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Coq10, a mitochondrial coenzyme Q binding protein, is required for proper respiration in *Schizosaccharomyces pombe*

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Running title,

CoQ binding protein in fission yeast

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Abbreviations: Coenzyme Q, Q; DM, dodecyl maltoside; Dlp1, D-less polyprenyl diphosphate synthase; Dps1, decaprenyl diphosphate synthase; GFP, green fluorescence protein, PHB, *p*-hydroxybenzoate, PM, Pombe Minimum

Summary

It has been widely accepted that most coenzyme Q (Q) exists freely in the mitochondrial membrane as a Q pool. However, the recent identification of a mitochondrial Q binding protein, termed Coq10, in budding yeast has the potential to change our current view of Q status in membranes. Here, we studied the counterpart of budding yeast Coq10 (also termed Coq10) in fission yeast. Fission yeast *coq10*-null mutants exhibited a similar, albeit less severe, phenotype as Q-deficient fission yeast, including the requirement for antioxidants for proper growth on minimal medium, increased sensitivity to H_2O_2, high levels of H_2S production, and a deficiency in respiration. The *coq10* null mutant produced nearly normal levels of Q10, suggesting that *coq10* does not belong to the group of Q biosynthetic genes. To elucidate the role of Coq10, we expressed recombinant *coq10* in *Escherichia coli*, and found that Q8 was present in purified recombinant Coq10. Mutational analysis of 13 conserved residues of Coq10 revealed that two hydrophobic amino acid residues, leucine 63 (L63) and tryptophan 104 (W104) play an important role in Coq10 binding to Q. An L63A/W104A double mutant of Coq10 exhibited lower Q-binding activity than either of the single mutants, and was unable to complement the *coq10* deletion in fission yeast. In light of the observation that a human Coq10 ortholog was able to functionally compensate for the absence of *coq10* in fission yeast, our results suggest that the role of Coq10 is important for proper respiration in a variety of organisms.

Introduction

Coenzyme Q (Q or ubiquinone) is an essential electron carrier in the respiratory chain of eukaryotic cells and most prokaryotes. Biosynthesis of Q occurs in the mitochondria in eukaryotes and in cytosolic membranes in prokaryotes. The biosynthetic pathway of Q has been elucidated primarily through genetic analysis of *Escherichia coli* and *Saccharomyces cerevisiae* mutants [1-3]. Q synthesis is initiated with the synthesis of polyisoprenyl diphosphate and the subsequent combination of polyisoprenyl diphosphate with *p*-hydroxybenzoate. The benzoquinone ring is then multiply modified to generate Q. To date, nine genes (*coq1-coq9*) have been identified that are involved in Q biosynthesis in *S. cerevisiae*, and orthologous genes in *Schizosaccharomyces pombe* [2, 4-7], *Caenorhabditis elegans* [8, 9] and humans [10-12] have been partly characterized. It has been suggested that Coq polypeptides form a complex in the mitochondria in *S. cerevisiae*, and complexes that lack any of the components are unstable [13, 14]. This is one reason why the functions of some of the components are as yet unknown.

The fission yeast *S. pombe* is a well-studied model organism. It is easy to handle, and many essential genes are conserved in fission yeast and mammals. *S. pombe* produces coenzyme Q10, a natural Q species that is also found in humans, and is thus viewed as a potential tool for the commercial production of Q10. Coenzyme Q10 is used world-wide as a food supplement, and increasing the production of Q10 through genetic engineering in a variety of hosts has been a strong focus in the field of applied biology [15-18]. There are some differences between the Q biosynthetic pathways of *S. pombe* and *S. cerevisiae*. First, polyisoprenyl synthase in *S. pombe* is a heterotetramer composed of two subunits, Dps1 and Dlp1 [7], similar to mouse and human [19], but different from the homodimeric (or homotetrameric) structure in *S. cerevisiae* [20]. Second, the disruption of certain Q synthetic genes in *S. pombe* results in the production of

different intermediates (our unpublished data) than those found in *S. cerevisiae*, in which the specific early intermediate 3-hexaprenyl-4-hydroxybenzoic acid (HHB) accumulates [21]. Third, the role of Q in some biological processes differs between *S. pombe* and *S. cerevisiae*. For example, Q reduction is coupled to sulfide oxidation in *S. pombe* [6, 22, 23], but not in *S. cerevisiae*. These results have prompted our interest in exploring the multiple functions of Q in *S. pombe*.

It is generally accepted that most Q (nearly 90%) is distributed freely in membranes as a Q pool [24]. However, Barros, M.H. et al [25] recently suggested that Coq10, a Q-binding protein that does not belong to succinate- and NADH-Q reductase nor the bc_1 complex in *S. cerevisiae*, might be involved in the transport of Q from its synthetic site to its functional site. These results not only strengthen our knowledge of the regulation of Q, but also challenge the current model that has existed for a long time that Q is a free lipid molecule in membranes. These findings prompted us to examine Coq10 function in other organisms to gain further insight into this important protein. Here, we have carried out a functional characterization of Coq10 in *S. pombe*. Our results indicate that *S. pombe* Coq10 is a Q binding protein, and is required for the proper function of Q in several processes. We propose that Coq10 is a universal binding protein of Q.

Results

Cloning of *coq10* and construction of *S. pombe* Δ*coq10* mutants

We identified an orthologous gene of *S. cerevisiae COQ10* in the *S. pombe* genome through the GeneDB hosted by the Sanger Institute [http://www.genedb.org/]. The predicted amino sequence of the gene (SPCC16A11.07) exhibited medium sequence identity (21%) with Coq10 of *S. cerevisiae*, and we designated it as *S. pombe* Coq10. We searched the National Center for Biotechnology Information (NCBI) database [http://www.ncbi.nlm.nih.gov/] and found that orthologous *coq10* genes are

widely distributed among a broad range of species and *S. pombe* Coq10 is the smallest in size (164 a. a.) (Fig. 1A). However, to date, only *coq10* of *S. cerevisiae* has been characterized [25].

To analyze the function of *S. pombe coq10*, we amplified *coq10* sequences from the *S. pombe* genome by PCR using specific primers (Table2), and used the amplified sequences to construct a deletion mutant strain, *S. pombe* Δ*coq10*, in which *coq10* was replaced by the *kanMX6* module. The strain resistance to G418 and analysis of the deletion using PCR and specific primers confirmed that *coq10* was successfully deleted.

Q production in Δ*coq10* cells

To investigate the role of *coq10* in Q biosynthesis, we compared the Q levels of Δ*coq10* and wild type strains. The levels of Q10 in *S. pombe* Δ*coq10* were comparable to the wild type strain (Fig. 2), which suggested that *coq10* is not required for Q biosynthesis in *S. pombe*. This result was consistent with a previous report in which the production of Q6 was nearly normal in a *coq10* deletion mutant of *S. cerevisiae* [25].

Phenotype of *S. pombe Δcoq10*

The growth of *S. pombe* Q-deficient mutants on minimal medium is slower than wild type cells, and the addition of an antioxidant, such as glutathione or cysteine, can overcome this growth defect [5, 6]. Growth on medium that contains a non-fermentable carbon source is minimally detected [5, 6]. Similar to the Q-deficient mutant Δ*coq8,* Δ*coq10* exhibited growth retardation on (PM)-based minimal medium, although to a lesser extent than Δ*coq8* cells (Fig. 3A). The addition of cysteine to the medium improved the growth of both strains (Fig. 3B). When we added glutathione to minimal medium, the growth of both Δ*coq10* and Δ*coq8* strains recovered, which confirmed the antioxidant-dependent phenotype of these

strains (Fig. 3C). These results suggested that the lack of Coq10 in Δ*coq10* mutants results in the impaired function of Q10 as compared to wild type cells. In support of this, Δ*coq10* mutants displayed an intermediate sensitivity to Cu^{2+} and H_2O_2 as compared to wild type and Δ*coq8* mutants (Fig. 4). However, unlike Δ*coq8* [6] and other Q-deficient mutants, which are highly sensitive to both Cu^{2+} and H_2O_2 (Fig. 4), Δ*coq10* exhibited a sharp sensitivity to H_2O_2, but not to Cu^{2+}, similar to the respiration mutant Δ*cyc1* (cytochrome *c*) [26]. This difference may help to distinguish Q-deficient mutants from respiration mutants. *S. pombe* possesses a Q sulfide reductase, heavy metal tolerance protein 2 (Hmt2), which links Q reduction and sulfide oxidation [23]. Due to the presence of this enzyme, Q-deficient mutants accumulate abnormally high amounts of sulfide. The level of sulfide in Δ*coq10* mutants was much higher (21nM/OD_{600}) than wild type cells (2nM/OD_{600}), although lower than Δ*coq8* cells (35nM/OD_{600}). These results confirmed that *coq10* is required for functional Q.

Since Q plays an essential role in respiration, we analyzed the respiration competency of Δ*coq10* mutants by measuring oxygen consumption (Fig. 5). The level of O_2 uptake by Δ*coq10* mutants was similar to Δ*cyc1* and Δ*dps1* (decarprenyl diphosphate synthase) mutants, which indicated that *coq10* is crucial for respiration. Taken together, these results suggested that *coq10* is required for multiple functions of Q, and that the role of *coq10* in Q-dependent biological processes is distinct from the one of other Q biosynthetic genes. The phenotype of the Δ*coq10* mutants is closer to that of respiration deficient mutants of *S. pombe* [26].

Identification of key functional amino acids in Coq10.

To identify the amino residues of Coq10 that might be involved in the binding of Coq10 to Q, we first identified 13 highly conserved hydrophobic amino acids by sequence alignment of Coq10 orthologs (Fig. 1A). We then constructed

individual alanine substitution mutants of the thirteen amino acids. Among the single mutants, the expression of L63A and W104A *coq10* mutants in a Δc*oq10* background resulted in defective respiration (Fig. 5) and growth retardation on minimal media as compared to wild type cells (Fig. 6A). The single L63A and W104A mutants and the double mutant exhibited other defects as well, similar to the phenotype of Δ*coq10* mutants, including the requirement for antioxidants for proper growth on minimal medium (Fig. 6A), and increased sulfide production (Fig. 6B). Thus, the L63A/W104A double mutant was nearly non-functional in the background of a *coq10* deletion mutant.

Complementation of the Δ*coq10* mutation by human *coq10*.

We searched the NCBI database and found that *coq10* is widely distributed in many organisms. To investigate the conservation of Coq10 function between fission yeast and humans, we constructed an expression plasmid for human *coq10A* (Note, there is a spliced variant termed *coq10B* in human) (Fig. 1). The recovery of growth of *Hucoq10A* transformants to the level of wild type cells suggested that *Hucoq10A* fully complements the *S. pombe* Δ*coq10* phenotype (Fig. 7). These results, together with the complementation data from *S. cerevisiae*, indicated that functional homologs of Coq10 are present in yeast and humans.

Coq10 localizes to mitochondria in *S. pombe*

Q synthetic proteins and Coq10 have been shown to localize to the mitochondria in *S. cerevisiae* [14]. To further investigate the role of Coq10 in respiration in fission yeast, we constructed an expression plasmid for a green fluorescent protein (GFP) fusion protein of Coq10 (Coq10-GFP), and analyzed the localization of Coq10-GFP in Δ*coq10* mutants. The expression of *Coq10-GFP* in *S. pombe* was confirmed by Western blot (Fig. 8A). The expression of Coq10-GFP complemented the phenotype of Δ*coq10* mutants (data not shown), which

indicated that that Coq10-GFP is functional in these cells. When we observed the cells by fluorescence microscopy, Coq10-GFP co-distributed with Mitotracker Red, a mitochondrial marker (Fig. 8B), indicating that Coq10 localizes to the mitochondria in *S. pombe*.

Expression, purification and analysis of recombinant Coq10 from *E. coli*

For the analysis of purified Coq10, we constructed a histidine (6xHis) fusion protein of Coq10 (*6xHis-Coq10*) and expressed it in *E. coli*. The addition of IPTG to transformed cells resulted in the expression of a 20 KDa protein (Fig. 9A). *E. coli* that expressed *coq10* showed a slightly faster growth rate as compared to wild type cells under low rotation speed (data not shown), which suggested that Coq10 functions in some way to assist the growth of *E. coli*. We were unable to purify 6xHis-Coq10 by affinity chromatography, which indicated that most of the fusion protein formed inclusion bodies, or was integrated into cell membranes. We then screened several detergents to try and purify the fusion protein, including Triton X-100, Tween-20, Chaps, NP-40 and dodecyl maltoside (DM). DM was the only detergent that enabled effective recovery of fusion protein (data not shown). While it is possible that DM decreased the specific binding of the fusion protein to Ni-NTA agarose, we were able to resolve the Coq10 fusion protein by SDS-PAGE as a single band (Fig. 9A). Purified Coq10 bound to Q8, a native *E. coli* Q species, as well as Q6, which was used as an internal control, whereas only Q6 was detected in purified samples from cells that expressed the control empty vector (Fig. 9B). Q6 was added in both samples just before extraction of Q as an internal control to calculate the quantity of Q8. The ratio of bound Q8 to Coq10 was 0.81 mol/mol, which is close to a 1:1 ratio, under our experimental condition (Fig. 9). These results confirmed that *S. pombe* Coq10 is a Q-binding protein and probably it binds to CoQ in the 1:1 ratio in nature.

We then tested the Q binding ability of mutated Coq10s, which were described

in Figs 5 and 6. We expressed each of the single mutants of L63A and W104A, as well as an L63A/W104A double mutant, as 6xHis-Coq10 fusion proteins in *E. coli*, and measured the amount of Q associated with the purified proteins. The L63A and W104A single mutants exhibited decreased binding to Q, to 60% and 43%, respectively, of that bound by wild type Coq10. Binding to Q was further decreased in the L63A/W104A double mutant, to 30% of wild type protein. These results indicated that L63 and W104 are critical residues in the binding of Q by Coq10 (Fig. 9), and are important for the functions of Coq10 as shown in Figs. 5 and 6.

Discussion

In the current study, we carried out a functional characterization of *S. pombe* Coq10, a Q-binding protein in yeast. Q was detected in complex with purified Coq10, consistent with the idea that *S. pombe* Coq10 is a Q-binding protein, similar to its role in *S. cerevisiae*. Disruption of *coq10* in *S. pombe* did not appear to affect Q10 levels in cells (Fig. 2), which ruled out a role of *coq10* in Q biosynthesis. However, Δ*coq10* mutants displayed a typical *S. pombe coq* mutant phenotype, including the requirement for antioxidants for growth on minimal medium (Fig. 3), increased sulfide accumulation, sensitivity to H_2O_2, and defects in respiration (Fig. 5). The underlying mechanisms of these phenotypes most likely do not involve the minor decrease in Q10 levels in Δ*coq10* mutants, because very low levels of Q were able to restore the growth of Δ*dps1* mutants that expressed orthologous genes from other organisms [19, 27]. Alternatively, the deletion of *coq10* may decrease the functional activity of Q, resulting in a Q-deficient phenotype. Q is not only an antioxidant, but also a pre-oxidant. Q becomes toxic in cells if the oxidized form of Q accumulates, thus Q-binding proteins must be needed to protect cells from damage due to this pre-oxidant property of Q. Our results suggest that Coq10 is a universal component in the

regulation of Q function. In other words, Q function in membranes is a protein-mediated process.

The over-expression of *COQ2*, *COQ7* or *COQ8* partially complements the Δ*COQ10* mutation in *S. cerevisiae*, and this is accompanied by higher levels of Q synthesis [25]. Because the overexpression of *ppt1(coq2)*, *coq7* or *coq8* in *S. pombe* is inhibitory for cell growth (our observation), it was difficult to assess the effect of these genes in the *Δcoq10* mutant background. When we grew *S. pombe* Δ*coq10* on Q10-containing minimal media, growth was restored (data not shown), indicating that higher amounts of Q10 can compensate for some of the defects in Δ*coq10* mutants, and that *coq10* might not be crucial for some functions of Q.

Through the use of a detergent-based purification scheme, we were able to purify sufficient amounts of recombinant Coq10 from *E. coli* for protein analysis. The binding ratio of purified Coq10 to Q was 0.81 mol Q/mol protein, which suggests the natural ratio of Coq10 to the bound Q is one to one. While the use of a detergent in the purification process most likely affected the ability of Coq10 to bind to Q, the Q-binding properties of wild type and mutant Coq10 could be distinguished. In addition, this ratio is an improvement over the reported binding of *S. cerevisiae* Coq10 to Q, in which the ratio of Coq10 bound to Q6 was 0.032-0.034 mol Q/mol protein. We also tried to express Coq10 in *S. pombe* in order to analyze the binding of purified Coq10 to Q10. However the over-expression of *coq10* inhibited the growth of *S. pombe*, and the yield of purified Coq10 was very low, making it difficult to assess the Q binding properties of Coq10 from *S. pombe*. Nevertheless we detected Q10 from partially purified Coq10 from *S. pombe,* but it was too low to discuss on stoichiometry.

We identified two amino acids of Coq10 that were important for Coq10 function. Mutation of L63 and W104 abolished the ability of Coq10 to complement the *coq10* deletion mutation, and lowered, but did not completely inhibit, the Q-binding activity of Coq10. The components of the electron transfer system

NADH-ubiquinone reductase [28], succinate-ubiquinone reductase [29] and ubiquione-cytochrome *c* reductase [30, 31] have been reported to have Q-binding subunits. Those are necessary for the electron transfer mediated by Q in each reaction. Other types of Q-binding proteins have also been identified in bacterial Gdh [32] and RegB [33], and recently in human urinal saposin B [34]. However, the alignment of all those Q binding proteins with Coq10 did not reveal any clear conserved amino acids motifs. Coq10 has been categorized in Pfam PF03364 [http://pfam.sanger.ac.uk/) as an enzyme involved in polyketide synthesis, and as a lipid binding and transport protein. Based on sequence alignment, there appears to be many eukaryotic orthologs of Coq10, as well as orthologous proteins in bacteria whose functions are not known.

What is the real function of Coq10? Because Coq10 is not included in any complex of the electron transfer system in *S. cerevisiae,* it was proposed that Coq10 may be function in transporting Q from its site of synthesis to the catalytic sites of the respiratory chain complexes or may play as a shuttle between NADH- and succinate-Q reductase complexes to the bc_1 complex [25]. Our results also support the possibility of Coq10 as a transporter or a shuttle of Q. In ether case, Coq10 is required for proper function or localization of Q under the physiological condition, because an excess amount of Q compensates the loss of function of Coq10 in both budding yeast [25] and fission yeast (our result). We do not know yet whether any preference of Coq10 to the binding of either oxidized or reduced form of Q occur or not. If there is a preference of either form of Q in binding with Coq10, transport of Coq10 mediated process to the complex must be in one direction. It is theoretically reasonable to think Coq10 mediates the process of transferring Q than to think Q freely moves from one complex to the other complex in the electron transport system.

Human coenzyme Q deficiency leads to neural and muscular diseases, and patients possessing a genetic disorder in Q biosynthetic genes have been

cumulatively discovered [10, 11, 35]. The characterization of *S. pombe coq10* provides new phenotypic characteristics for analysis, and we have shown that *coq10* function is conserved between yeasts and human (Fig. 6). Thus, our results should help identify and characterize medical cases related to genetic deficiencies of *coq10.*

In conclusion, Coq10 is a conserved Q-binding protein that is essential for proper function of the electron transfer system, possibly by assisting in the transfer of Q from one site to another in the mitochondrial membranes of eukaryotes.

Experimental procedures

Materials

Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Bio Inc. (Kyoto, Japan) and New England Biolab Japan, Inc. (Tokyo, Japan). *n*-Dodecyl ß-D-maltoside was purchased from Sigma Chemical Co. (St Louis, MO, USA). Ni-NTA agarose was purchased from QIAGEN Inc. (Tokyo, Japan). The human *coq10A* cDNA clone was purchased from Invitrogen Corp. (Tokyo, Japan).

Strains, plasmids and media

E. coli strain DH5α and the vectors pBluescript II SK+/-, pT7Blue-T (Novagen, Darmstadt, Germany), pET28c (Novagen), pREP1 [36], pREP81 and pSLF272 [37] were used for the construction of plasmids. The *S. pombe* strains used in this study are listed in Table 1. Yeast were grown in YE (0.5% yeast extracts, 3% glucose) or PM minimal medium with appropriate supplements, as previously described [38, 39]. The concentration of supplemented amino acids (adenine and uracil) was 75μg/ml.

DNA manipulations

Cloning, restriction enzyme analysis and the preparation of DNA plasmids were performed as described previously [40]. PCR was carried out as previously described [41]. Nucleotide sequences were determined using the dideoxynucleotide chain-termination method and an ABI377 DNA sequencer. The primers used in this study are shown in Table 2. For the expression of *coq10* in S. *pombe* and *E. coli*, two oligonucleotides, coq10-N and coq10-C, were used to amplify a full-length *coq10* cDNA from the *S. pombe* genome. The resulting *coq10* sequence was cloned into pT7Blue-T to generate pT7-coq10. An *Nde* I - *Bam* HI fragment containing *coq10* was subcloned into the corresponding sites of pREP1, pREP81 and pET28c to generate pREP1-coq10, pREP81-Scoq10 and pET28c-coq10, respectively (Fig. 1B). For the expression of *coq10-GFP*, two primers, coq10-Ntag and coq10-Ctag, were used to amplify the *coq10* cDNA, and then the amplified fragment was cloned into the *Xho* I and *Bgl* II sites of pSLF272a to generate pSLF272a-coq10. To construct pREP1-Hucoq10A, two oligonucleotides, Hucoq10-N and Hucoq10-C, were used to amplify the predicted *Hucoq10A* open reading frame (ORF) from a human *coq10A* cDNA clone. The resulting fragment was cloned into the *Sal* I and *Bam* HI sites of pREP1 to generate pREP1-Hucoq10A (Fig. 1B).

Gene disruption

PCR-based gene targeting was performed as previously described [42, 43]. Briefly, two pairs of primers, coq10-A and coq10-B, and coq10-C and coq10-D, were used to amplify the upstream and downstream regions of *coq10*. The amplified *coq10* fragments were used as long primers to amplify a *kanMX6* module with flanking *coq10* homologous sequences. The amplified fragment was introduced into *S. pombe* cells by homologous recombination and cells were screened by G418

(Sigma, Chemical Co., St Louis, MO, USA) resistance. A pair of checking primers, nb2 and coq10-check, was used to confirm that the chromosomal *coq10* gene was replaced. The resulting deletion mutant was termed CZ48 (Table 1).

Extraction and measurement of Q from *S. pombe*

Q was extracted as described previously [44]. Briefly, crude lipid extracts were analyzed by normal phase thin layer chromatography (TLC) with authentic coenzyme Q10 as the standard. Normal-phase TLC was carried out on a Kieselgel 60 F_{254} plate with benzene/acetone (97:3 v/v). The band containing coenzyme Q10 was collected from the TLC plate following UV visualization and extracted with chloroform/methanol (1:1 v/v). The samples were dried and resolved in ethanol. Purified Q was further analyzed by HPLC with ethanol as the solvent.

Measurement of extracellular sulfide

Hydrogen sulfide was measured as the production of PbS from lead acetate. A quantitative determination of sulfide was performed by the methylene blue method, with minor alterations. Briefly, *S. pombe* was grown in YES or PMU medium (50ml) to late log phase. Cultures (0.5 ml) were mixed with 0.1 ml of 0.1% dimethylphenylenediamine (in 5.5 N HCl) and 0.1 ml of 23 mM $FeCl_3$ (in 1.2 N HCl). The samples were incubated at 37°**C** for 5 minutes (min). Following centrifugation at 13,000 rpm for 5 min, the supernatant was removed, and absorbance at 670 nm was measured using a blank consisting of reagents alone.

Mitochondrial staining and fluorescence microscopy

Mitochondria were stained using the mitochondria-specific dye MitoTracker Red FM (Invitrogen). Cells were suspended in 10 mM HEPES (pH7.4) containing 5% glucose, and then MitoTracker Red FM was added to a final concentration of 50 nM. After standing for 15 min at room temperature, cells were visualized by

fluorescence microscopy using a BX51 microscope (Olympus, Tokyo, Japan) (1,000 x magnification). Fluorescence of GFPS65A was observed at an excitation wavelength of 485 nm. Fluorescence images were obtained using a digital camera (DP70, Olympus) connected to the microscope.

Purification of recombinant 6xHis-Coq10 fusion protein

An overnight culture (10 ml) of *E. coli* BL21(DE3)pLysS harboring pET28c-coq10 was used to inoculate a 500 ml culture of peptone-phosphate enriched medium containing 10 mM Mg^{2+}, 440 mM sorbitol, 2.5 mM betaine, 50 μg/ml kanamycin and 36 μg/ml chloromycetin. Cultures were incubated at 37˚C with vigorous shaking until the optical density (OD_{660nm}) reached 0.6. The culture was cooled to 25˚C and isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Growth was allowed to continue for 3 hours at 25˚C and then the cells were collected by centrifugation at 6,000 g for 10 min. The cell pellets were stored at -20˚C until use. Cell paste was suspended in 5 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Protease inhibitor cocktail (EDTA free) (Roche Corp., Basel Switzerland) was added to the cell suspension, according to the manufacturer's instructions, and then the cells were disrupted by sonication (Cosmo Bio, Co. Ltd., Tokyo, Japan) for 8 min with 50% duty cycles. After sonication, unbroken cells and inclusion bodies were removed by centrifugation at 12,000 g for 20 min, and the supernatant was centrifuged at 120,000 g for 90 min. The pellets (membrane fraction) were suspended at 5 mg of protein/ml in lysis buffer. After the addition of DM at a final concentration of 0.4%, the sample was incubated for 30 min with slow stirring and centrifuged at 120,000 g for 90 min. The supernatant was removed and mixed with an equal volume of pretreated Ni-NTA agarose. The gel mixture was gently shaken for 2 h at 4˚C and then packed into a column. The beads were washed using 3x10 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH

8.0) containing 20-40 mM imidazole. The 6xHis-Coq10 fusion protein was eluted from the column using 5 x 0.5 column volumes of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The Coq10 mutants were purified using the same procedure.

Extraction and measurement of Q from purified proteins

The amount of Q bound to purified Coq10 was determined according to the method of Yamashita *et al* [28], with some modifications. Briefly, 0.25 μg of Q6 were added to purified protein as an internal control. The mixture was treated with a 5-fold volume of methanol and incubated at 70˚C for 10 min with frequent agitation. Denatured protein was removed by centrifugation at 13,000 rpm for 3 min. The resulting supernatant was mixed with one-fold hexane. The upper phase was dried and resolved in 100 μl ethanol for HPLC analysis.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technol. Inc., CA, USA), according to the manufacturer's instructions. Briefly, for the construction of the single mutants, pT7-coq10 was used as the template for the PCR reactions. The amplified PCR products were self-ligated in *E. coli* and the sequences of the *coq10* mutants were confirmed. The plasmids were digested with *Nde*I and *Bam*HI and the resultant fragments were subcloned into the corresponding sites of pREP81 and pET28c to generate the mutant *coq10* expression plasmids for *S. pombe* and *E. coli*, respectively. pT7-coq10^{L63A} was used as the template instead of pT7-coq10 to construct the double mutant, *coq10* $^{L63A\text{-}W104A}$. The sequences of all the substitution mutants were confirmed by sequence analysis.

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Figure legends

Fig. 1. (A) Alignment of Coq10 homologs from *Homo sapiens* (H. s.) Coq10A, *Xenopus laevis* (X. l.) Coq10, *Drospohila melanogaster* (D. m.) Coq10A, *Arabidopsis thaliana* (A. t.) Coq10 (AT4G17650), *S. cerevisiae* (S. c.) Coq10, and *S. pombe* (S. p.) Coq10. Identical amino acids are highlighted in black. Asterisks indicate substituted amino acids. (B) Schematic representation of the plasmids used in this study. pT7-coq10, pREP1-coq10 and pREP81-coq10 contained full length *coq10*. pET28c-coq10 contained full length *coq10* fused to 6xHis at the N-terminus. pSLF272a-coq10 contained full length *coq10* fused to GFP at the C-terminus. pREP1-Hucoq10A contained the putative ORF of *Hucoq10A*. Among the *nmt1*, *nmt1**, and *nmt1*** promoters, *nmt1* is the strongest and *nmt1*** is the weakest. Abbreviations: N, *Nde* I; Ba, *Bam* HI; X, *Xho* I; B, *Bgl* II.

Fig. 2. Coenzyme Q10 levels in *S. pombe*. Cultures (100 ml) in mid-log phase of wild type (PR110), Δ*coq10* (CZ48) and Δ*coq8* (NBp17) strains were subjected to extraction for the analysis of Q levels. Q was first separated by TLC, and then 1:20 of each extract was further analyzed by HPLC.

Fig. 3. Growth recovery of Δ*coq10* mutants on minimal media by the addition of antioxidants. Wild type, Δ*coq10* and Δ*coq8* strains were grown on (A) PM medium supplemented with 75 μg/ml leucine and uracil, or (B) PM medium supplemented with leucine, uracil and 200 μg/ml cysteine. (C) The indicated strains were grown as described for (B), with the exception that glutathione was used instead of cysteine. All strains were grown for 2 days at 30˚C.

Fig. 4. Sensitivity of Δ*coq10* mutants to oxygen radical producers.

Wild type, Δ*coq10* and Δ*coq8* strains were pre-grown, and then placed in fresh YES media with 2 mM Cu^{2+}, 1.5 mM H_2O_2, or nothing. Cell number was counted at 4 h intervals.

Fig. 5. *coq10* is required for respiration in *S. pombe*.

Oxygen consumption was measured in cells taken from YES cultures at mid-log phase. Cells were washed twice with phosphate buffered saline (PBS), and then 1 x 10^8 cells were resuspended in 100 ul of PBS containing 2% glucose. Analysis was carried out using a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Inc.). Oxygen consumption was measured as the loss of oxygen in 3 ml of PBS. For the analysis of the point mutants of Δ*coq10*, *S. pombe* Δ*coq10* was transformed with multi-copy expression plasmids for $coq10^{L63A}$, $coq10^{W104A}$ or $coq10^{L63A\text{-}W104}$ (pREP81). The data represents the averages of three measurements.

Fig.6. L63 and W104 are required for the function of Coq10. (A) Wild type cells harboring pREP81, or Δ*coq10* mutants harboring pREP81-coq10, pREP81-$coq10^{L63A}$, pREP81-$coq10^{W104A}$, pREP81-$coq10^{L63A\text{-}W104A}$ or pREP81, as indicated, were spotted onto PMU minimal medium with or without glutathione. The cells were incubated at 30˚C for 3 days and growth was observed. (B) Cells were grown on PMU medium and extracellular sulfide was measured as described in 'Experimental procedures'. The strains are as described for (A). The data represents the averages of three measurements.

Fig. 7. Complementation of Δ*coq10* by *Hucoq10A*. Wild type cells harboring pREP1, or Δ*coq10* mutants harboring pREP81-coq10, pREP1-Hucoq10A and pREP1, as indicated, were spotted onto PMU minimal medium. The cells were incubated at 30˚C for 3 days and growth was observed.

Fig. 8. Co-localization of Coq10-GFP with a mitochondria-specific dye. (A) The expression of Coq10-GFP was confirmed by Western blot analysis using an anti-GFP antibody (Roche). (B) Phase contrast image of Δ*coq10* mutant cells expressing Coq10-GFP (Phase), and fluorescence images of the same cells showing the location of Coq10-GFP (GFP), and mitochondrial staining by Mito-Tracker red (MitoTracker).

Fig. 9. L63 and W104 of Coq10 contribute to Q-binding. (A) 12.5% SDS-PAGE analysis of isolated recombinant Coq10. *E. coli* BL21(DE3)pLysS harboring pET28c-coq10 were induced by the addition of IPTG (0.5 mM) at 25˚C for 3 h (lane 2). The addition of DM resulted in the purification of soluble 6xHis-Coq10 (lane 3). M, protein marker; Lane 1, crude lysate before induction; Lane 2, crude lysate after induction; Lane 3, purified 6xHis-Coq10. (B) Coq10 binds to Q8. Purified His-Coq10 was used for the extraction of Q. The extracts were analyzed by HPLC (Shimadzu Co., Japan) at a flow rate at 1 ml/min. As a negative control, *E. coli* BL21(DE3)pLysS harboring pET28c (control empty vector) were analyzed in parallel. To calculate the binding ratio of Coq10 to Q, authentic Q6 as an internal control was mixed with purified protein before the extraction of Q8. (C) L63 and W104 are involved in the binding of Coq10 to Q. Wild type and the indicated point mutants of Coq10 were purified from *E. coli* BL21(DE3)pLysS using 0.4% DM. Bound Q8 was analyzed by HPLC using authentic Q6 as the internal control. The data represents the averages of three measurements.

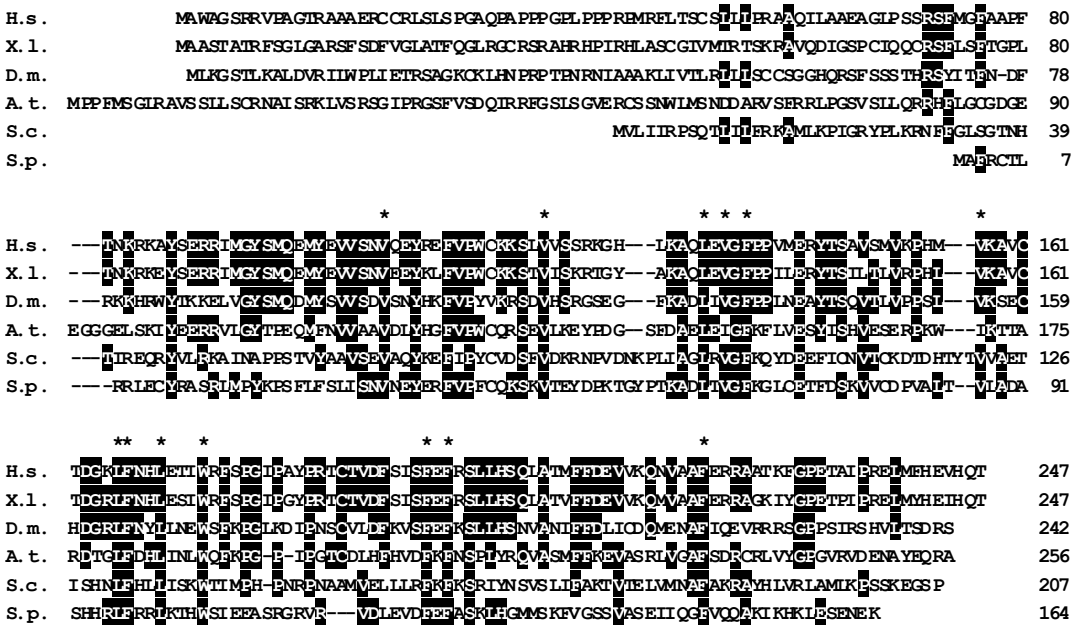
Table 1. Fission yeast strains used in this study.

Strain	Genotype	Source
PR110	*h*$^{+}$*, leu1-32, ura-D18*	Lab. stock
CZ48	*h*$^{+}$*, leu1-32, ura-D18, coq10::kanMX6*	This study
NBp17	*h*$^{-}$*, leu1-32, ura-D18, abc1Sp::LEU2*	[6]
KS10	*h*$^{+}$*, leu1-32, ade-M216, ura-D18, dps1::ura4*	[4]
RM3	*h*$^{+}$*, leu1-32, ura-D18, cyc1::kanMX6*	Lab. stock

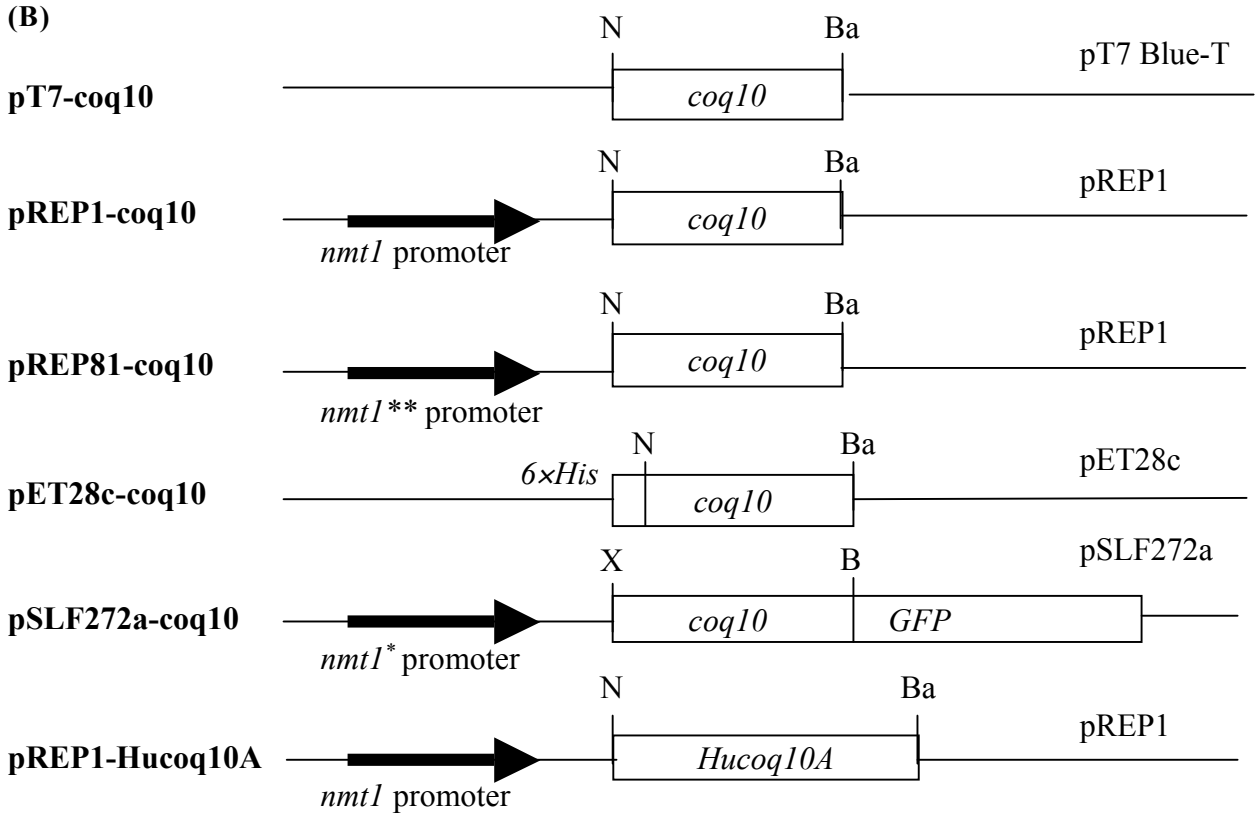
Table 2. Primers used in this study. Primers are shown from 5' to 3'. For mutagenesis, only the forward primers are shown and the substituted codons are underlined.

Name	**Sequence**	**(Created sites)**
coq10-A	CGTTGCTCAGGAGAAGAG	
coq10-B	GGGGATCCGTCGACCTGCAGCGTACGAACATCGAAAAGCCATGAG	
Coq10-C	GGTTATCAGTGACTCAAG	
Coq10-D	GTTTAAACGAGCTCGAATTCATCGATGCTAGAATCA GAAAATGAG	
nb2	GTTTAAACGAGCTCGAATTC	
Coq10-check	CAGTTGACCTCCAAGGACTC	
Coq10-N	GGCATATGGCTTTTCGATGTACG	Nde I
Coq10-C	AAGGATCCTTATTTCTCATTTTCTG	BamH I
Coq10-Ntag	CTCGAGCATGGCTTTTCGATGTA	Xho I
Coq10-Ctag	CGAGATCTCCTTTCTCATTTTCTG	Bgl II
HuCOQ10-N	GTCGACATGAGGTTTCTGACC	Sal I
HuCOQ10-C	GGATCCTCAAGTCTGGTGCAC	Bam I
V33A	GTTCAGTTTAATAAGCAATGCCAACGAATACGAACGGTTTG	
V48A	GTCAAAAGTCCAAAGCCACTGAATACGATCC	
L63A	ATCCTACAAAGGCCGACGCTACAGTTGGTTTTAAAG	
V65A	CCGACCTTACAGCTGGTTTTAAAGG	
F67A	CTTACAGTTGGTGCTAAAGGATTGTG	
V87A	GCCCTAACCGCGTTGGCAGAC	
L96A	CCATCATCGTGCGTTCCGGCGG	
F97A	CGTCCCATCATCGTCTGGCCCGGCGGCTTAAAACAC	
L100A	CTGTTCCGGCGGGCTAAAACACATTG	
W104A	GGCTTAAAACACATGCGAGTATTGAAGAGG	
F122A	GTGTTGATTTAGAGGTGGATGCTGAATTTGCAAGCAAACTCC	
F124A	GGTGGATTTTGAAGCTGCAAGCAAACTC	
F148A	CATTCAAGGAGCCGTCCAACAG	

(A)


H.s.
X.l.
D.m.
A.t.
S.c.
S.p.

(B)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N
Ba
coq10
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nmt1 promoter
pREP1
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nmt1** promoter
pREP1
pET28c-coq10
6×His
pET28c
pSLF272a-coq10
X
B
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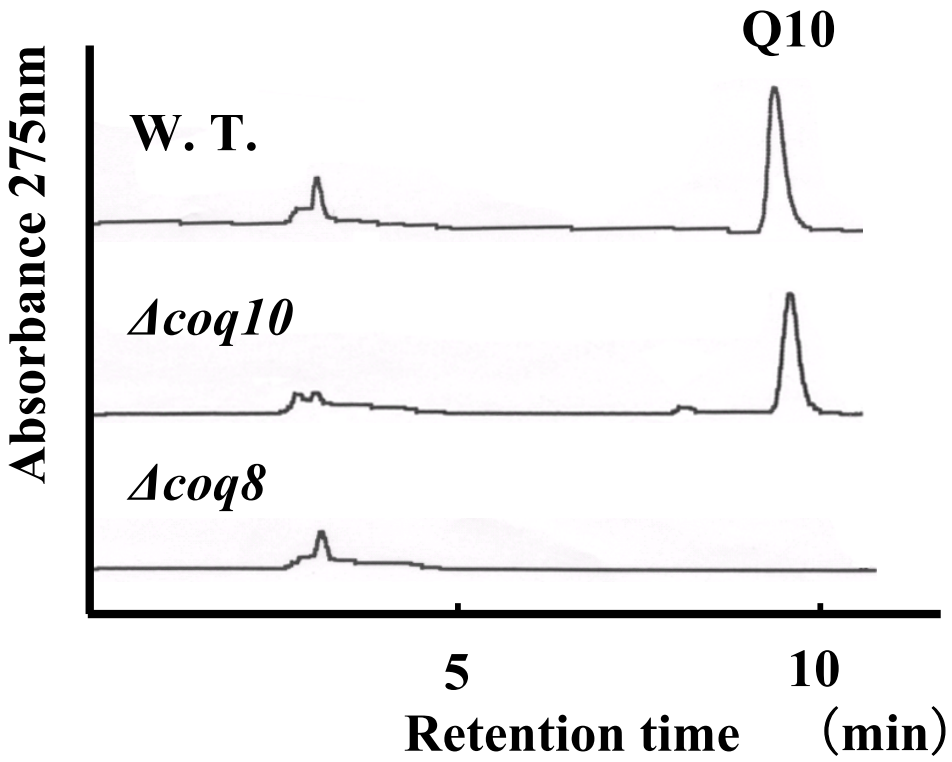
Hucoq10A
pREP1
nmt1 promoter

Fig. 1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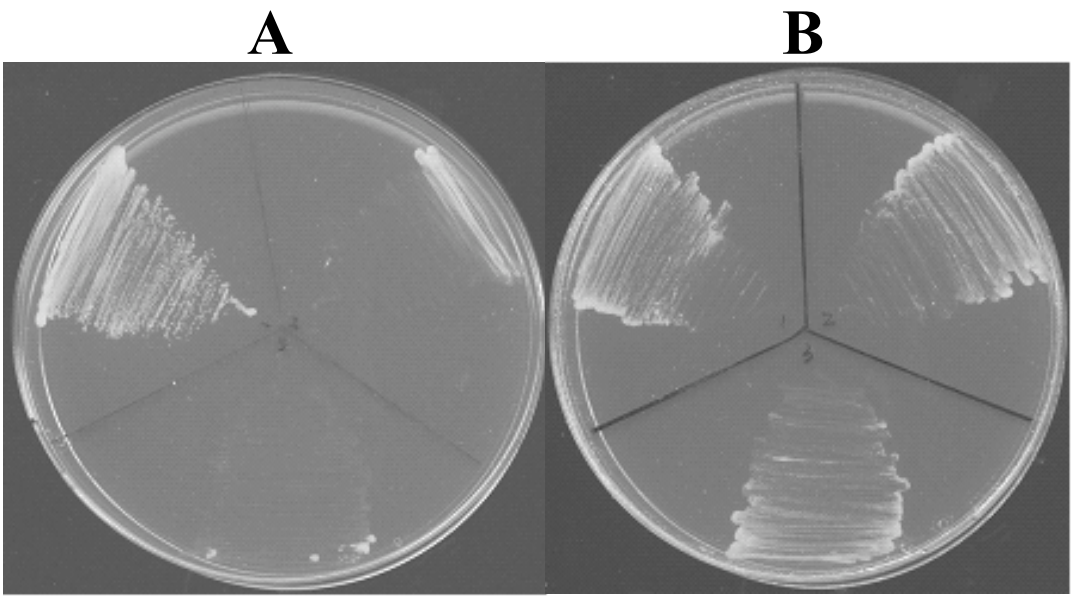
W. T.
Δcoq10
Δcoq8
Absorbance 275nm
5
10
Retention time （min）

Fig. 2



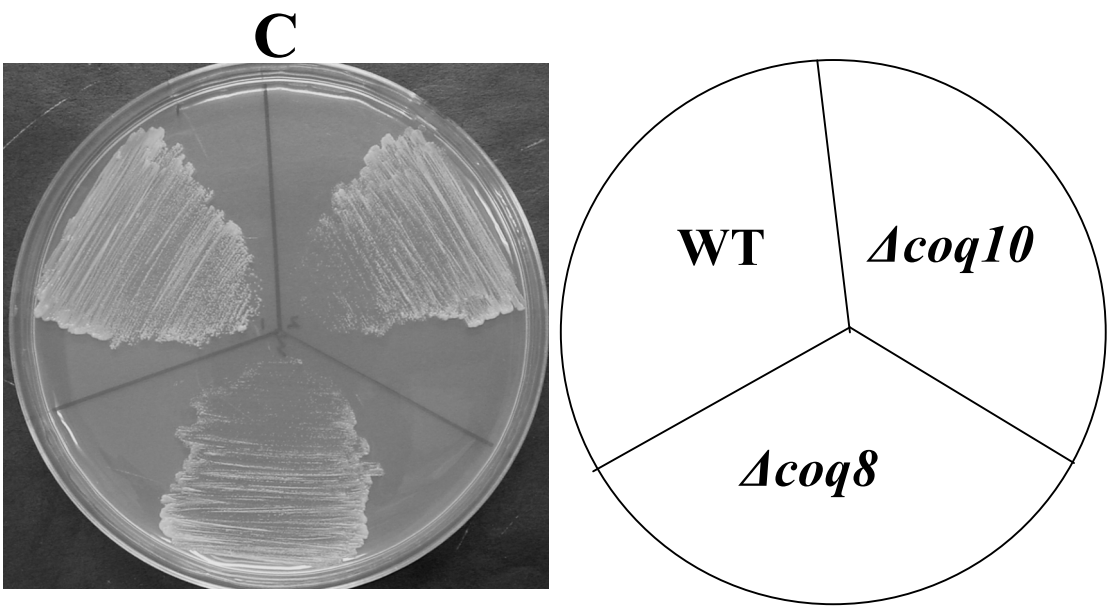
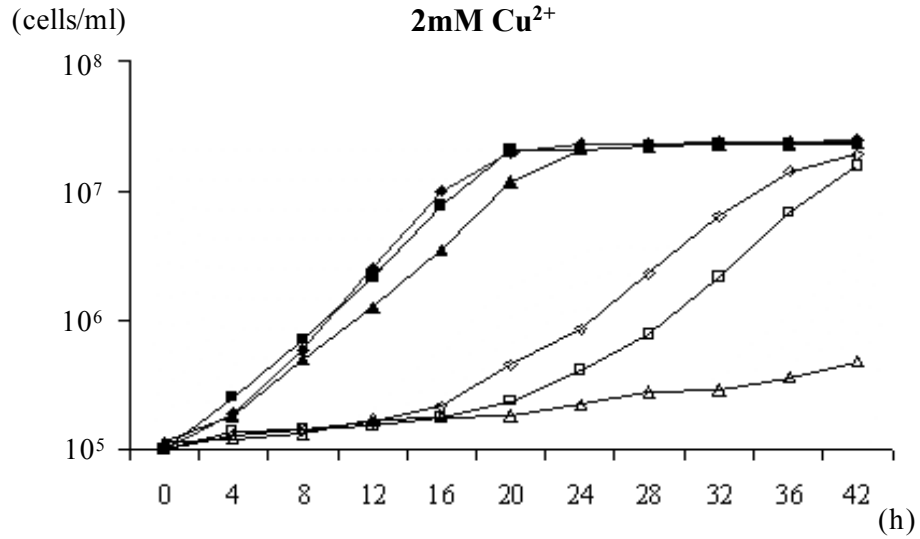


Fig. 3


(cells/ml)
2mM Cu2+
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10^7
10^6
10^5
0
4
8
12
16
20
24
28
32
36
42
(h)

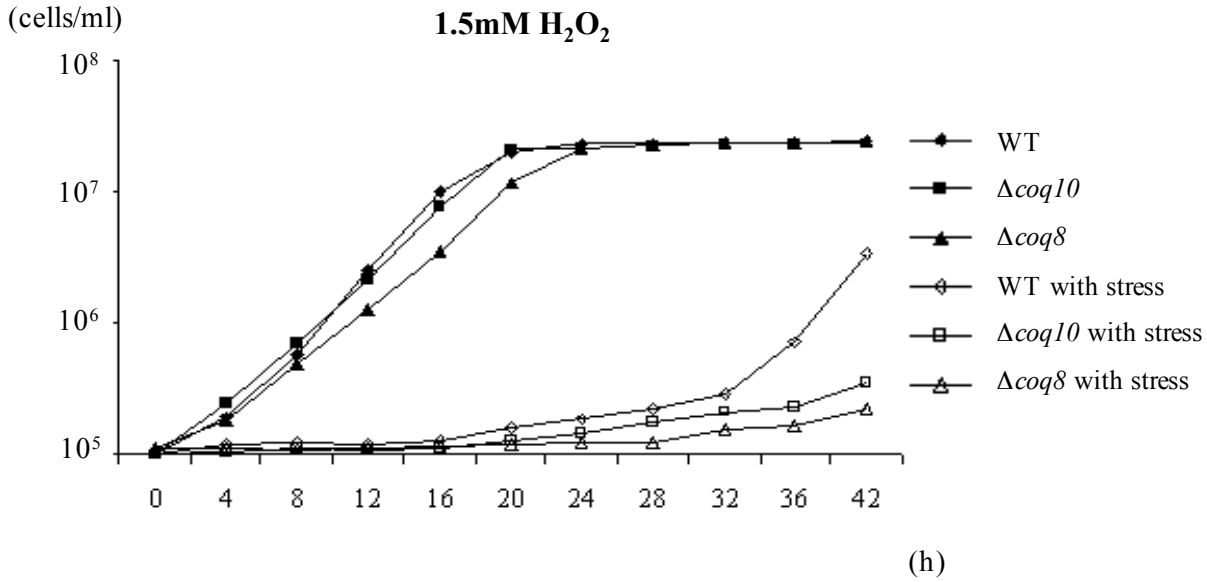
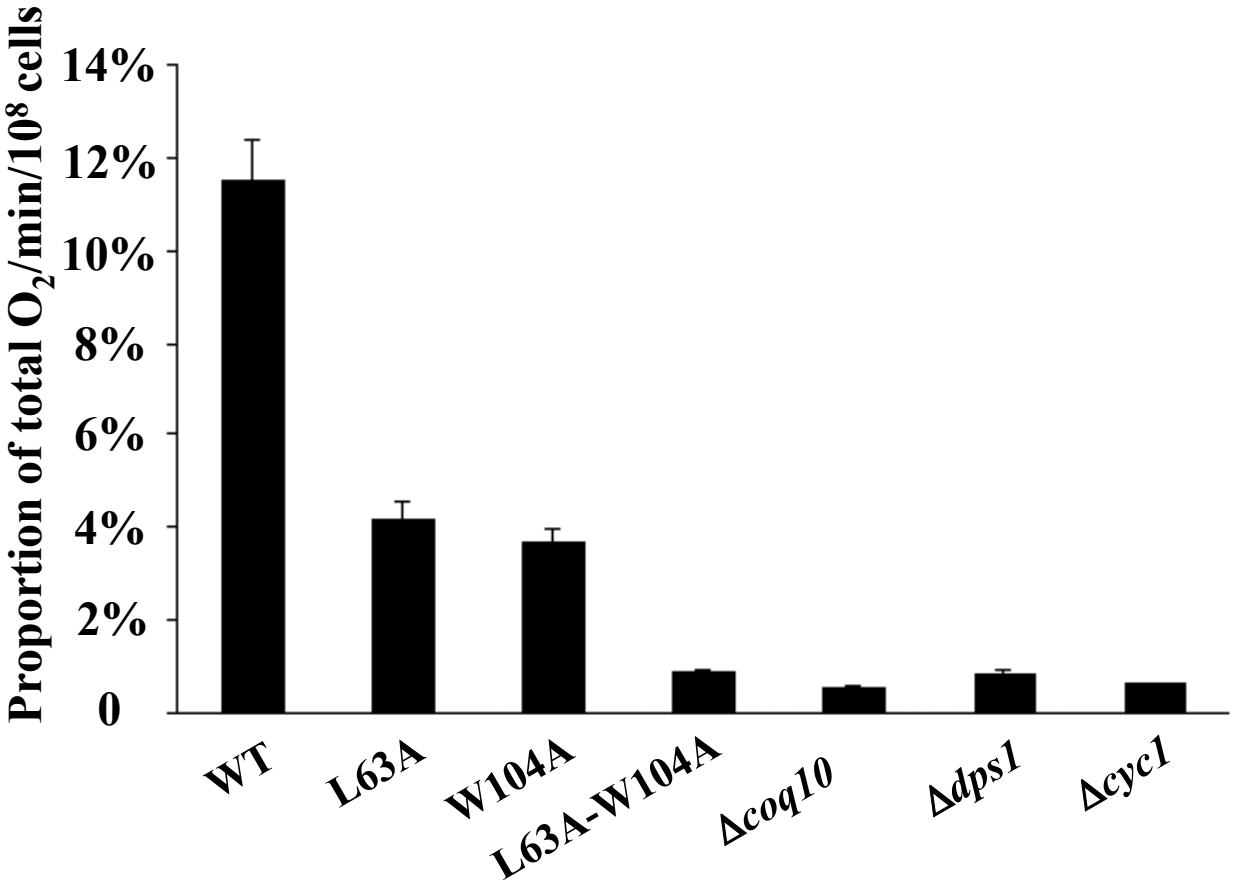

(cells/ml)
1.5mM H2O2
10^8
10^7
10^6
10^5
0
4
8
12
16
20
24
28
32
36
42
(h)
WT
Δcoq10
Δcoq8
WT with stress
Δcoq10 with stress
Δcoq8 with stress

Fig. 4


Proportion of total O_2/min/10^8 cells
14%
12%
10%
8%
6%
4%
2%
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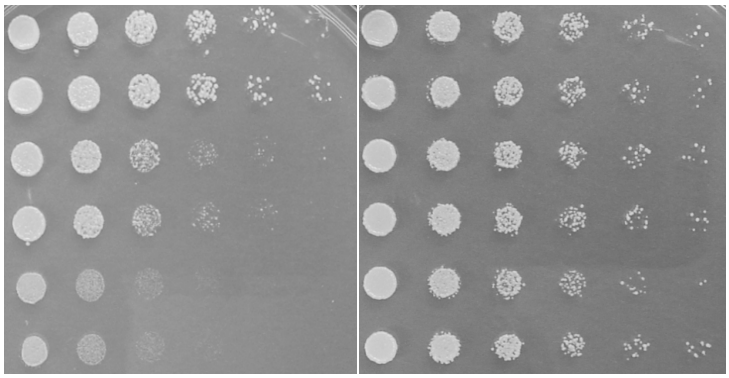
W104A
L63A-W104A
Δcoq10
Δdps1
Δcyc1

Fig. 5

(A)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L63A
W104A
L63A-W104A
Vector
PM + ura
PM + ura + glu

(B)

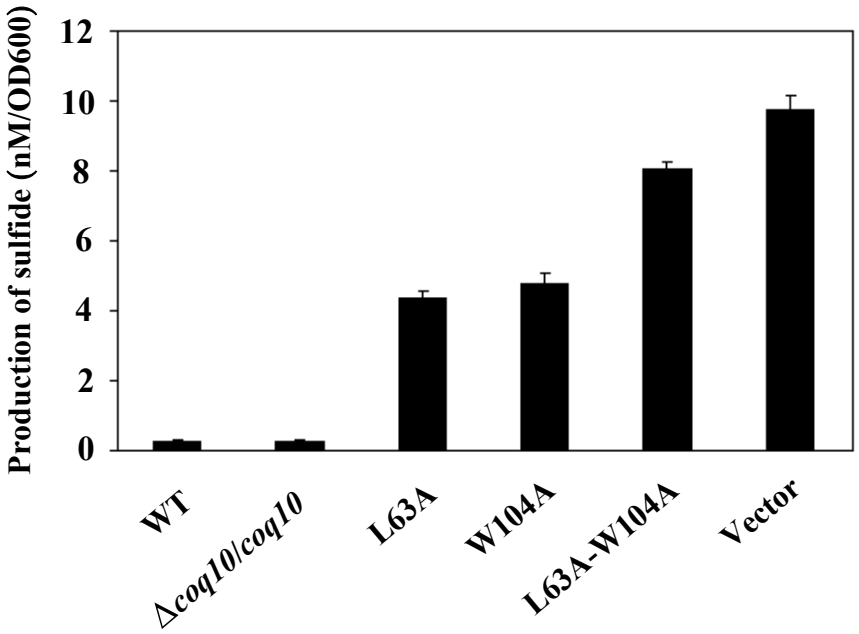
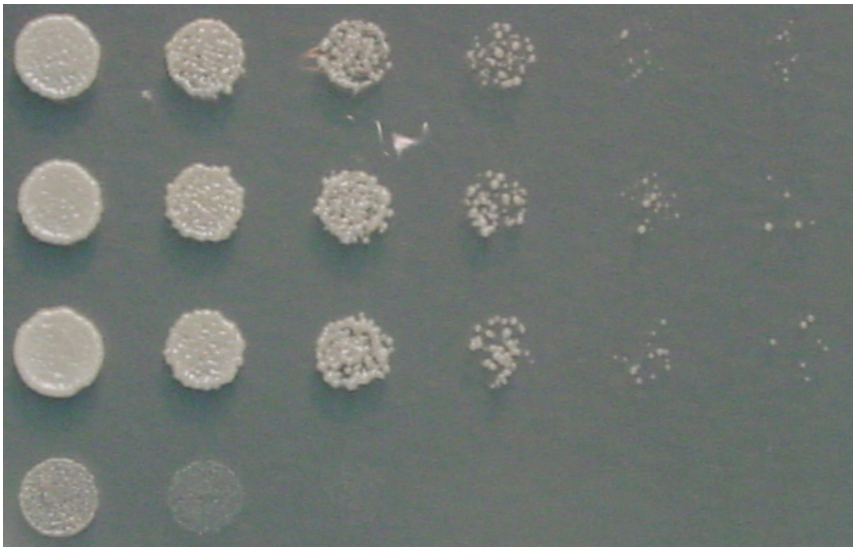

Production of sulfide (nM/OD600)
12
10
8
6
4
2
0
WT
Δcoq10/coq10
L63A
W104A
L63A-W104A
Vector

Fig. 6



WT/vector

Δ*coq10*/*coq10*

Δ*coq10*/*Hucoq10A*

Δ*coq10*/vector

PM + ura

Fig. 7

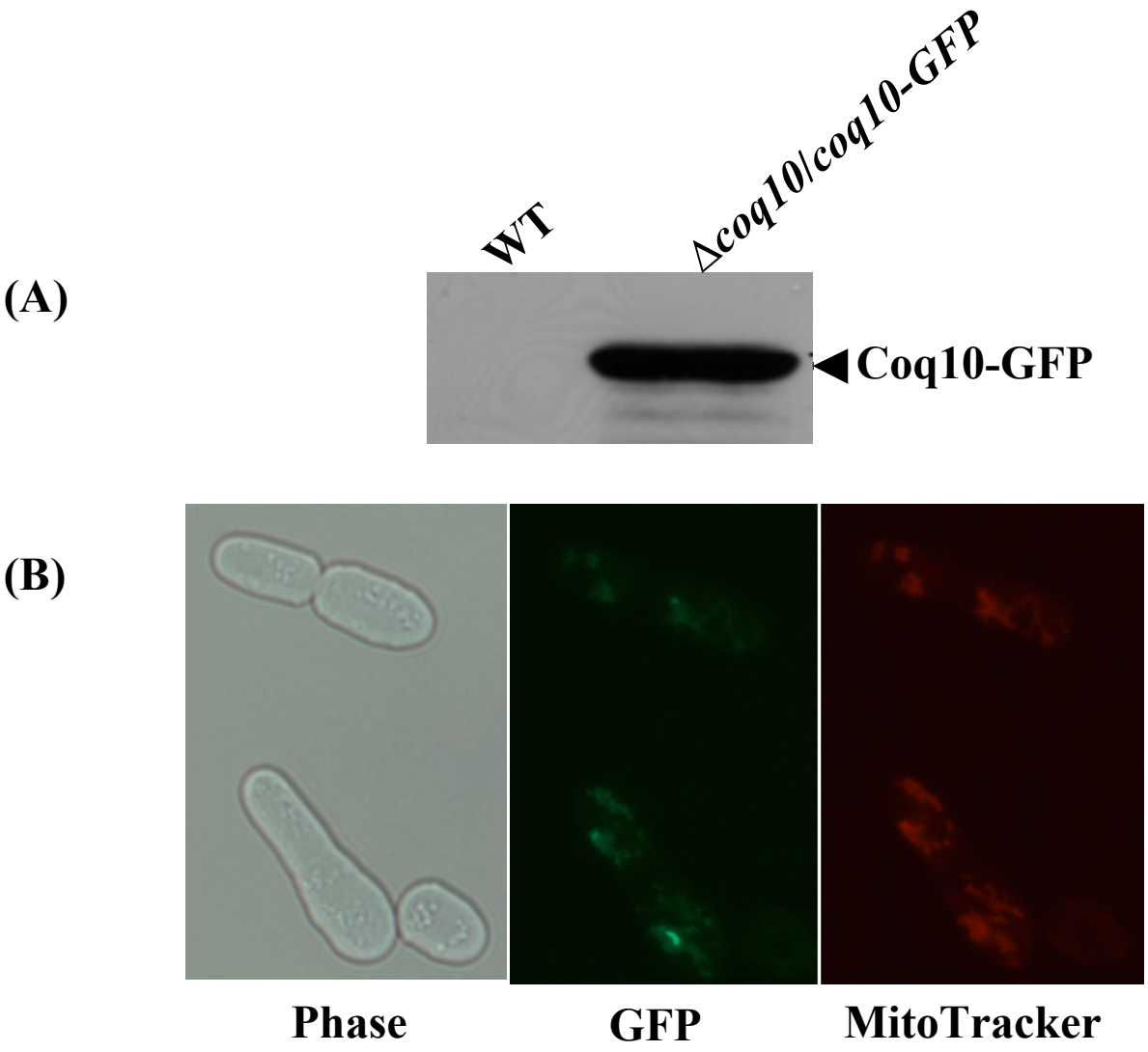


Fig. 8

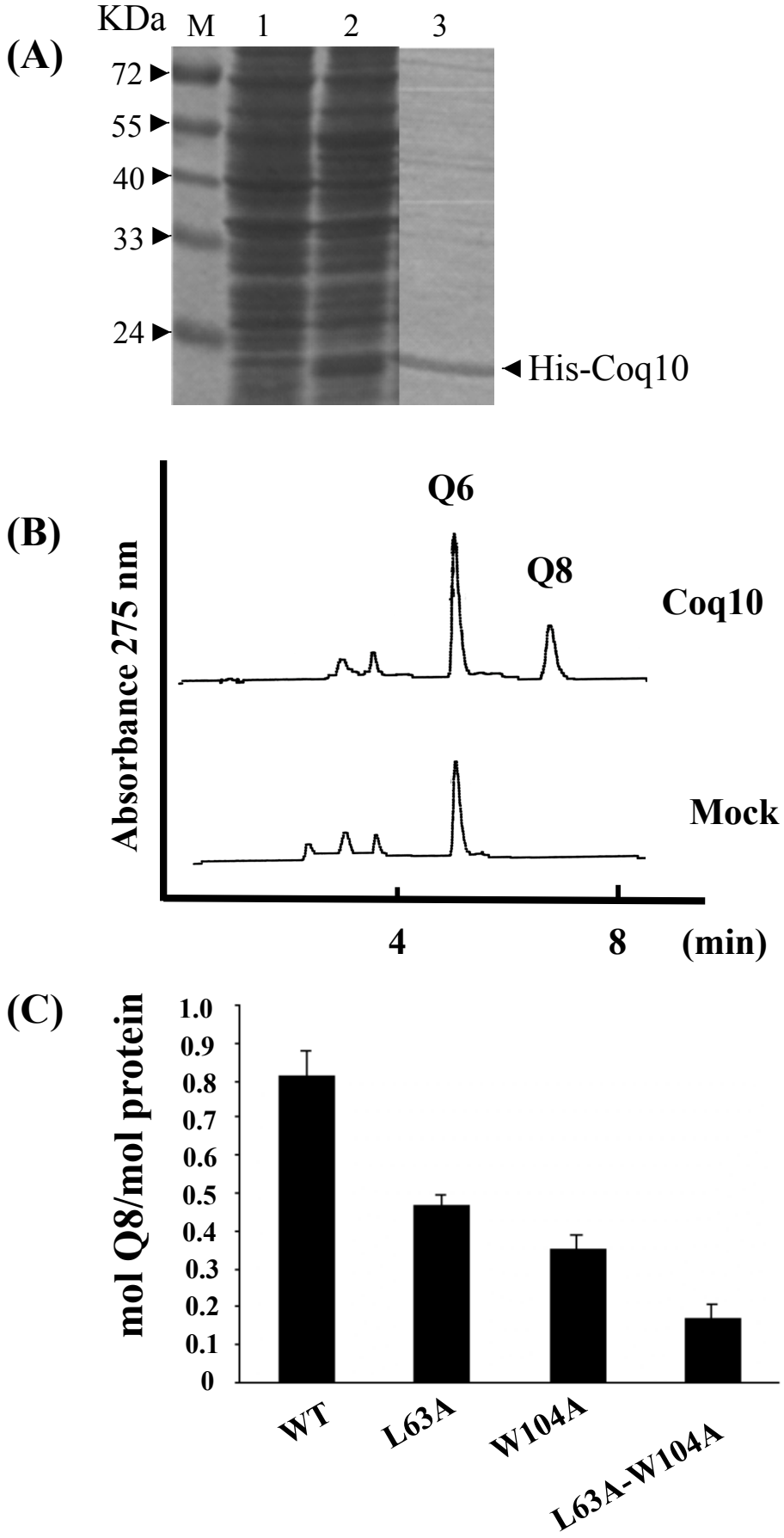

(A)
KDa
M
1
2
3
72
55
40
33
24
His-Coq10
(B)
Q6
Q8
Coq10
Mock
Absorbance 275 nm
4
8
(min)
(C)
mol Q8/mol protein
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
L63A
W104A
L63A-W104A

Fig. 9