the deletion of one gene necessary for CoQ biosynthesis (*coq2Δ*) in conjunction with the deletion of another gene hypothesized to be involved with CoQ transport. The inability of some of these *ORFΔcoq2Δ* double mutants to recover growth in YPG even in the presence of CoQ_6 allowed us to identify six genes that are important for CoQ_6 trafficking, highlighting key components of the intracellular CoQ transport pathway. Intriguingly, our results showed that single *coqΔ* mutants differ in their ability to be rescued with exogenous CoQ_6 and demonstrated that the presence of CoQ_6-hexaprenylated biosynthetic intermediates impairs the assimilation of exogenous CoQ_6 in yeast cells.

Materials and Methods

Coenzyme Q isoforms

CoQ_2 was obtained from Cayman Chemical, CoQ_6 from Avanti, and CoQ_4 and CoQ_{10} from Millipore-Sigma. Each was dissolved in ethanol and the concentration was calculated using the absorbance at 275 nm and the corresponding molar extinction coefficients (13,700 $M^{-1}cm^{-1}$ for CoQ_2; 14,200 $M^{-1}cm^{-1}$ for CoQ_4; 14,700 $M^{-1}cm^{-1}$ for CoQ_6 and 15,200 $M^{-1}cm^{-1}$ for CoQ_{10}).

Determination of CoQ_6 movement in mixed vesicle populations

The methodology used was a combination of previously described methods [39–41]. Briefly, LUVETs (large unilamellar vesicles produced by extrusion) were created from 25 mg/mL L-α-phosphatidylcholine (PC) from soybean (Type III-S, Millipore-Sigma) in chloroform, supplemented with 1-pyrene dodecanoic acid (Pyr_{12}, Santa Cruz Biotechnology) to achieve a final concentration of 4 μM. The mixture was evaporated until dry under a stream of nitrogen gas, re-hydrated with MBSE buffer (0.15 M NaCl/ 0.01 M MOPS pH7/ 0.1 M EDTA) to a final PC concentration of 1 mg/mL, and subjected to ten freeze-thaw cycles in a solid CO_2/ethanol bath. The multilamellar vesicles were extruded through two polycarbonate filters in tandem using the Avestin Lipofast system (Avestin, Inc). A second population was created as described above, with the addition of CoQ_2, CoQ_4, CoQ_6, or CoQ_{10} to the chloroform to achieve a final concentration of 200 μM after re-hydration. To monitor the ability of the different CoQ isoforms to move between vesicles, Pyr_{12} quenching was monitored. Two mL of the initial LUVETs population was placed in a stirred cuvette in a Photon Technology International QuantaMaster Spectrofluorimeter (Horiba) (λ_{ex} 346 nm and λ_{em} 377 nm) at 30 °C. After 90 s, 0.5 mL of a CoQ-containing vesicle population was added and the fluorescence was monitored for a total of 10 min. CoQ collisionally quenches Pyr_{12} fluorescence if both are present in the same vesicle and are capable of physical interaction [39, 42]. On mixing the two populations of vesicles, a further decrease in the fluorescence signal can only occur if the CoQn isoform is able to move from vesicle to vesicle. This is because the gain in fluorescence in the CoQn vesicle population from CoQ loss is less than the loss in fluorescence in the previously CoQn-less population from the gain of CoQ (for a discussion see [39]). Data are expressed as the ratio of fluorescence at a given point in time (I) to initial fluorescence just before the addition of the second population of vesicles (I_0).

Yeast strains, growth media, and verification of the mutants

S. cerevisiae strains used in this study are described in Table S1. Standard growth media for yeast included YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose) and YPG (1% Bacto yeast extract, 2% Bacto peptone, 3% glycerol). Solid plate medium contains additional 2% Bacto agar. To select for mutants that contain a *kanMX* cassette, YPD + 200 mg/L G418 (Santa Cruz Biotechnology, Inc) plates were used. To select for *ORFΔcoq2Δ* mutants obtained from a previously created library [39], synthetic dextrose media without arginine and histidine (SD –His –Arg) (20 g/L glucose, 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 1 g/L monosodium glutamic acid, and 2 g/L of amino acid supplement lacking histidine and arginine) plates were used. These SD –His –Arg plates were supplemented with 200 mg/L G418, 70 mg/L canavanine (Millipore-Sigma) and 100 mg/L nourseothricin sulfate (ClonNat) (Gold Biotechnology). For mutants of new creation (see method below), SD –Leu (0.18% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% $(NH_4)_2SO_4$, 0.14% NaH_2PO_4, 2% dextrose and amino acid supplement lacking leucine) plate medium was used. For plasmid selection (see below), SD –Ura (0.18% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% $(NH_4)_2SO_4$, 0.14% NaH_2PO_4, 2% dextrose and amino acid supplement lacking uracil) liquid and plate media were used.

Deletions in each and every strain (both *ORF* and *COQ2* deletions) were confirmed by PCR, using primer pairs A - KanB or D - KanC. Primer A and D are specific for each *ORF* while KanB and KanC are located whithin the *kanMX* cassette. Primers were obtained from the *Saccharomyces* Genome Deletion Project database (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). T_m ranged between 57 and 62 °C. The *rho* status of the cells was also confirmed using JM6 and JM8 as *rho0* test strains [34]. Additionally, prior to the initiation of CoQ_6 rescue experiments, we confirmed the ability of each of the corresponding single selected *ORFΔ* mutants to grow on YPG. The deletion of a single gene could compromise the ability of a mutant to grow on YPG if the gene is involved in respiratory activity. Demonstrated growth of the single *ORFΔ* on YPG ensures that recovery of respiration of the corresponding *ORFΔcoq2Δ* mutants with exogenous CoQ_6 is theoretically possible. Any single mutant unable to grow on YPG automatically eliminated the corresponding *ORFΔcoq2Δ* from the study.

Generation of double deletion mutants

Some non-available mutants of interest for the study were created *de novo* using a PCR-based gene deletion strategy [43]. In all cases, the *ORF* of interest was replaced with a *LEU2* auxotrophic marker amplified from the pRS305 vector using primers *Leu forward: 5'-TGCCCTCCTCCTTGTCAATA-3'* and *Leu reverse: 5 '-GGCGCCTGATTCAAGAAATA-3'*, to which homologous sequences to the upstream of *ORF* start codon and downstream of the *ORF* stop codon were attached. Details about these homologous sequences are described in Table S2. Following PCR clean-up (Thermo Fisher Scientific), the PCR products were used to transform corresponding *ORFΔ* mutant strain using the lithium acetate/single-stranded carrier DNA/Polyethylene Glycol method [44], and transformed cells were selected twice on SD –Leu plates. Genomic DNA isolation following by PCR and DNA sequencing (Laragen, Inc.) were used to verify the correct integration of the auxotrophic marker at the correct gene locus. In these verification steps, specific primers A and D from the *Saccharomyces* Genome Deletion Project database were used, together with two internal primers inside the LEU vector (5'-CCATCACCATCGTCTTCCTT-3' and 5'-CTGTGGGTGGTCCTAAATGG-3') for DNA sequencing.

Generation of *rho* negative yeast

To generate *rho0* BY4741 yeast, cells were incubated on YPD media plus 25 μg/mL ethidium bromide for two subsequent overnight periods. After that, cells were diluted (1:20,000 from a 1 A600/mL solution) and plated on YPD. After 1-2 days, cells were replica plated to YPG plate medium to test their ability to respire. Colonies unable to grow on YPG were considered *rho0*. To further confirm the absence of mtDNA, two distant mitochondrial DNA loci, *COX3* and *OLI1*, were checked by PCR (**COX3-F**: 5'-GGTAATATGAATATGGTATATTTAGC-3'; **COX3-R**: 5' -GTTACAGTAGCACCAGAAGATAATAAG-3'; **OLI1-F**: 5'-ATGCAATTAGTATTAGCAGCTAAAT-3'; **OLI1-R**: 5'-CCGAATAATAATAAGAATGAAACCATTA-3') [45]. The *ERG2* locus was included as a positive control for a nuclear gene, using erg2A and erg2D primers from the *Saccharomyces* Deletion Project Database.

Exogenous CoQ rescue assays

Yeast mutants were grown overnight in YPD at 30 °C 250 rpm. The next day, the A_{600nm}/mL was measured and yeast strains were sub-inoculated into tubes containing 5 mL of YPG supplemented with CoQ (2 μM of CoQ_6 or 100 μM of CoQ_2) or with the corresponding amount of ethanol at a cell density of 0.1 A_{600nm}/mL. In all experiments the amount of ethanol added to the culture (containing CoQ or as vehicle) was less than 1% of the culture volume. Yeast were allowed to grow at 30 °C 250 rpm for 7 days. To construct the growth curves (see examples in Fig. S4), A_{600nm} measurements were taken at approximately 0, 7, 24, 30, 48, 54, 72, 78, and 144 hours. An aliquot of each culture (200 μL) was transferred to a 96-well plate and A_{600nm} was measured in a TECAN M1000 plate reader. 1/10 dilutions were made if necessary. With the exception of Fig. S4, CoQ rescue is always represented as a percentage of a designated control, considered as 100% and represented as a dashed line in the graphics. A_{600nm} from the last time point, 144 h, was used to calculate the degree of rescue.

Determination of CoQ_6 and CoQ_6 intermediates

To determine the content of CoQ_6 and CoQ**6** intermediate in *coqΔ* mutants, 15 mL of yeast grown on YPD were harvested at approximately 1 A_{600nm}/mL. Lipid extractions and the following analysis of CoQ content in a 4000 QTRAP linear MS/MS spectrometer (Applied Biosystems) were performed as described [46]. Aliquots of the culture were saved prior to lipid extraction to determine protein concentration using Bradford assay [47].

Immunoblot analysis

Three mL of yeast grown on YPD were harvested at approximately 1 A_{600nm}/mL. Pellets were resuspended in 100 μL 2M lithium acetate and incubated on ice for 5 min. Samples were centrifuged and pellets were resuspended in 100 μL 0.4M NaOH, then incubated on ice for 5 min. After this step, a 10 μL aliquot of each sample was saved for further protein quantification. Samples were resuspended in 100 μL 1X SDS sample buffer (50 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 1.33% β-mercaptoethanol) and boiled for 5 min. After centrifugation, supernatants were moved to clean tubes and stored at −20 °C until further use. 30-50 μg of protein was loaded in individual lanes and separated by SDS gel electrophoresis on 12% Tris-glycine polyacrylamide gels. Proteins were subsequently transferred to 0.45 μm Immobilon-P membrane (Bio-Rad). Blots were stained with Ponceau S dye for visualization of protein lanes and assessment of equal protein loading. After blocking (blocking buffer: 0.5% BSA, 0.1% Tween 20, 0.02% SDS in phosphate-buffered saline), blots were incubated with 1:1,000 primary anti-Porin 1 (obtained from Dr. Carla Koehler, University of California, Los Angeles) and 1:20,000 secondary IRDye 680LT goat anti-rabbit IgG antibody (LiCOR). Immunoblot images were visualized with a LiCOR Odyssey infrared scanner (LiCOR), and relative protein levels were quantified by band densitometry using both Image Studio Lite 5.2 and ImageJ.

Complementation of *ORFΔcoq2Δ* mutants with yeast *COQ_2*

To re-introduce *COQ_2* in the *ORFΔcoq2Δ* double mutants, the vector pRSQ2 was constructed. The sequence of yeast *COQ_2* plus 350 bp of the upstream sequence was cloned

into the vector pRS316 [48] using the BamHI restriction site. Then, the empty vector, as well as pRSQ2, were each used to transform *coq2Δ, cdc10Δcoq2Δ, rts1Δcoq2Δ, rvs161Δcoq2Δ, rvs167Δcoq2Δ* and *nat3Δcoq2Δ*. Transformation of WT and the corresponding single *ORFΔ* with pRS316 served as controls. Cells containing the plasmid were double selected on SD −Ura plates. The ability of the *ORFΔcoq*$_2$*Δ* to recover their respiratory capacity after *COQ*$_2$ was analyzed using spot plate assays. Briefly, plasmid-containing cells were grown overnight in SD −Ura at 30 °C 250 rpm. The next morning, A_{600nm}/mL was measured, cells were diluted to 0.2 A_{600nm}/mL in PBS, and a series of 5-fold dilutions were prepared. 2 μL of each dilution were plated onto YPD, YPG and SD −Ura plates. Plates were incubated at 30 °C and after 3-4 days, pictures were taken.

Statistical analysis

Data shown in this work represent mean ± standard deviation (SD) from at least three biological replicates. Statistical analyses and graphics were performed with Graphpad Prism 7 (Graphpad Software Inc., San Diego, CA, USA). When a specific mutant is compared to WT or to a designated normalization variable (specific *coqΔ* + CoQ_6 or *ORFΔ*) a *paired t-test* analysis was performed. When comparing two specific mutants between each other, an *unpaired t-test* analysis was used. Significant differences were referred as *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

Results

1 CoQ_6 does not move passively between non-contiguous membranes

Growth of *coqΔ* strains on YPG with exogenous CoQ_6 [33, 34] indicated that a sufficient quantity of the supplied CoQ_6 reached the mitochondrial inner membrane. However, the uptake of a particular CoQ isoform depends on the length of the isoprenoid chain. A previous study assessed the ability of different CoQ isoforms to diffuse between non-contiguous membranes [39]. Short CoQ isoforms such as CoQ_2 and CoQ_4 that are more water soluble, freely exchanged between phospholipid bilayers, while a long CoQ isoform (CoQ_9) did not due to its extreme hydrophobicity, and thus required a mechanism of transport [39]. The behaviour of the *S. cerevisiae* isoform CoQ_6 has not been evaluated. Therefore, to assess the ability of CoQ_6 to move between non-contiguous membranes, fluorescence quenching experiments were performed with two populations of phosphatidylcholine vesicles. Both populations contained equal concentrations of the fluorophore 1-pyrene dodecanoic acid (Pyr_{12}), but only the second population contained the specified isoform of CoQn. In this second population, the presence of CoQn fully quenches Pyr_{12} fluorescence by collisional quenching. On mixing the two populations of vesicles, a further decrease in the fluorescence signal can only occur if the CoQn isoform is able to move from vesicle to vesicle [39, 49]. Results showing the behavior of CoQ_6 in comparison with CoQ_2, CoQ_4 and CoQ_{10} are depicted in Fig. 2. When the population of CoQ-loaded vesicles was added, the fluorescence decreased dramatically when CoQ_2 or CoQ_4 were present, while fluorescence was maintained when the vesicles were loaded with either CoQ_6 or CoQ_{10}. These results indicated that CoQ_2 and CoQ_4 were able to move rapidly between the different vesicles, in agreement with previous results [39], while CoQ_{10} and CoQ_6 were non-diffusible. The inability of CoQ_6 to move between synthetic phospholipid membranes

supports the idea that a transport/distribution mechanism is needed due to its negligible water solubility.

2 Yeast mutants harboring deletions in distinct *COQ* genes show different degrees of rescue in response to exogenous CoQ_6

Previous work indicated that at least four genes of the endocytic pathway are required for rescue of *S. cerevisiae* by exogenous CoQ_6 [33]. In that study, *ERG2, TLG2, PEP12*, or *VPS45* deleted in combination with a *COQ3* deletion inhibited growth of the resulting double mutants on YPG supplemented with CoQ_6. CoQ_6 is produced in the mitochondrial inner membrane by a large multiprotein complex (the CoQ synthome) formed by several Coq proteins (Coq3-Coq9, Coq11) [1]. The absence of one Coq polypeptide can result in the loss of other Coq proteins [1, 50]. Given the effects of distinct *COQ* gene deletions on the CoQ synthome composition, we tested whether there is a difference in the ability of CoQ_6 to rescue strains carrying different *COQ* deletions in combination with the endocytic pathway mutations. Deletions of either COQ_2 or *COQ3* were combined with *erg2Δ, tlg2Δ, pep12Δ*, or *vps45Δ*, and the resulting strains were assessed for the degree of rescue by CoQ_6 (Fig. 3A, Table S3). When normalized to the wild-type parental strain (WT), rescue of the four *ORFΔcoq*$_2$*Δ* double mutants by exogenous CoQ_6 was more robust than rescue of the *ORFΔcoq3Δ* counterparts. Similar results were observed if the degree of rescue was compared to the corresponding positive control (either *coq2Δ* + CoQ_6 or *coq3Δ* + CoQ_6), instead of to WT growth (Fig. 3A, Table S3). These results suggested that *coq3Δ* cells are sensitized to the effects of endocytic pathway mutations on CoQ_6 rescue compared to *coq2Δ* cells. This was most apparent in the case of *tlg2Δ*, which severely inhibited rescue in *coq3Δ* cells but had no effect on rescue in *coq2Δ* cells. It thus seemed possible that the presence of different CoQ_6 synthesis intermediates might influence the rescue with exogenous CoQ_6. In a *coq3Δ* mutant, the Coq2 polypeptide is present and active, resulting in the production of two hexaprenylated CoQ_6 intermediates: 3-hexaprenyl-4-hydroxybenzoic acid (HHB), and 3-hexaprenyl-4-aminobenzoic acid (HAB) (Fig. 1) [1]. On the other hand, in a *coq2Δ* mutant, CoQ biosynthesis is blocked at the hexaprenyl transfer step, and neither HHB nor HAB can be formed.

To further examine whether the accumulation of early CoQ_6 synthesis intermediates influences the degree of rescue by exogenous CoQ_6, we focused on a particular endocytosis-defective mutant, *tlg2Δ*, and constructed *COQ1* and *COQ7* deletion strains. Coq1 synthesizes the CoQ polyisoprenoid tail, so its deletion prevents the formation of any hexaprenylated CoQ precursors (Fig. 1) [1]. Coq7 is a hydroxylase responsible for catalyzing the penultimate step of the CoQ biosynthetic pathway. However, because it is also a required structural component of the CoQ synthome, its deletion leads to the accumulation of HHB and HAB [1]. As shown in Fig. 3B, the degree of rescue of either *tlg2Δcoq3Δ* or *tlg2Δcoq7Δ* was significantly different from the mutants carrying *tlg2Δcoq1Δ* or *tlg2Δcoq2Δ*. It is notable that the *tlg2Δcoq3Δ* exhibits the most severely impaired degree of rescue (Fig. 3B, Table S3). The same general trend was observed when the degree of rescue is compared to each of the corresponding positive controls (e.g. *coq1Δ* + CoQ_6; *coq2Δ* + CoQ_6; *etc.*), instead of to WT growth (Fig. 3B, Table S3). These results suggest that the

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accumulation of CoQ hexaprenylated intermediates can affect the restoration of respiratory chain function by exogenous CoQ_6.

We then assessed the degree of rescue of each of the *coq1Δ* to *coq9Δ* single mutants in presence of exogenous CoQ_6 (Fig. 3C). The *coq1Δ* mutant grew to WT levels under these conditions (Fig. 3C, Table S3A). Although exogenous CoQ_6 does not completely restore the *coq2Δ* growth to WT levels, the response of the *coq2Δ* mutant is significantly better as compared to each of the other mutants, from *coq3Δ* to *coq9Δ* (Fig. 3C, Table S3). Interestingly, the *coq8Δ* mutant is more impaired than each of the other mutants, except for *CoQ_4Δ* (Fig. 3C, Table S3). This finding might suggests an active role of Coq8 in CoQ_6 transport. As *coq3Δ* - *coq9Δ* mutants are reported to accumulate hexaprenylated CoQ_6 intermediates, these results further support the hypothesis that the presence of such intermediates inhibits rescue of respiratory defective cells by CoQ_6.

3 The accumulation of early CoQ_6 hexaprenylated intermediates impairs CoQ_6 rescue

The presence of HHB and HAB in *coq3Δ*-*coq9Δ* mutants has been described previously [1, 2, 5], but a direct comparison of intermediates between the different strains is not available. We now show this comparison in Fig. 4A. These experiments revealed the loss of CoQ_6 in each of the *coqΔ* mutant strains (Fig. 4B) and a similar accumulation of HHB and HAB in *coq3Δ* - *coq9Δ* mutants (Fig. 4A, Fig. S2). None of the *coqΔ* mutants accumulated late-stage CoQ_6 intermediates (Fig. S2). In all cases, several controls were included: WT, which defines normal content of CoQ_6 and CoQ_6 biosynthetic intermediates in yeast cells; *corlΔ*, as an example of a mutant with normal CoQ_6 content but an inability to respire due to the deletion of *COR1*, an essential component of complex III; and a *rho*0 mutant on the same genetic background, to evaluate the absence of mitochondrial DNA (mtDNA) on CoQ_6 content. The absence of mtDNA can be considered a secondary CoQ deficiency [2, 51], and accordingly a dramatic decrease of CoQ_6 is observed (Fig. 4B). Additionally, to investigate whether altered mitochondrial mass might affect the degree of rescue of *coq3Δ*-*coq9Δ* mutants, we measured levels of the porin 1 polypeptide as a mitochondrial mass marker. Mitochondrial mass was not altered in any of the nuclear mutants (Fig. S3).

If accumulation of hexaprenylated CoQ_6 intermediates HAB and HHB caused by *coq3Δ* - *coq9Δ* mutations is responsible for impaired rescue by exogenous CoQ_6, then deletion of either *COQ1* or *COQ2*, which prevent the prenylation, should improve growth of *coq3Δ* - *coq9Δ* in the presence of CoQ_6. As predicted, deletion of *COQ1* or *COQ_2* in a *coq3Δ* mutant provided a degree of CoQ_6 rescue comparable to either the *coq1Δ* and *coq2Δ* single mutant, and approximately double that of a *coq3Δ* mutant (Fig. 4C). Similarly, deletion of *COQ_2* in either a *coq7Δ* or a *coq8Δ* mutant enhanced rescue by exogenous CoQ_6 as compared with the rescue of either the *coq7Δ* or *coq8Δ* single mutants (Fig. 4D). Interestingly, the rescue of the *coq8Δcoq2Δ* mutant was still lower than the rescue of a single *coq2Δ* strain (Fig. 4D). Together, our data indicate that the accumulation of HHB and HAB intermediates in *coq3Δ*-*coq9Δ* mutants impairs rescue by exogenous CoQ_6.

4 Genes that diminish growth response to exogenous CoQ_6

To identify genes involved in CoQ_6 uptake and trafficking to mitochondria, we used a library of double *ORFΔcoq2Δ* mutants, and tracked their ability to recover growth on YPG when supplemented with exogenous CoQ_6. This library was previously developed to identify proteins or pathways that might be involved in CoQ_2 or CoQ_4-dependent growth on non-fermentable substrates [39]. Based on the results described above, the use of *coq2Δ* in the library avoids potential complications from accumulation of HHB and HAB. Rescue with exogenous CoQ_6 requires vigorous aeration of liquid cultures, which does not occur in high throughput multi-well assays [52]. Therefore, we selected 48 candidate genes that previous literature suggests may be involved in CoQ_6 uptake/distribution [32, 33, 39]. The genes can be grouped into seven categories (Table 1): 16 genes described to have diminished response to exogenous CoQ_2 and CoQ_4 [39] plus two extra genes involved in phospholipid and sterol transport (*CSR1* and *HES1*) with limited growth on exogenous CoQ_2 and CoQ_4; 15 genes implicated in yeast clathrin-mediated endocytosis [53]; four genes that assemble the endoplasmic reticulum (ER)-mitochondria encounter structure (ERMES) [54]; two genes that encode the nucleus-vacuole junction (NVJ); three genes that represent the vacuole-mitochondria patch (vCLAMP) [55, 56]; four endocytosis genes already described to play a role in CoQ_6 transport [33]; and two genes that form the N-terminal acetyltransferase B (Nat B) complex. Some *ORFΔcoq2Δ* double mutants were not recoverable from the library (see Table 1), so a total of 40 mutants were ultimately analyzed.

The ability of each *ORFΔcoq2Δ* double mutant to recover respiratory growth on YPG in the presence of 2 μM CoQ_6 was monitored for seven days. In all experiments, WT, *coq2Δ* and *cor1Δ* strains were included as controls in the presence and absence of CoQ_6. This screen identified 23 mutants that showed significantly diminished rescue compared to the positive control (*coq2Δ* + 2 μM CoQ_6) (Fig. 5A), indicating that the presence of these particular *ORF*s may be necessary for optimal CoQ_6 rescue. A representative *ORFΔcoq2Δ* mutant growth curve from each group is displayed in Fig. S4. Almost all double mutants from the heterogeneous Group 1 had diminished ability to be rescued by CoQ_6, in agreement with the deficient response to addition of CoQ_2 or CoQ_4 [39]. Four affected candidates were yeast endocytic factors (Group 2), while six other members of this group retained the ability to be rescued with exogenous CoQ_6. This result suggests that the entire endocytic pathway may not be necessary for CoQ_6 transport, or that alternative or redundant mechanisms of CoQ_6 transport may compensate for the deletion of a particular gene. A strong deficiency in rescue was also observed in three of the four gene deletions previously studied [33] (Group 6) and in the members of the Nat B complex. Interestingly, the groups representing the connections between organelles were in general not affected. These results may either exclude gene products involved in organelle connections as participants in the mechanism of CoQ_6 transport, or suggest that CoQ_6 transport has more than one path and so the elimination of one organelle connection is not sufficient to produce a defective phenotype.

Deletions of certain genes may not completely abolish the ability of mutants to grow on YPG, but can still compromise growth. To examine this, a second round of screening was performed and the ability of the *ORFΔcoq2Δ* mutants to grow in the presence of exogenous CoQ_6 was compared with the corresponding single *ORFΔ*. We analyzed the 23 candidates

identified in Fig. 5A for their degree of rescue when compared to the single *ORFΔ*. It should be noted that two double mutants were excluded: *qcr9Δcoq2Δ* and *emilΔcoq2Δ*. The growth of the single *qcr9Δ* in YPG was highly inconsistent, for reasons that are unclear. In the case of the *emilΔcoq2Δ* mutant, the phenotype observed was unlikely to be related to CoQ_6 transport, and is being investigated separately. Of the remaining 21 *ORFΔcoq2Δ* mutants, 17 candidates were significantly different from the corresponding *ORFΔ* (Fig. 5B), suggesting they are involved in CoQ_6 transport. The four *ORFΔ* that were eliminated were each rescued to their maximum potential level, indicating that the slow YPG growth is unrelated to CoQ_6 transport. A good example of this phenomenon can be observed in Fig. S4B, where the growth of *ede1Δcoq2Δ* + CoQ_6 is similar to the growth of *ede1Δ*, and both display decreased growth as compared to the positive control.

5 Comparison of the CoQ_2 and CoQ_6 rescue phenotypes identifies six candidates necessary for CoQ_6 transport

In order to identify the most relevant genes in the transport of CoQ_6, we analyzed the rescue phenotype of the 17 previously identified candidates in response to exogenous CoQ_2. We hypothesized that if the growth defect of the *ORFΔcoq2Δ* mutants is in fact due to the uptake and/or distribution of CoQ_6, a shorter, diffusible isoform of CoQ such as CoQ_2 should allow for a better degree of rescue than CoQ_6. In this scenario, CoQ_2 will be able to reach the mitochondria of the mutants even when the mechanisms of transport are impaired. It is important to note that the degree of rescue of the *coq2Δ* mutant in the presence of CoQ_2 was significantly lower than that observed in the presence of CoQ_6 (Fig. S5A). It is possible that the more rapid diffusion of CoQ_2 between membranes due to its far lower hydrophobicity limits its accumulation in the mitochondria where it must partition to recover respiratory function.

Comparison between CoQ_2 and CoQ_6 rescue phenotypes of each of the 17 *ORFΔcoq2Δ* mutants is depicted in Fig. 6A. The CoQ_2 rescue phenotype of the mutants on their own can be observed in Fig. S5B. Of the 17 *ORFΔcoq2Δ* mutants analyzed, eight showed statistically significant differences between CoQ_2 and CoQ_6 rescue. However, only five *ORFΔcoq2Δ* candidates fit the criterion of having a degree of CoQ_2 rescue significantly greater than that by CoQ_6. Thus, these five genes (*CDC10, RTS1, RVS161, RVS167*, and *NAT3*) seem good candidates to encode proteins that are required for CoQ_6 uptake and/or distribution to mitochondria within yeast cells. The known functions of these proteins are summarized in Table 2. In Fig. S6, we showed that the transformation of the final five *ORFΔcoq2Δ* candidates with the plasmid pRSQ2 (containing the *COQ2* gene) restores the growth of the *ORFΔ* on YPG plates.

Two of the five identified proteins, Rvs161 and Rvs167, function together in the vesicle scission step of the clathrin-mediated endocytosis pathway. Multiple studies have identified Vps1 as a third player in this mechanism [61, 62]. Moreover, Vps1 and Rvs167 have been reported to function together during endocytosis [63]. *VPS1* was not part of our initial selection of candidates, but due to its functional relationship with Rvs161 and Rvs167, we hypothesized that it might also function in CoQ_6 transport. The degree of rescue with exogenous CoQ_6 of the *vps1Δcoq2Δ* mutant, in comparison to the positive control and also

with *vps1Δ*, is represented in Fig. 6B-C. In both cases, the degree of CoQ_6 rescue is dramatically impaired. In addition, the degree of rescue with exogenous CoQ_2 was analyzed, and the comparison with the CoQ_6 rescue phenotype showed that the "CoQ_2 rescue > CoQ_6 rescue" criteria is fulfilled (Fig. 6D). These results confirmed *VPS1* as an additional gene required for CoQ_6 transport.

Discussion

CoQ_{10} is among the most widely used dietary and nutritional supplements on the market, ranging from an over-the-counter supplement to more specific administration for particular disorders [64]. Numerous disease processes associated with CoQ_{10} deficiency may benefit from CoQ_{10} supplementation, including primary and secondary CoQ_{10} deficiencies, mitochondrial diseases, fibromyalgia, cardiovascular disease, neurodegenerative diseases, cancer, diabetes mellitus, infertility, and periodontal disease [17, 65, 66]. However, CoQ_{10} is not an efficient oral supplement. Its high lipophilicity and poor solubility lead to low bioavailability due to limited absorption into systemic circulation, making the effective delivery of CoQ_{10} to the mitochondria a major challenge [23, 67]. Understanding the mechanism(s) used to uptake and distribute CoQ_{10} within cells is of great relevance to help CoQ_{10} become an efficient therapy. Towards this end, the studies in yeast reported here uncovered an unexpected inhibitory effect of prenylated CoQ intermediates on exogenous CoQ assimilation and identified six genes required for functional rescue of CoQ-deficiency by exogenous supplementation with the normal cellular form of CoQ. While we have utilized respiration as a means to identify genes that transport CoQ, CoQ supplementation via these transport pathways could offer antioxidant protection against mitochondrial-derived oxidants.

Multiple lines of evidence support the existence of one or more import pathways for exogenous CoQ_6 and CoQ_{10}, as well as an intracellular distribution mechanism for *de novo* synthesized CoQ from the mitochondria to other cellular membranes [30–34]. Results presented in Fig. 2 establish the necessity of transport systems for movement of CoQ_{10} and CoQ_6 between membranes, supporting the view that CoQ transport requires the endomembrane system [32, 33]. To address the mechanism of transport, we adopted a genetic strategy in which gene deletions were screened for effects on the ability of exogenous CoQ_6 to rescue the growth of a *coqΔ* strain deficient in CoQ synthesis (ref 30). During the course of these experiments, we observed differential responses of *coqΔ* mutants to exogenous CoQ_6 due to reduced rescue in strains (*coq3Δ*-*coq9Δ*) that accumulate early-stage prenylated CoQ_6 intermediates, HHB and HAB (Figs. 3 and 4). This likely also explains the previous observation that exogenous decylubiquinone blunts rescue by exogenous CoQ_2 in *coq2Δ* mutants [39].

The exact reason why the presence of early CoQ_6 hexaprenylated intermediates prevents the rescue with exogenous CoQ_6 is not clear, but different hypotheses can be proposed: CoQ_6 intermediates could (i) produce a certain level of toxicity that affects the normal operation of the cells; (ii) mimic the behavior of detergents by trapping CoQ_6 in micelles and prevent its correct delivery to the mitochondrial inner membrane; or (iii) impede incorporation of CoQ_6 into the mitochondrial inner membrane by saturating the bilayer or transport proteins with

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polyisoprenoids; or (iv) interact with CoQ-utilizing proteins and prevent the proper function of imported exogenous CoQ_6. It is also possible that the slight rescue deficiency observed in *coq2Δ* (Fig. 3C) is due to the presence of hexaprenyl-diphosphate in the membranes. The accumulation of CoQ prenylated intermediates is not as well explored in human cells as it is in yeast cells. However the accumulation of DMQ_{10} has been reported in fibroblasts derived from patients with inherited COQ4, COQ7, and COQ9 deficiencies [64, 68, 69], as well as in fibroblasts where *COQ3, COQ5*, or *COQ6* were knocked down with siRNAs [64]. DMQ is considered a late-stage CoQ intermediate and it is not clear whether this precursor negatively affects CoQ rescue. However, if this is the case, patients with CoQ_{10} deficiencies that accumulate DMQ10 might not respond well to CoQ_{10} supplementation and alternative treatments, such as the induction of internal CoQ_{10} synthesis, should be considered instead. Based on our results, in addition to CoQ_{10} intermediates serving as a potentially important biomarker for the diagnosis of CoQ_{10} deficiency disorders [64], they may also impact selection of an appropriate treatment.

Our use of *coq2Δ* cells to screen for genes involved in CoQ_6 transport avoids complications that could arise from accumulation of prenylated CoQ_6 intermediates. This was evident in our analyses of *TLG2*, previously identified as required for CoQ_6 uptake using *coq3Δ* cells. In our experiments, whereas deletion of *TLG2* in a *coq3Δ* strain impaired rescue by exogenous CoQ_6, the same deletion in a *coq2Δ* mutant had no significant impact on rescue, indicating that *TLG2* does not play a significant role in CoQ_6 transport in cells that do not accumulate HHB and HAB (Fig. 3).

Comparison of rescue by CoQ_6 and CoQ_2 provided a way to distinguish mutations that specifically impeded CoQ_6 transport (preferentially rescued by CoQ_2) from those that generally prevented utilization of CoQ. For example, in addition to *TLG2*, three other genes were identified using *ORFΔcoq3Δ* mutants: *ERG2*, *PEP12* and *VPS45* (30). Unlike deletion of TLG2, deletion of these genes in *coq2Δ* strains still reduced CoQ_6 rescue, though not to the extent observed with *coq3Δ* cells. However, the *coq2Δ* strains harbouring deletions of *ERG2, PEP12 or VPS45* were not preferentially rescued by CoQ_2 (Fig. 6A), suggesting that defective transport of CoQ_6 is not the underlying issue. Like the *erg2Δcoq2Δ* mutant, many other *ORFΔcoq2Δ* strains in Fig. 6A responded similarly to exogenous CoQ_2 and CoQ_6, indicating that the defect in these strains is also more likely to be related to CoQ utilization. Notably, *pep12Δcoq2Δ* and *vps45Δcoq2Δ*, as well as *rpl31AΔcoq2Δ*, responded better to CoQ_6 than to CoQ_2. The basis for this phenotype remains to be determined, but may be due to lower levels of delivery to the vacuole and degradation.

The Coq8 polypeptide is a member of an ancient atypical kinase family [70], with several conserved kinase motifs that are essential for CoQ biosynthesis [71, 72]. Unique characteristics have been attributed to Coq8 like the fact that its overexpression in *coqΔ* mutants augments the levels of several Coq polypeptides, stabilizes CoQ synthome formation and restores the formation of CoQ domains [73–77]. We find it intriguing that the *coq8Δ* mutant yeast also displayed an unique phenotype with regard to rescue by exogenous CoQ_6. Its degree of rescue is lower as compared to the other *coqΔ* mutants (*coq3Δ, coq5Δ* - *coq7Δ* and *coq9Δ*; Fig. 3C), and the additional deletion of COQ_2 only partially augmented rescue (Fig. 4D). Together, these observations might suggest an active role of Coq8 in CoQ_6

transportation. Such a role is in agreement with previous studies, where Coq8 has been proposed to function as a chaperone that facilitates the partial extraction of lipophilic CoQ intermediates out of the inner mitochondrial membrane and into the aqueous matrix environment promoting the enzymatic reactions catalyzed by other Coq proteins [78].

Due to their endosymbiotic origin, mitochondria are not embedded within the vesicular trafficking system [79]. Instead, they are connected to other cellular organelles by multiple membrane contact sites [79, 80]. Recently, it has been described that the CoQ synthome is localized to distinct puncta within the inner mitochondrial membrane, termed CoQ domains [76]. The CoQ domains are non-randomly localized to adjacent ER-mitochondria contact sites [76, 81]. Moreover, it was observed that the disruption of ERMES alters the distribution of CoQ_6 and its precursors [81]. These observations suggest a role for ER-mitochondria contacts in CoQ domain formation and CoQ_6 production and distribution from mitochondria to the rest of the cell. Our screen to identify genes involved in CoQ_6 transport revealed that *ORFΔcoq2Δ* strains missing an ERMES gene were not affected in their ability to be rescued by exogenous CoQ_6. Deletion of genes belonging to the vCLAMP or the NVJ families also had no significant impact on rescue by exogenous CoQ_6. These results may suggest that exogenous CoQ_6 is reaching the mitochondria by a mechanism unrelated to these organelle connections, perhaps by means of the novel clathrin-mediated vesicle transport from the plasma membrane to the mitochondria [82], or via a still-unknown transport process. It is also possible that transport of CoQ_6 through organelle connections occurs via redundant steps. It has been shown previously that for phospholipid exchange, mitochondria are dependent on at least one functional contact site with the endomembrane system: ERMES or vCLAMP, whose functions are tightly co-regulated [83]. In this case, the loss of one system elicits a compensatory response in the other, and loss of both is lethal. Thus, the cell is able to adapt and, in the absence of one structure, an increase in the other serves as a “backup” path for small-molecule transport [83].

We identified six *ORFΔcoq2Δ* double mutants that are compromised in their CoQ_6 rescue, and perform better when diffusible CoQ_2 is supplied instead (Figs. 5 and 6). These six *ORFs* (*CDC10, RTS1, RVS161, RVS167, NAT3*, and *VPS1*) encode proteins that are necessary for CoQ_6 transport and identify essential steps that cannot be compensated by other proteins or pathways. Three of these proteins (Rvs161, Rvs167, and Vps1) are directly involved in the endocytosis process at the plasma membrane. Rvs161 and Rvs167 are Bin/amphiphysin/Rvs (BAR) domain proteins that form a heterodimeric protein complex capable of deforming the membrane, and are necessary for invagination and scission of vesicles in the final step of clathrin-mediated endocytosis [84]. In mammalian cells, in addition to Rvs161/167 homologues, the GTPase dynamin (Vps1 human homolog) is essential for scission of clathrin-coated vesicles. In yeast, the role of Vps1 in yeast endocytosis has been controversial [84], but recent reports demonstrated that Vps1 functions with the Rvs161/167 heterodimer to facilitate scission and release of the vesicles [61–63, 85]. Indeed, Vps1 and Rvs167 were found to physically interact with each other [63]. The *rvs161Δ* and *rvs167Δ* mutants have a specific defect: a significant fraction of the endocytic patches begins to be internalized and move inward from the cell surface but are then retracted toward the cortex [86]. This defective phenotype might impair the uptake of exogenous CoQ_6, resulting in the poor rescue phenotype observed in *rvs161Δcoq2Δ* and *rvs167Δcoq2Δ* double mutants (Fig.

5 and 6A). Invaginations do not form correctly in the absence of Vps1 either [61]. Vps1 plays a significant role in endosomal trafficking, and the *vps1Δ* mutant accumulates endosomes in the cytoplasm [60]. Perhaps these attributes of the *vps1Δ* mutant account for the extremely deficient rescue phenotype of the *vps1Δcoq2Δ* double mutant (<5% of the positive control) (Fig. 6B).

Cdc10 was also identified in our screens as a protein involved in CoQ_6 transport (Fig. 5 and 6A). Cdc10 is one of the four core septin proteins in *S. cerevisiae*, a group of GTPases that assemble into filaments and higher-order structures [87–89]. In yeast cells, septin filaments assemble at the bud neck in a 'septin ring', which is essential in cytokinesis during which it acts as a scaffold to recruit cytokinetic factors to the site of cell division [88, 90]. In addition to scaffolding, the other main function of septins is to serve as cortical barriers to prevent the free diffusion of membrane proteins between different compartments [88, 90]. Septins may play a role in endocytosis since they interact with a subset of endocytic proteins, including Vps1, and the accessory proteins Syp1 and Sla2 [91]. SEPT9, the human homolog of Cdc10, has been implicated in endosomal sorting [92]. Interaction partners previously identified suggested a role for SEPT9 in vesicle transport to and from the plasma membrane [93]. The other three core septins (Cdc3, Cdc11 and Cdc12) are encoded by essential genes and therefore the null mutants cannot be interrogated [94]. Whether the involvement of Cdc10 (or potentially other septins) in CoQ_6 uptake/transport is related to the role of septins in endocytosis or a different septin function, will require further experimentation.

The remaining two proteins identified as necessary for CoQ_6 transport, Rts1 and Nat3, may function as regulators. Rts1 is one of the two regulatory subunits of protein phosphatase 2A (PP2A), a major class of serine/threonine protein phosphatases that play an important role in many biological processes [95, 96]. The regulatory subunit is thought to determine substrate specificity, targeting $PP2A^{Rts1}$ for regulation of the mitotic spindle orientation checkpoint, cell size control, and septin ring organization and disassembly during cytokinesis [96]. Phosphoproteomic analyses in *rts1Δ* cells reveals 156 hyperphosphorylated proteins, highlighting the multitude of $PP2A^{Rts1}$ complex targets [97]. The cytokinesis defect observed in *rts1Δ* cells is in agreement with the role of $PP2A^{Rts1}$ in the regulation of septin dynamics, possibly by dephosphorylating some factor(s) at the bud neck [98]. $PP2A^{Rts1}$ reverses phosphorylation of (at least) septin Shs1 and contributes to timely septin disassembly after cytokinesis [90]. Yet-to-be-identified relationships between septins and $PP2A^{Rts1}$ are possible as suggested by data from other fungal species. As*pB* (the *CDC3* ortholog in *Aspergillus fumigatus*) is dephosphorylated in a PP2A-dependent manner [99]. To the best of our knowledge, there is no direct association between $PP2A^{Rts1}$ and Cdc10, although Rts1 has a strong potential to serve as a regulator during CoQ_6 transport even if it is not related with septins. Almost all endocytic proteins participating in clathrin-mediated endocytosis are phosphorylated [100], including Rvs161, Rvs167, and Vps1 [100–102], and are excellent targets for phospho/dephosphorylation regulation.

The final gene influencing CoQ_6 uptake or transport is Nat3, the catalytic subunit of the NatB complex. Together with its auxiliary subunit (Mdm20), Nat3 mediates the N-terminal acetyl transfer of about 20% of proteins in yeast and humans [103–105]. In yeast, there are four functional NATs, and each has distinct substrate specificity [105, 106]. Could N-

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acetylation modifications play a role in some of the proteins involved in CoQ_6 transport? This seems less likely given that *mdm20Δcoq2Δ* had profound rescue defects in response to both exogenous CoQ_2 and CoQ_6 (Fig. 6A), excluding *MDM20* from consideration as an *ORF* related with CoQ_6 transport. Thus, we believe that Nat3 and CoQ_6 may be connected through an unknown, but NatB-independent, function of this protein. Although *nat3Δ* and *mdm20Δ* share several phenotypes, *nat3Δ* mutants possess additional unique phenotypes [104]. Moreover, Mdm20 has recently been related with several cellular functions independent of Nat3 [107, 108], supporting the idea that Nat3 participates in additional pathways, either by itself or in conjunction with another auxiliary protein. It is also possible that the deficient rescue displayed by *mdm20Δcoq2Δ* (Fig. 6A) is a consequence of multiple defects in this strain, and that N-acetylation modifications somehow regulate CoQ_6 transport.

Conclusion

Our studies have revealed deleterious effects of CoQ_6 hexaprenylated intermediates on rescue with exogenous CoQ_6. Additionally, our research defines six novel proteins as necessary for appropriate CoQ_6 uptake and transport, and strongly connects CoQ_6 distribution with endocytosis and membrane trafficking pathways.

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Abbreviations

CoQn	coenzyme Q containing a polyisoprenyl tail of *n* isoprene units
CoQ_6	oxidized coenzyme Q_6
CoQ_6H_2	reduced coenzyme Q_6H_2
Coq1	polypeptide encoded by the *COQ1* gene in *Saccharomyces cerevisiae*
coqlΔ	yeast mutant harboring a deletion in the *COQ1* gene
$DMQH_2$	demethoxy-QH_2
ERMES	endoplasmic reticulummitochondria encounter structure
HAB	3-hexaprenyl-4-aminobenzoic acid

4HB	4-hydroxybenzoic acid
HHB	3-hexaprenyl-4-hydroxybenzoic acid
IDMQH$_2$	4-imino-demethoxy-QH$_2$
LUVET	large unilamellar vesicles produced by extrusion
mtDNA	mitochondrial DNA
NatB	N-acetyltransferase B complex
NVJ	nucleus vacuole junction
ORFΔcoq2Δ	yeast double mutant harboring a gene deletion in a designated open reading frame plus a deletion in *COQ2*
pABA	para-aminobenzoic acid
PC	phosphatidylcholine
Pyr$_{12}$	1-pyrene dodecanoic acid
SD	synthetic dextrose medium
vCLAMP	vacuole-mitochondria patch
WT	wild-type parental yeast strain
YPD	rich growth medium containing dextrose as a fermentable carbon source
YPG	rich growth medium contain glycerol as the sole non-fermentable carbon source

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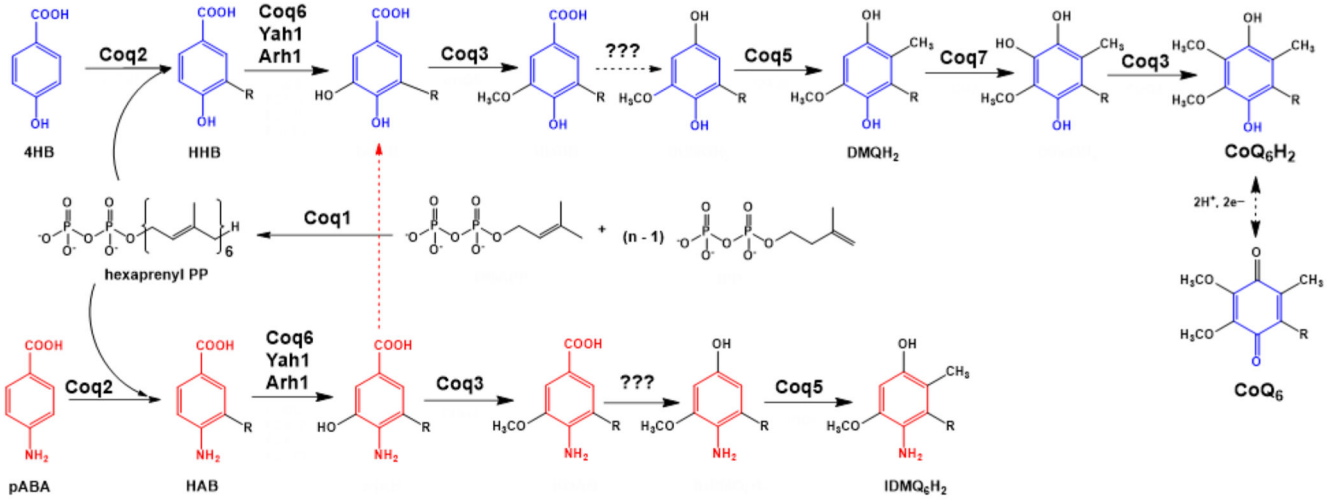

Figure 1. Coenzyme Q biosynthetic pathway in *S. cerevisiae*.

Adapted from [1]. At least 13 proteins are necessary for efficient CoQ biosynthesis in *S. cerevisiae* (Coq1-11, Yah1 and Arh1) [1]. The polyprenyl diphosphate is produced by a hexaprenyl diphosphate synthase (Coq1) [6]. Coq2 mediates the condensation of the isoprenoid tail with the aromatic ring precursor, generating a membrane-bound CoQ intermediate. In yeast, both 4HB and pABA can be used as ring precursors for CoQ_6 biosynthesis [7, 8]. Intermediates originating from 4HB are depicted in *blue*, while intermediates originating from pABA are depicted in *red*. Several Coq proteins (Coq6, Coq3, Coq5 and Coq7) modify the hexaprenylated CoQ precursor to form the final product. Other Coq proteins (Coq4, Coq8, Coq9, CoQ_{10} and Coq11) are essential for efficient CoQ production. For simplicity, only the relevant intermediates to understand further analysis have been named. 4HB = 4-hydroxybenzoic acid; pABA = para-aminobenzoic acid; HHB = 3-hexaprenyl-4-hydroxybenzoic acid; HAB = 3-hexaprenyl-4-aminobenzoic acid; $DMQH_2$ = demethoxy-QH_2; $IDMQH_2$ = 4-imino-demethoxy-QH_2; CoQ_6H_2 = reduced coenzyme Q_6H_2; CoQ_6 = oxidized coenzyme Q_6.

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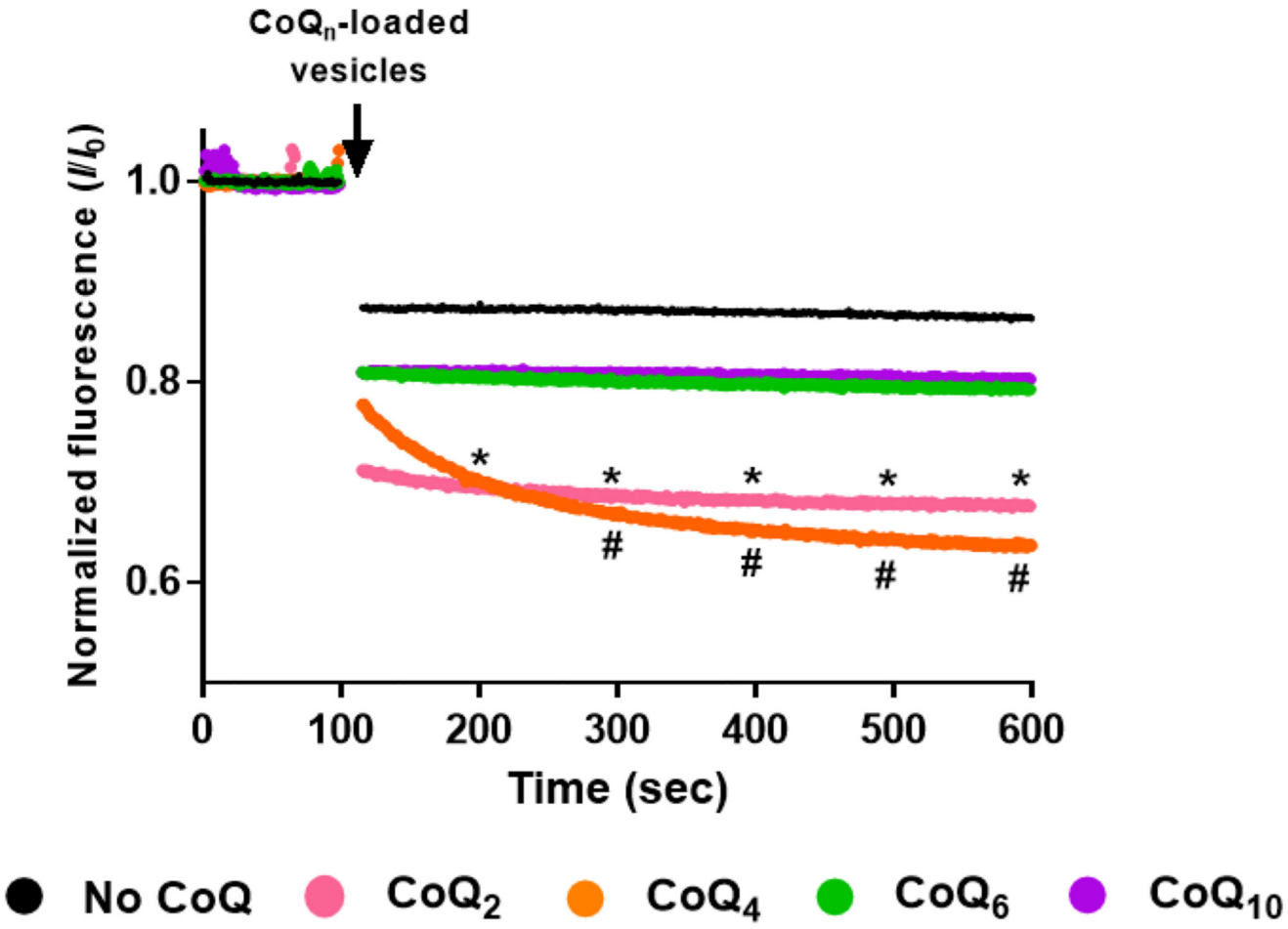

Figure 2. CoQ_6 and CoQ_{10} isoforms fail to move freely between phosphatidylcholine vesicles.

The membrane behavior of different CoQ_n isoforms was analyzed using a fluorescence-quenching assay. An initial population of vesicles containing 4 μM Pyr_{12} was mixed with 500 μL of a second population of vesicles that contained 4 μM Pyr_{12} and either a control, with no addition (*black*), or a CoQ isoform: CoQ_2 (*pink*), CoQ_4 (*orange*), CoQ_6 (*green*) or CoQ_{10} (*purple*). Fluorescence was monitored for 600 seconds and data points were collected every second. The data are expressed as the relative amount of fluorescence at a given point in time (I) to the initial fluorescence just before the addition of the CoQ_n-loaded vesicles (I_O). Dots represent the average, at each point, of at least three independent experiments. Standard deviations have been omitted for clarity in this figure but they are provided in Fig. S1. At intervals of 100 sec, the (*) symbol represents significant differences (p<0.05) between CoQ_2 and the no-CoQ condition, while (#) represents differences between CoQ_4 and the no-CoQ condition.

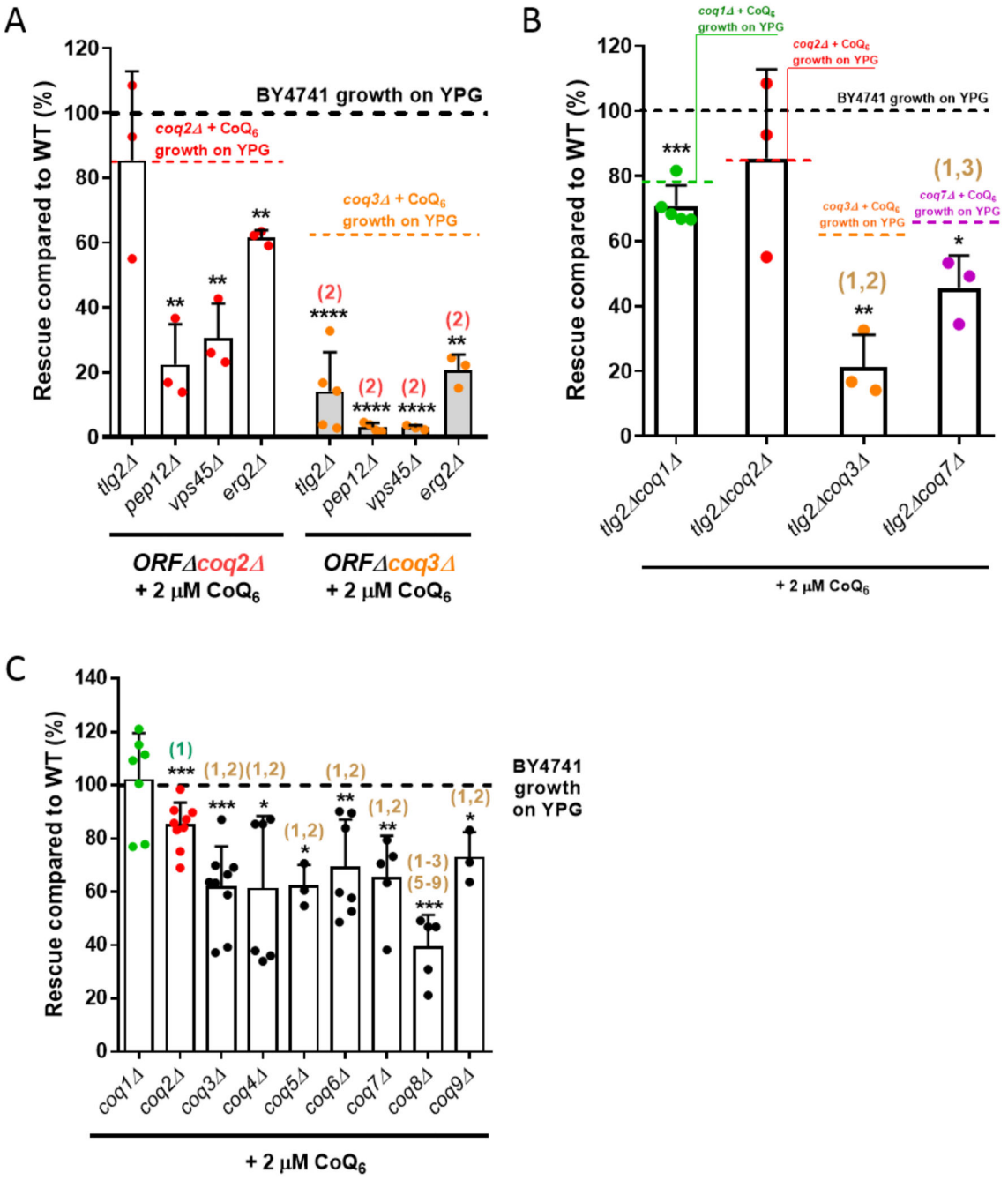

Figure 3. Yeast mutants harboring deletions in distinct *COQ* genes show different degrees of rescue in response to exogenously added CoQ_6.

(A) Yeast *coq2Δ* mutants harboring additional deletions in *tlg2Δ, pepl2Δ, vps45Δ* or *erg2Δ* showed a more pronounced YPG growth rescue by CoQ_6 than do the comparable *coq3Δ* double mutants. **(B)** The *tlg2Δcoqlδ, tlg2Δcoq3Δ* and *tlg2Δcoq7Δ* double mutants presented an impaired degree of rescue as compared to WT. The decrease of rescue was more pronounced in *tlg2Δcoq3Δ* and *tlg2Δcoq7Δ*, but especially in the *tlg2Δcoq3Δ* mutant. **(C)** Yeast mutants harboring deletions in either the *COQ1* or *COQ2* gene showed more robust

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rescue in response to exogenous CoQ_6 treatment than do the other single *coq* null mutants (*coq3Δ* - *coq9Δ*). In all cases, columns represent the degree of rescue (in %) ± SD of a strain compared to WT, which is defined as 100% and represented as a dashed line. In A and B, the average of rescue of the specific positive controls (*coq1Δ* + CoQ_6, *coq2Δ* + CoQ_6, *coq3Δ* + CoQ_6, or *coq7Δ* + CoQ_6), which were analyzed in parallel with the samples, is represented as a dashed line. Three or more independent rescue experiments were performed for every strain. Asterisks on top of the columns represent significant differences when compared to WT (*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001). Statistically significant differences between a specific *coqΔ* mutant (or *ORFΔcoqΔ* mutant) and one of its counterparts (another *coqΔ* or *ORFΔcoqΔ* mutant) are denoted with numbers in parentheses on top of the columns. (1) represents differences comparing to *coq1A*, (2) represents differences comparing to *ORFΔcoq_2Δ* (panel A) or *coq2Δ* (panel B-C), (3) represents differences comparing to *coq3Δ*, etc. Statistical comparisons not detailed in the figure are provided in Table S3.

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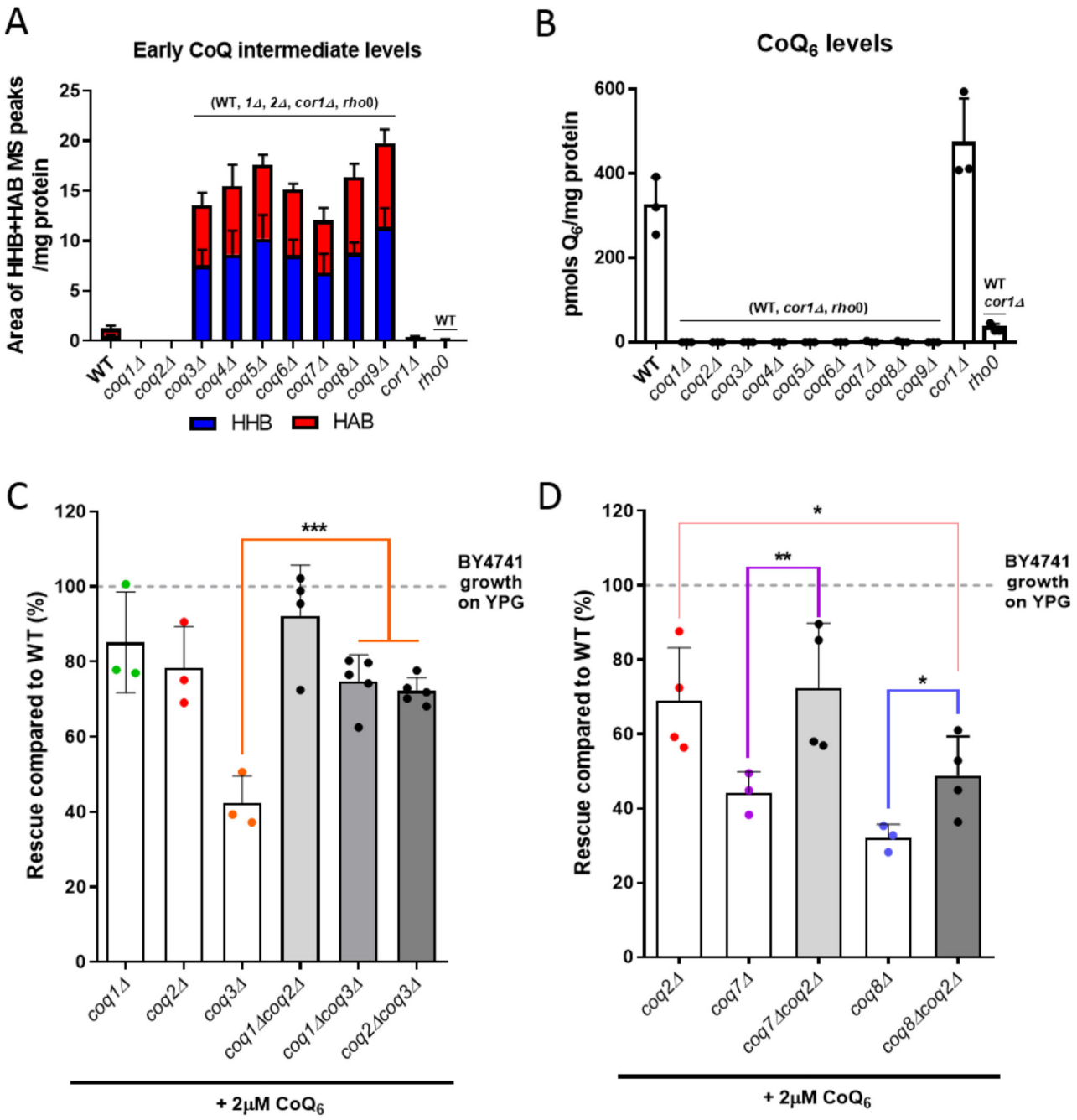

Figure 4. The elimination of HHB and HAB intermediates augments the degree of rescue with exogenous CoQ_6.

(A) Each of the *coq* mutants, from *coq3Δ* to *coq9Δ*, accumulated early CoQ_6 intermediates (HHB and HAB). The amount of HHB and HAB was calculated as the area of the MS peak/mg protein. **(B)** CoQ_6 was not detected in any of the *coqΔ* mutants. CoQ_6 (pmol/mg protein) was determined based on a CoQ_6 standard curve as described in [46]. **(C)** Additional deletions of either *COQ1* or *COQ2* restored the deficient CoQ_6-rescue of a *coq3Δ* mutant, but do not affect the phenotype observed in *coq1Δ* or *coqΔ2* strains. **(D)**

Similar results were observed when *COQ2* is deleted in a *coq7Δ* strain, but only a partial improvement was observed in a *coq8Δ* mutant. In A and B, columns represent mean ± SD and strains named in parentheses on top of the columns denote statistically significant differences ($p<0.01$). In C and D, columns represent the degree of rescue (in %) ± SD of a strain in comparison to WT (considered 100% and represented as a dashed line). Asterisks denote significant differences as compared to the single *coqΔ* strain (*$p<0.05$, **$p<0.01$, and *** $p<0.001$). In all cases, three or more independent biological replicates were performed.

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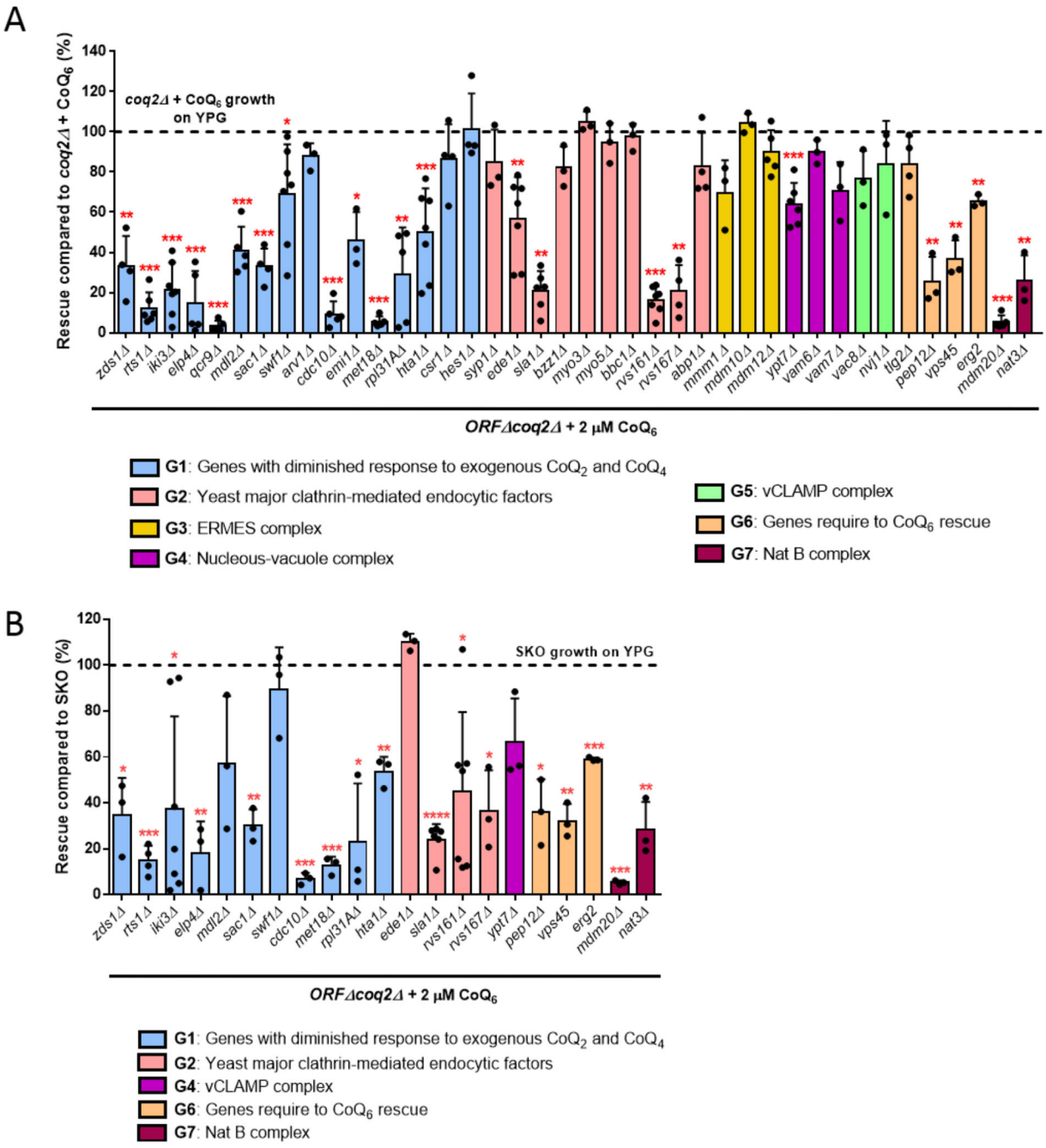

Figure 5. Two rounds of screening identified 17 *ORFΔcoq2Δ* double mutants to have a diminished response to exogenous CoQ_6.

(A) First, the degree of rescue of the *ORFΔcoq2Δ* candidates was calculated in comparison with the positive control: *coq2Δ* + CoQ_6 **.** Of a total of 40 candidates, 23 showed a significantly decreased ability to rescue in presence of exogenous CoQ_6. **(B)** Since a single deletion can compromise the ability of a mutant to grow on YPG, the degree of rescue of the previous significant candidates was calculated in comparison to the corresponding *ORFΔ* in the second phase of the screening. The *emilΔcoq2Δ* and *qcr9Δcoq2Δ* mutants were

eliminated for further analysis due to special phenotypes (see main text). 17 candidates display impaired rescue in response to treatment with exogenous CoQ_6. In both cases, columns represent the average degree of rescue (in %) ± SD of mutants growing on YPG in presence of 2 μM CoQ_6 for 7 days. Three or more replicas were included for all candidates. The normalization variable is considered 100% and represented as a dashed line. Asterisks denote the different degrees of significance: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

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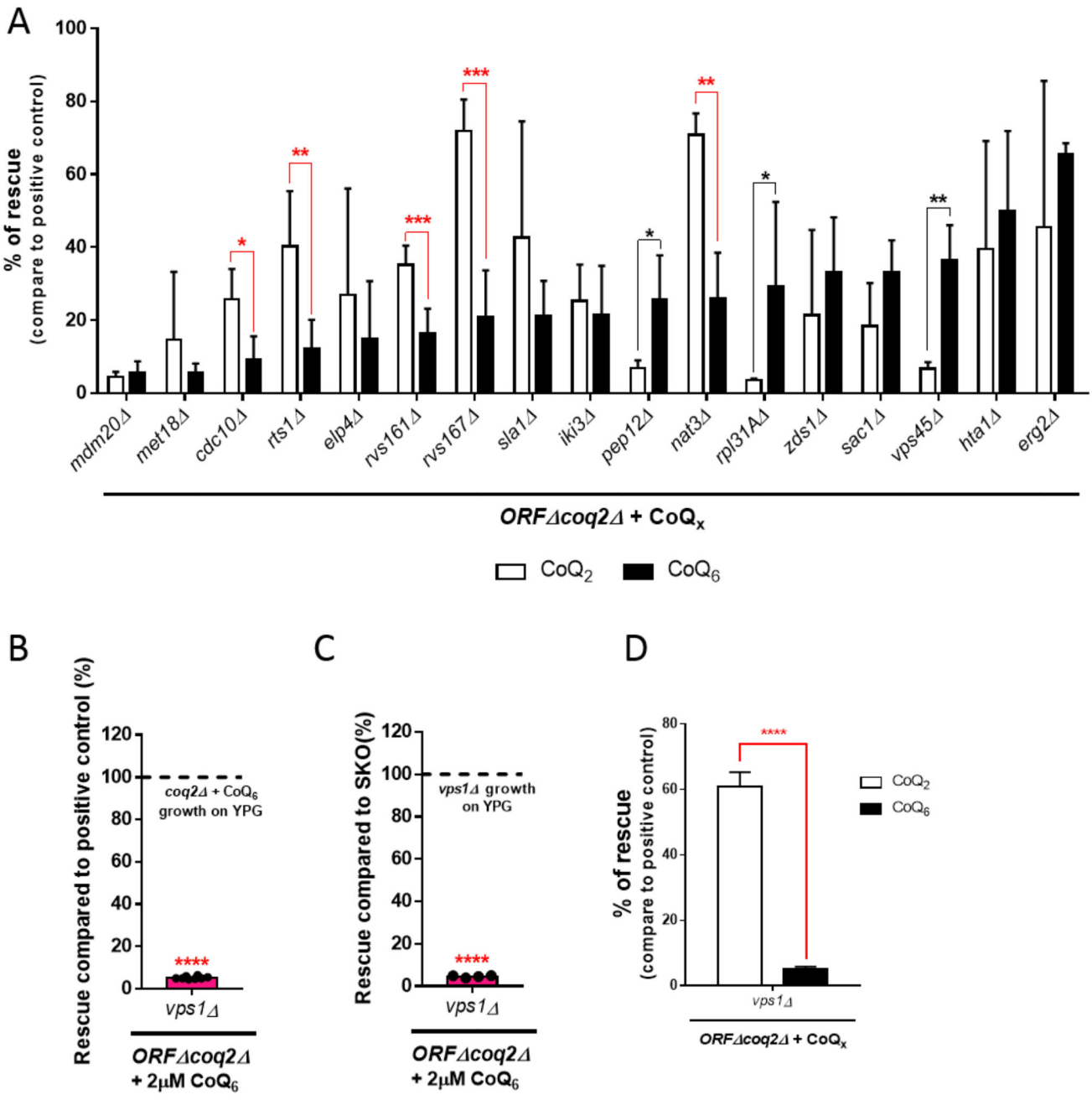

Figure 6. Comparison of the CoQ_2 and CoQ_6 rescue phenotypes identifies six genes necessary for CoQ_6 transport.

(A) The degree of CoQ_2 and CoQ_6 rescue, compared to the corresponding positive control (*coq2Δ* + CoQ_2 or *coq2Δ* + CoQ_6), are represented side by side (from low to high degree of CoQ_6 rescue). Five candidates (denoted with red lines) fulfilled the "CoQ_6 rescue < CoQ_2 rescue" criteria and have been identified as involved in CoQ_6 transport. This panel merges data from Figure 5A and Figure S5B to allow for a straightforward comparison. **(B)** The degree of rescue of *vps1Δcoq2Δ* was observed to be dramatically low when compared to the positive control. **(C)** Similar results were observed when the rescue of the *vps1Δcoq2Δ*

mutant was compared to *vps1Δ*. **(D)** The degree of rescue with the addition of exogenous CoQ_2 was superior to the one observed with the addition of CoQ_6. Degree of rescue is referred to the corresponding positive control (*coq2Δ* + CoQ_2 or *coq2Δ* + CoQ_6). In all cases, columns represent the average degree of rescue (in %) ± SD of mutants growing on YPG in presence of CoQ for 7 days. At least three replicas were included in all determinations. The normalization variable is considered 100% and represented as a dashed line. Asterisks denote: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

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Table 1
Yeast gene candidates selected for screening

Group 1	**Genes with diminished response to exogenous CoQ_2 and CoQ_4** [39]	***ZDS1, RTS1, IKI3, ELP4, QCR9, ACO1, MDL2, SAC1, SWF1, ARV1, CDC10, KCS1, EMI1, MET18, RPL31A, HTA1, CSR1, HES1***
Group 2	**Yeast major clathrin-mediated endocytic factors** [53]	***SYP1, EDE1, CHC1, CLC1, SLA2, END3, SLA1, VRP1, BZZ1, MYO3, MYO5, BBC1, RVS161, RVS167, ABP1***
Group 3	**ER-mitochondria encounter structure (ERMES)**	***MMM1, MDM10, MDM12, MDM34***
Group 4	**Nucleous-vacuole junction (NVJ)**	***VAC8, NVJ1***
Group 5	**Vacuole-mitochondria patch (vCLAMP)**	***YPT7, VAM6, VAM7***
Group 6	**Genes previously described to be required for CoQ_6 rescue** [33]	***TLG2, PEP12, ERG2, VPS45***
Group 7	**Nat B complex**	***MDM20, NAT3***

Genes depicted in light grey represent the *ORFΔcoq2Δ* double mutants that were not recoverable for the *ORFΔcoq2Δ* library.

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Table 2
Genes identified to be related to CoQ_6 transport

ORFΔ (protein name)	*ORF* description (www.yeastgenome.org)	Human homolog
YCR002C (Cdc10)	Subunit of the septin complex with GTPase activity. Localizes to the bud neck septin ring, and can function as scaffolds for recruiting cell division factors and as barriers to prevent diffusion of specific proteins.	***SEPT9***
YOR014W (Rts1)	Protein phosphatase regulator involved in protein dephosphorylation, mitotic spindle orientation checkpoint, and septin ring organization and disassembly.	***PPP2R5C***
YCR009C (Rvs161)	N-BAR domain protein that interacts with Rvs167 and regulates polarization of the actin cytoskeleton, endocytosis, cell polarity, cell fusion and viability following starvation or osmotic stress.	***BIN3***
YDR388W (Rvs167)	N-BAR domain protein with roles in endocytic membrane tabulation, constriction and exocytosis. Interacts with Rvs161 to regulate actin cytoskeleton, endocytosis and viability following starvation or osmotic stress.	***BIN1, AMPH*** [57–59]
YPR131C (Nat3)	Catalytic subunit of the NatB N-terminal acetyltransferase complex, which is in charge of the N-acetylation of the amino-terminal methionine residues of all proteins beginning with Met-Asp or Met-Glu and of some proteins beginning with Met-Asn or Met-Met (approximately 20% of proteins in yeast).	***NAA20***
YKR001C (Vps1)	Dynamin-like GTPase required for vacuolar sorting, protein retention in Golgi apparatus, peroxisome organization and fission, endocytosis, and actin cytoskeleton organization.	***Dynamin*** [57, 60]

Unless otherwise is indicated, the closest human homolog genes were located in the *HomoloGene* tool from the NCBI database.