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RESPIRATORY-INDUCED COENZYME Q BIOSYNTHESIS IS REGULATED BY A PHOSPHORYLATION CYCLE OF Cat5p/Coq7p.

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Abstract

Coenzyme Q₆ biosynthesis in yeast is a well regulated process that requires the final conversion of the late intermediate demethoxy-Q₆ (DMQ₆) to Q₆ in order to support respiratory metabolism in yeast. The gene *CAT5/COQ7* encodes the Cat5/Coq7 protein that catalyzes the hydroxylation step of DMQ₆ conversion to Q₆. In this study, we demonstrated that yeast Coq7 recombinant protein purified in bacteria can be phosphorylated *in vitro* using commercial PKA or PKC kinases in S20, S28 and T32 predicted phospho-amino acids. The total absence of phosphorylation in a Coq7p version containing alanine instead of these phospho-amino acids, the high extent of phosphorylation produced and the saturated conditions maintained in the phosphorylation assay indicate that probably no other putative amino acids are phosphorylated in Coq7p. *In vitro* assays have been corroborated in a phosphorylation assay performed in purified mitochondria without external or commercial kinases. Coq7p remains phosphorylated in fermentative conditions and becomes dephosphorylated when respiratory metabolism is induced. The substitution of phosphorylated residues to alanine dramatically increases Q₆ levels (256%). Conversely, substitution with negatively charged residues decreases Q₆ content (57%). These modifications produced in Coq7p also alter the ratio between DMQ₆ and Q₆ itself, indicating that Coq7p phosphorylation state is a regulatory mechanism for Q₆ synthesis.

Introduction

Coenzyme Q (CoQ or Q) is an essential isoprenylated benzoquinone component of mitochondria. Coenzyme Q resides in the phospholipid bilayer of mitochondrial membranes [1] where acts as an electron carrier from complex I or II to complex III at the inner membrane [2]. Coenzyme Q also works as an antioxidant agent quenching free radicals [3] and is required for other cellular functions that have been extensively reviewed [4].

Down-regulation of Q biosynthesis in humans causes a specific type of mitochondrial disease known as Q deficiency syndrome [5]. These patients show neuromuscular defects caused by very low Q levels. Q supplementation usually improves their clinical condition [6]. However, Q administration sometimes fails to alleviate the symptoms of the patients [7] likely due to the low and irregular distribution of exogenous Q in organs and tissues [8]. An alternative strategy to relieve Q deficiency is through the activation of its *de novo* synthesis. In order to up-regulate Q biosynthesis a better understanding of Q biosynthesis and its regulation in eukaryotic cells is required.

In *Saccharomyces cerevisiae*, at least ten genes (*COQ1-10*) are involved in Q biosynthesis. Although most of the Coq proteins catalyze known reactions [9], other Coq proteins such Coq4p and Coq9p do not seem to be involved directly and must participate in the regulation of coenzyme Q₆ biosynthesis [9]. Several lines of evidence suggest the existence of a coenzyme Q biosynthetic complex. In general null mutants in *COQ* genes accumulate the same early precursor known as hexaprenyl hydroxybenzoate (HHB) [10]. These data imply that Q biosynthesis is produced by a protein complex that catalyzes the modification of the benzenic ring [11]. Some *COQ* genes, such as *CAT5/COQ7*, display a different response when point mutations are introduced. *CAT5* is the initial name given for this gene but the name *COQ7* is also accepted because it has been demonstrated that the requirement for gluconeogenesis corresponds to the inability to respire due to the lack of Q biosynthesis [12]. Previous studies have demonstrated that Coq7p is a mono-oxygenase that catalyzes the hydroxylation of DMQ₆ to demethyl-Q₆ (Figure 1) [12], but some point mutants of *COQ7*, such as *e2519*, [13] accumulate the precursor DMQ₆ while more severe truncated alleles, such as *qm51*, only accumulate the early precursor HHB. Since DMQ₆ does not support either the electron transport respiratory chain nor prevent oxidative stress [13], it is considered that DMQ₆ accumulation in wild-type strains acts as a reservoir. These results support the idea that Coq7p is a key player in Q₆ biosynthesis, since DMQ₆ is accumulated during fermentative growth in glucose but is quickly modified to Q₆ in two steps following the respiratory induction or when cells are subjected to oxidative stress [14]. Furthermore, this hypothesis is supported by the action of Coq8p. This protein belongs to a family of putative protein-kinases known as ADCK (aarF domain containing kinase) [15] although its alleged kinase activity remains to be confirmed. Interestingly, over-expression of Coq8p in yeast lacking Coq7p allows the production of the intermediate demethoxy-Q₆ (DMQ₆), indicating a regulatory role of Coq8p in Q₆ biosynthesis complex assembly [14]. *COQ5* encodes a C-methyl transferase that converts 2-hexaprenyl-6-methoxy-1,4-benzoquinone to DMQ₆ [16-17]. Some *COQ5* point mutations (*Coq5-2* and *Coq5-5*) fail to support C-methyl transferase activity but show a steady state expression similar to wild-type Coq5. These data demonstrate that Coq5p carries out two different functions; first, in modifying the benzenic ring and second, as a structural component of the CoQ synthesis complex [18]. Recently, there have been physical evidences reported of the coenzyme Q biosynthetic complex existence by size exclusion chromatography and blue native electrophoresis [19-22] involving most of Coq proteins. Coq5p and Coq7p are also involved in the up-regulation of coenzyme Q₆ biosynthesis induced by oleic acid [23], glycerol [14, 23], oxidative stress [14] and the retrograde response [24].

The role of phosphorylation in mitochondrial signalling transduction was initially poorly understood but its knowledge has been increased in the last few years [25-26]. Phosphorylation by PKA has been related to mitochondrial biogenesis [27] and modulation of growth during post diauxic shift in yeast [28], although many other kinases may also be involved in mitochondrial metabolism [25]. Coq7p amino acid sequence shows several putative phospho-

sites suggestive of post-translational modification to regulate its activity. The regulation of mitochondrial complexes activity by phosphorylation is a known process as described for the pyruvate dehydrogenase complex (PDH) [29]. Phosphorylation is likely to be involved in the control of many other metabolic processes within the mitochondria as depicted by the number of phospho-proteins detected in yeast mitoproteome [30]. In the case of Q biosynthesis, recent studies have reported some indirect evidences for the phosphorylation of several catalytical Coq proteins by Coq8p [19, 31], although the kinase activity of Coq8p has not been demonstrated to date.

Here we show that Coq7p is phosphorylated both *in vitro* and *in vivo*. The Coq7p phosphorylated state is induced during fermentative metabolism and becomes inhibited under respiratory conditions. Substitution of phospho-amino acid residues in Coq7p to non-phosphorylatable ones induces an increase of Q₆ levels but the presence of negative charges at these residues, mimicking a Coq7p phosphorylated state, produces a decrease of Q₆ levels. These results provide direct evidence that Coq7p phosphorylation is a regulatory mechanism of Q₆ biosynthesis in yeast.

Experimental

Yeast strains and growth media

Yeast strains used in this study are listed in Table S1. Growth media for yeast and bacteria were prepared as described previously [32]. Yeasts were grown at 30°C with shaking (200 rpm).

Plasmid construction

Plasmid pNMQ7 was created by digesting the low number copy plasmid pRS316 with HindIII and Xhol and subsequent ligation of the fragment carrying *COQ7* plus one kb both up-stream and down-stream in order to maintain the natural promoter and terminator sequences. The pL series were created by digesting the plasmid with KpnII and HindIII and ligating the digested *COQ7* versions obtained by mega-primer polymerase chain reaction mutagenesis [33]. pG series were obtained using QuikChange II XL Site-directed Mutagenesis kit (Stratagene) using the pNMQ7 plasmid as template. The primers used are shown in Table S2. GST recombinant proteins were obtained amplifying the sequences by PCR following the ligation in the pGEX bacterial expression vector. PCR was performed using Fw-GEX and Rv-GEX primers. DNA sequencing was carried out by the MWG-Biotech AG Sequencing Service (Ebersberg, Germany).

Quinone identification and quantification

Total Q₆ quantification in mitochondrial membrane samples was performed by HPLC-ECD. Description of equipment and methods has been previously published [34].

Protein purification and in vitro phosphorylation assay

The *E. coli* strain BL21 pLys was used for expression of Coq7p in pGEX4T1 plasmid (GE Healthcare Life Sciences) using GST Sepharose beads to purify Coq7p recombinant protein as described by the manufacturer. Protein phosphorylation assay was performed [35-36] with recombinant active PKA (1 µg) (Sigma) or PKC from rat brain (1µg) (Sigma) incubated with 0.1µCi γ-[³²P]ATP, (Perkin Elmer) 200 µM ATP (Sigma) and 2 µg recombinant Coq7p protein in 50 µl of kinase buffer (50 mM Tris HCl pH 7.8, 25 mM β-glycerophosphate, 5 mM MgCl₂, 5 mM MnCl₂, 1 mM dithiothreitol) and Sigma phosphatase inhibitor cocktail 1, 1/100 v/v. Reactions were incubated at 30°C for 2 hours. In some experiments, GST moiety was released after thrombin digestion with 0.5 units of thrombin (Sigma) at 4°C overnight. Proteins were separated by 12% SDS-PAGE, and results were visualized by Pro-Q Diamond gel stain (Molecular Probes), autoradiography, silver staining and anti-GST tag immunodetection.

Phosphorylation assay in purified mitochondria

Fresh mitochondria from cells expressing V5 tagged Coq7p (100 µg protein) were permeabilized in 50 µl kinase buffer (50 mM Tris HCl, 25 mM β-glycerophosphate, 5 mM MgCl₂, 1 mM MnCl₂, 1 mM dithiothreitol and 0.3 g glass beads 106 µm) plus 1:100 w/v digitonin and Sigma phosphatase inhibitor cocktail 1, 1/100 v/v. Samples were subjected to vortex (15 min at 4°C). After centrifugation (1000 r.p.m. for 5 min at 4°C) the supernatant was incubated with 0.1 µCi γ-[³²P]ATP (Perkin Elmer) and 200 µM ATP at 30°C for 2 hours. Proteins were separated by 12% SDS-PAGE, and results were visualized by Pro-Q Diamond gel stain (Molecular Probes), autoradiography, Sypro Ruby Red (Sigma) and anti-V5 tag immunodetection.

Immunoprecipitation and 2-DE

Mitochondrial samples from cells expressing V5 tagged Coq7p were permeabilized and phosphorylated as indicated above with non-radioactive ATP. Immunoprecipitation was performed with Anti-V5 Agarose Gel Affinity beads (Sigma) according to the manufacturer's procedure. Proteins were subjected to 2-DE as follows: agarose beads were resuspended in rehydration buffer (DeStreak, GE Healthcare). Supernatants were applied to Immobiline DryStrips (GE Healthcare, 7 cm, pH 3–11 NL) and incubated overnight at room temperature.

IEF was performed in the Protean IEF Cell Biorad in four steps (1-h constant at 200 V, 1-h gradient up to 500 V, 6-h gradient up to 10000 V and further 0.4-h constant at 10000 V) at 20 °C with 50 µA per strip. Strips were equilibrated for 15 min in SDS buffer (375 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS) containing 2% (w/v DTT) and then for 15 min in SDS buffer with 2.5% (w/v) iodoacetamide. For the second dimension, the strips were applied on 12% SDS gels and sealed with 0.5% agarose in running buffer and 0.002% bromophenol blue.

Other methods

Mitochondrial DNA integrity was checked in all strains using JM6 and JM8 strains, two ρ^0 strains. Steady-state levels of specific peptides were measured by densitometry analysis, carried out with a GS800 densitometer (Bio-Rad) with ImageJ (v1.41o) as software analysis. All results are expressed as the average \pm SD. Statistical analyses were carried out using the SigmaStat 3.0 (SPSS) statistical package (*t*-test). Phosphorylation analysis in immobilized GST tagged proteins was performed with a scintillation counter Beckman-Coulter using Ready Protein Plus scintillator cocktail.

Results

Recombinant Coq7p is phosphorylated in three predicted phospho-amino acids

The *in silico* analysis of Coq7p amino acid sequence performed using different algorithms (PPSearch and Netphos 2.0) [37-38] predicts that three amino acid residues can be phosphorylated by protein kinases [39]. These putative phospho-sites are located at residues 20 (Ser), 28 (Ser) and 32 (Thr) (Figure 2A). In the *COQ7* gene sequence, codons encoding the predicted phospho-amino acids were changed to alanine by recombinant PCR to obtain a non-phosphorylatable version of Coq7p (AAA) (Figure 2A). Wild-type and non-phosphorylatable versions of Coq7p GST-tagged proteins were purified by size exclusion chromatography (Figure S1) and were phosphorylated with commercial protein kinases (PKA and PKC) plus $\gamma^{32}\text{P}$ ATP while immobilized on glutathione Sepharose beads. Phosphorylated proteins were released from beads by thrombin digestion and analyzed by SDS-PAGE (Figure 2B). ^{32}P labeling was detected only in samples corresponding to wild-type Coq7p (Coq7p-SST-GST), but not in the Coq7p-AAA-GST version (S20A, S28A, T32A). Recombinant protein was subjected to 2-DE separation and SDS-PAGE (see *Experimental*). Spots corresponding to these proteins were digested (ProGest, Genomic Solutions) and peptides were analyzed by MS-MS/MS (MALDI TOF/TOF) in a MALDI-TOF/TOF Mass Spectrometer (4700 Proteomics Analyzer, Applied Biosystems) in the proteomic service of the University of Córdoba (Figure S2). These spots can be produced by different extent of phosphorylation. Data analysis performed by SEQUEST search engine software on several protein databases confirmed both proteins as Coq7p from *Saccharomyces cerevisiae*. False discovery rate was 1%. The coverage for Coq7p-SST for the analyzed spot was between 60 to 63% with a score between 128.52 and 134.76.

To calculate the stoichiometry of Coq7p phosphorylation, 4 μg of Coq7p-SST-GST, 4 μg of Coq7p-AAA-GST and 2 μg of free GST were phosphorylated as previously indicated with $\gamma^{32}\text{P}$ ATP and 200 μM non-radiolabeled ATP. The amount of GST included was half of the amount of Coq7p-GST protein in order to maintain the same number of molecules in the assay since the molecular weight of GST is 26 kDa while Coq7p-GST is 52 kDa. All proteins were immobilized in GST sepharose beads and washed several times. The samples were divided in two aliquots and one was treated with λ -phosphatase. Finally, both aliquots were analyzed in a scintillation counter (Figure 2C). Coq7p-GST-SST shows the highest amount of bound-phosphate compared with Coq7p-GST-AAA and free GST. The treatment with 5U λ -phosphatase completely removed the bound-phosphate in all proteins analyzed. GST is a protein that can be phosphorylated by PKC in S93 [35]. Phosphorylation is similar in free GST and Coq7p-GST-AAA proteins indicating that the phosphorylation detected in Coq7p-GST-AAA is produced in the tag but not in the Coq7p component. On the contrary, Coq7p-GST-SST is significantly phosphorylated in the Coq7p component, supporting the previous data obtained by SDS-PAGE and ^{32}P detection.

In the case of Coq7p-GST-SST the net phosphate (total phosphorylation minus free GST phosphorylation) detected was 1951 cpm. When a 96% of efficiency of the scintillation cocktail and a concentration of non-radiolabeled ATP 1000 times higher than $\gamma^{32}\text{P}$ ATP were taken into account, the number of phosphate molecules bound to Coq7p-GST-SST was approximately 5.56×10^{13} . In the assay there were 2 μg of Coq7p-GST-SST included, which contains approximately 2.31×10^{13} molecules based on the MW of the recombinant protein (52 kDa). The ratio is 2.41 molecules of phosphate per molecule of protein fit with the number of phosphoaminoacids expected, but is not possible assure that all phospho-amino acids were phosphorylated in the same proportion *in vitro*. Interestingly, we did not obtain any net phosphorylation in the Coq7p-GST-AAA version. The phosphorylation of the wild-type Coq7p (Coq7p-GST-SST) is also supported by the saturated conditions of the assay; ATP and the commercial kinases are present in excess in the assay. In this sense, the absence of phosphorylation in Coq7p-GST-AAA is produced by the lack of phospho-sites accessible for commercial kinases although it is not possible to dismiss other phospho-sites for mitochondrial

kinases. At least, these two and probably three putative phospho-amino acids are phospho-amino acids *in vitro*.

In vitro Coq7p phosphorylation is performed by mitochondrial kinases

Coq7p phosphorylation was corroborated in purified mitochondria (Figure 3A). Wild-type (Coq7p-SST V5) and non-phosphorylatable Coq7p (Coq7p-AAA V5) epitope tagged versions of Coq7p were expressed in a null *COQ7* strain cultured with 2% galactose. Coq7 versions were detected by western blotting (anti-V5). A slight shift in migration was determined in wild-type vs. Coq7p-AAA. Autoradiography clearly detected ^{32}P labeling in wild-type Coq7p-SST. However, only a faint signal was detected in the Coq7p-AAA version. Pro-Q Diamond staining, a fluorochrome with affinity for phospho-amino acids, showed an intense band for Coq7p-SST and only a weak band in the Coq7p-AAA version indicating that wild-type Coq7p is phosphorylated in these conditions.

As further evidence of the differential phosphorylation state of Coq7p-SST vs. Coq7p-AAA protein, a similar amount of mitochondria was subjected to immunoprecipitation and 2-DE (Figure 3B). Both proteins migrated at 26 kDa consistent with the predicted size. Spots were analyzed by MS (see *Experimental*). False discovery rate was 1%. The coverage for Coq7p-SST for the analyzed spot was between 78 to 82% with a score between 178.45 and 191.34. In Coq7p-AAA there were observed several spots stained with silver and anti-V5, but none of them were stained with Pro-Q Diamond, consistent with a lack of phosphorylation in this version. However, these data do not exclude that other phospho-amino acids different from the ones that we analyzed were phosphorylated in these conditions but the phosphorylation could not be detected by Pro-Q Diamond. On the other hand, the Coq7p-SST showed at least two additional forms separated in IEF at the same size that were stained with silver, anti-V5 as well as Pro-Q Diamond, consistent with multiple protein phosphorylation.

Coq7p phosphorylation is modulated by the cell metabolic state

Respiratory growth is established during the postdiauxic shift phase (PDS). When yeast changes their metabolism from glucose-fermentation to ethanol-respiration the metabolic shift requires Q₆ biosynthesis as an essential component of mitochondrial respiratory chain. Coq7p is considered a protein that regulates the final steps of the biosynthesis (Figure 1) from the accumulated DMQ₆.

V5 tagged Coq7p-SST was expressed in a null *COQ7* mutant strain. Cells were transferred to several culture media with different concentrations of fermentable (0.5, 2 and 10% glucose) and non-fermentable carbon sources (ethanol and glycerol). Mitochondria were permeabilized and analyzed by SDS-PAGE (Figure 4A). Pro-Q Diamond showed differential staining of Coq7-V5 protein according to the carbon source included in the culture. The ratio of phosphorylated Coq7p/total Coq7p-V5 (Figure 4B) normalized with the total protein indicated that higher glucose concentrations (2 and 10%) yielded a higher level of phosphorylation in Coq7p compared to cells grown with 0.5% glucose. More dramatic changes were observed in non-fermentable carbon sources such as ethanol or glycerol grown cells (33 and 37% compared to 10% glucose respectively). The levels of coenzyme Q were determined in order to relate changes in Coq7p phosphorylation state to the regulation of CoQ synthesis in mitochondrial samples used previously (Figure 4C). Samples incubated for two hours in non-fermentable carbon sources or in 0.5% glucose produced a small but significant increase in coenzyme Q₆ level compared to yeast cultured in 2% glucose. This increase was also accompanied by a parallel decrease of DMQ₆ content. The modification produced in 10% glucose is the opposite but it is not significant when compared to 2% glucose. The change of carbon source modifies the phosphorylation extent of Coq7p as well as the Q₆/DMQ₆ ratio although it does not demonstrate a direct relationship between these two facts.

Removal of Coq7p-phospho-sites increases coenzyme Q₆ levels

COQ7 site-directed mutagenesis was performed in a single copy cloning vector (pRS316) that maintains *COQ7* expression under the control of its natural promoter (pNMQ7) [39]. The modifications from phosphorylatable amino acids (SST) to alanine (A) are named pL since it is considered a loss-of-function (L). We have obtained several pL alleles by a combination of single, double and triple mutations. The expression of pL alleles support growth in liquid and glycerol plates (Figure S3 and S4) indicating the ability to maintain the respiratory metabolism. Yeast harboring pL mutant versions of Coq7p contained significant higher amounts of Q₆ compared to the wild-type control, from 157 to 256% (Figure 5, Figure S5 and Table S3). The amount of Q detected in yeast expressing the triple pL mutant (pL-AAA) was similar to the amount produced by a strain expressing the wild-type *COQ7* in a multicopy plasmid (pmQ7). Although versions of mutated Coq7p lacking one or more phospho-sites produced a Q₆ increase, data suggest that the removal of the second and third phospho-sites results in a higher Q₆ accumulation (Table S3).

Phosphomimetic changes in Coq7p-phospho-sites decrease coenzyme Q₆ levels

We introduced negatively charged amino acids to replace serine and threonine phospho-sites in Coq7p. Both glutamic and aspartic acids have been reported as a useful modification to mimic the phosphorylated state of proteins [40]. For example, the change of serine to alanine abolishes the ability of CKII to activate p53, but the change of alanine to aspartic acid partially restores this activity [41]. Serine 20 was changed to aspartic acid by the mutation TC58GA. Serine 28 was changed to glutamic acid by the mutation TC82GA and threonine 32 was changed to aspartic acid by the mutation AC94GA (Figure 2A). These plasmids were named pG to denote a gain-of-function, in contrast to the pL loss-of-function plasmids. We prepared different pG alleles with a combination of single, double and triple mutations. The expression of pG alleles supported the growth of yeast in liquid and glycerol plates (Figure S3 and S4). Mitochondrial Q₆ levels were significantly lower compared to the positive control, from 57 to 72%, and also compared with loss-of-function Coq7p mutants (Figure 5, Figure S5 and Table S3). As previously mentioned, the most severe changes were obtained in mutants including the second and third phospho-sites.

The evolution of coenzyme Q₆ and DMQ₆ ratio is modified in phosphomimetic and permanent dephosphorylated Coq7p versions in glucose based medium.

We have previously demonstrated that DMQ₆ levels were increased in wild-type cells during growth in glucose [14]. Initially, DMQ₆ concentration was increased but quickly decreased when the culture reached the postdiauxic growth phase. Coenzyme Q₆ displays the opposite pattern. These changes support the hypothesis of Coq7p participation on the coenzyme Q₆ biosynthesis regulation and it was also the reason why we performed the same study in yeast expressing both modified Coq7p versions (Figure 6). The expression of wild-type Coq7p (pNMQ7) produces a similar profile of Q₆/DMQ₆ ratio that has been described previously in whole cell lysates from wild-type yeast [14]. In the first phase there is an initial increase of DMQ₆ followed by a subsequent decrease accompanied by increased levels of Q₆. The same experiment was performed in the non-phosphorylatable version of Coq7p (pL-AAA). The amount of DMQ₆ was significantly lower compared to coenzyme Q₆, even when the total amount of quinones was dramatically higher compared to wild-type cells. In the phosphomimetic version of Coq7p (pG-DED), there are not severe changes between Q₆ and DMQ₆ during growth in glucose. These results support the hypothesis in which Coq7p regulates coenzyme Q₆ biosynthesis, since DMQ₆ and Q₆ content and the DMQ₆/Q₆ ratio depend on the phosphorylation state of Coq7p.

Discussion

After postdiauxic shift, transcription factor *HAP4* [42] controls the expression of several mitochondrial genes [43] and appears to be involved in the expression of *COQ2* and *COQ7/CAT5* genes [44]. The assembly of Coq polypeptides into a complex leading to Q₆ biosynthesis is another level of regulation [19, 22]. Some studies have demonstrated that either Q₆ addition or Coq8p over-expression produces DMQ₆ accumulation by promoting full complex assembly in a *COQ7* null mutant [14]. These data suggest that Coq7p could be a control point for the regulation of Q₆ biosynthesis in yeast [14]. Given that Coq7p contains three putative phospho-sites, we have studied whether Coq7p activity is regulated by phosphorylation. In this report we show that Coq7p is indeed a phospho-protein: *in vitro* phosphorylation performed with commercial PKA or PKC is not found after the removal of three potential phospho-amino acids. Recombinant Coq7-SST is phosphorylated by different methods which demonstrate the presence of two and probably three phospho-amino acids in Coq7p. These data make difficult the idea that phosphorylation takes place in any other Coq7p amino acids, but our study is inconclusive for this point since other possible kinases present in yeast mitochondria are not analyzed; multiple phospho-sites are possible given the large number of isoelectric points found by mass spectrometry in Coq7p recombinant protein in the *in vitro* assay.

We also determined that Coq7p is phosphorylated within the mitochondria, supporting the *in vitro* experiments. These data involve kinase(s) naturally expressed in mitochondria that phosphorylate wild-type Coq7p but not the non-phosphorylatable Coq7p version and it also supports that these putative phospho-sites are biologically active. Obviously, these phosphorylation conditions in the assay are not *in vivo* conditions but still demonstrate that natural kinase(s) perform this reaction.

Moreover, we have shown evidence that phosphorylation of Coq7p is physiologically relevant since it is affected by changes in carbon source that are known to alter the metabolism of yeast. Fermentable carbon sources, such as high glucose, increase Coq7p phosphorylation while non-fermentable carbon sources such as glycerol or ethanol or even lower glucose level (0.5%) reduce Coq7p phosphorylation. Given that non-fermentable carbon sources require the activation of mitochondrial respiration, these conditions are similar to the postdiauxic shift (PDS) changes occurring when glucose is exhausted in glucose based culture media [45]. In that sense, it has been reported that glycerol growth induces the expression of COQ genes and Coq proteins [23]. It is also known that low glucose culture media promote Q₆ accumulation [46] and finally, during stationary growth phase a high amount of Q₆ is produced when compared to the exponential growth phase [47]. Our data support the hypothesis that Coq7p phosphorylated state correlates with fermentation while the non-phosphorylated state is associated with respiration. We have shown that changes in Coq7p phosphorylation induce modifications in the amount of Q₆ and DMQ₆ produced, supporting the idea that the non-phosphorylated state of Coq7p leads to higher Q₆ levels in non-fermentable carbon sources as demonstrated in the analysis of Q₆ levels in the phosphomimetic Coq7p version (Coq7-pG-DED) and the non-phosphorylatable version (Coq7-pL-AAA).

Phosphorylation is a well-known process that regulates the activity of other mitochondrial enzymes; for instance, phosphorylation leads to lower activity, while dephosphorylation activates the PDH complex [29, 48]. We developed site-directed mutations in Coq7p in order to produce either a permanent dephosphorylated state of Coq7p (Ala substitutions of Ser and Thr) or the phosphorylated state of this protein (Asp or Glu substitution of Ser and Thr) [41]. These forms of Coq7p provided us with a tool to determine the effects of Coq7p phosphorylation on Q₆ biosynthesis. Our data demonstrate that dephosphorylated Coq7p causes a significant increase of Q₆ that is similar to the increase produced by the *COQ7* gene over-expression with a multicopy vector supporting the hypothesis that the dephosphorylated form of Coq7p is the more active one. Conversely, the phosphomimetic form of Coq7p produces a decrease of Q₆ biosynthesis. The effects of both Coq7p versions include a shift on the Q₆/DMQ₆ ratio during the growth in glucose as previously described [14]. This suggests that phosphorylated state of the phospho-sites in Coq7p reduces its activity. It is also important to note that even if we did

not determine exactly the number and location of the *in vivo* phospho-sites in Coq7p it does not compromise the fact that the phosphorylation state of this protein is biologically relevant in yeast.

Phosphorylation is a poorly known post-translational modification process in mitochondria since only 15 kinases and 5 phosphatases are present in yeast mitochondria [49-50]. It has been suggested that most of the kinase phosphorylation processes take place in the cytosol (cAMP-dependent, MAP, TOR, cyclin and Snf1p kinases) that finally affect the mitochondrial metabolism through the phosphorylation of mitochondrial or mitochondria-associated proteins. This may explain the low number of kinases/phosphatases observed in mitochondria [51]. In order to find the kinase responsible for Coq7 phosphorylation we could speculate a general regulation process based in a general kinase or kinases that send a repression signal into the mitochondria. On the other hand, the existence of specific mitochondrial kinases devoted to regulate coenzyme Q biosynthesis is also possible. In the first case, a lot of regulatory pathways that detect the nutrient availability have been described such as TOR1, SCH9, RAS1/2 and SNF1 [52-53] which are related energy metabolism. On the contrary there are examples within the mitochondria such as the PDH complex regulation. In this case, PDH activity is specifically regulated by two pairs of kinase/phosphatases (PKP1/2 and PTC5/6) [54]. Coq8p is a protein required for coenzyme Q biosynthesis [55] with unknown functions although it belongs to a family of putative kinases (ADCK kinases) [15]. Some studies relate Coq8p to the phosphorylation of several Coq proteins such as Coq3p [19] which has been previously pointed out to be a target of phosphorylation [46]. Coq5p and Coq7p have also been predicted to be phosphorylatable by indirect methods [31].

We propose a novel model of Coq7p-based regulation of coenzyme Q biosynthesis where participation of at least two new enzymes are required. A mitochondrial kinase and a mitochondrial phosphatase modify Coq7p activity through phosphorylation. Identifying these enzymes and linking their activity to the metabolic state of the cell is the new challenge in better understanding the regulation of Q₆ biosynthesis.

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References

- 1 Lenaz, G., Fato, R., Di Bernardo, S., Jarreta, D., Costa, A., Genova, M. L. and Parenti Castelli, G. (1999) Localization and mobility of coenzyme Q in lipid bilayers and membranes. *BioFactors* (Oxford, England). 9, 87-93
- 2 Trumper, B. L. (1990) The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome *bc₁* complex. *J. Biol. Chem.* 265, 11409-11412
- 3 Crane, F. L. and Navas, P. (1997) The diversity of coenzyme Q function. *Molec. Aspects Med.* 18, s1-s6
- 4 Bentinger, M., Tekle, M. and Dallner, G. (2010) Coenzyme Q-biosynthesis and functions. *Biochem Biophys Res Commun.* 396, 74-79
- 5 DiMauro, S., Quinzii, C. M. and Hirano, M. (2007) Mutations in coenzyme Q₁₀ biosynthetic genes. *J. Clin. Invest.* 117, 587-589
- 6 Salviati, L., Sacconi, S., Murer, L., Zucchetto, G., Franceschini, L., Laverda, A. M., Basso, G., Quinzii, C., Angelini, C., Hirano, M., Naini, A. B., Navas, P., DiMauro, S. and Montini, G. (2005) Infantile encephalomyopathy and nephropathy with CoQ₁₀ deficiency: a CoQ₁₀-responsive condition. *Neurology.* 65, 606-608
- 7 Quinzii, C. M., Hirano, M. and DiMauro, S. (2007) CoQ₁₀ deficiency diseases in adults. *Mitochondrion.* 7 Suppl, S122-126
- 8 Bentinger, M., Dallner, G., Chojnacki, T. and Swiezewska, E. (2003) Distribution and breakdown of labeled coenzyme Q₁₀ in rat. *Free Radic Biol Med.* 34, 563-575
- 9 Tran, U. C. and Clarke, C. F. (2007) Endogenous synthesis of coenzyme Q in eukaryotes. *Mitochondrion.* 7 Suppl, S62-71
- 10 Clarke, C. F. (2001) New advances in Coenzyme Q biosynthesis. *Protoplasma.* 213, 134-147
- 11 Gin, P. and Clarke, C. F. (2005) Genetic evidence for a multi-subunit complex in coenzyme Q biosynthesis in yeast and the role of the Coq1 hexaprenyl diphosphate synthase. *J. Biol. Chem.* 280, 2676-2681
- 12 Jonassen, T., Proft, M., Randez-Gil, F., Schultz, J. R., Marbois, B. N., Entian, K. D. and Clarke, C. F. (1998) Yeast Clk-1 homologue (Coq7/Cat5): a mitochondrial protein in coenzyme Q synthesis. *J. Biol. Chem.* 273, 3351-3357
- 13 Padilla, S., Jonassen, T., Jimenez-Hidalgo, M. A., Fernandez-Ayala, D. J. M., Lopez-Lluch, G., Marbois, B., Navas, P., Clarke, C. F. and Santos-Ocana, C. (2004) Demethoxy-Q, An Intermediate of Coenzyme Q Biosynthesis, Fails to Support Respiration in *Saccharomyces cerevisiae* and Lacks Antioxidant Activity. *J. Biol. Chem.* 279, 25995-26004
- 14 Padilla, S., Tran, U. C., Jimenez-Hidalgo, M., Lopez-Martin, J. M., Martin-Montalvo, A., Clarke, C. F., Navas, P. and Santos-Ocana, C. (2009) Hydroxylation of demethoxy-Q₆ constitutes a control point in yeast coenzyme Q₆ biosynthesis. *Cell Mol Life Sci.* 66, 173-186
- 15 Leonard, C. J., Aravind, L. and Koonin, E. V. (1998) Novel Families of Putative Protein Kinases in Bacteria and Archaea: Evolution of the "Eukaryotic" Protein Kinase Superfamily. *Genome Res.* 8, 1038-1047
- 16 Dibrov, E., Robinson, K. M. and Lemire, B. D. (1997) The *COQ5* gene encodes a yeast mitochondrial protein necessary for ubiquinone biosynthesis and the assembly of the respiratory chain. *J. Biol. Chem.* 272, 9175-9181

- 17 Barkovich, R. J., Shtanko, A., Shepherd, J. A., Lee, P. T., Myles, D. C., Tzagoloff, A. and Clarke, C. F. (1997) Characterization of the *COQ5* gene from *Saccharomyces cerevisiae*. Evidence for a C-methyltransferase in ubiquinone biosynthesis. *J. Biol. Chem.* 272, 9182-9188
- 18 Baba, S. W., Belogrudov, G. I., Lee, J. C., Lee, P. T., Strahan, J., Shepherd, J. N. and Clarke, C. F. (2004) Yeast Coq5 C-methyltransferase is required for stability of other polypeptides involved in coenzyme Q biosynthesis. *J. Biol. Chem.* 279, 10052-10059
- 19 Tauche, A., Krause-Buchholz, U. and Rodel, G. (2008) Ubiquinone biosynthesis in *Saccharomyces cerevisiae*: the molecular organization of O-methylase Coq3p depends on Abc1p/Coq8p. *FEMS yeast research.* 8, 1263-1275
- 20 Marbois, B., Gin, P., Gulmezian, M. and Clarke, C. F. (2009) The yeast Coq4 polypeptide organizes a mitochondrial protein complex essential for coenzyme Q biosynthesis. *Biochim. Biophys. Acta.* 1791, 69-75
- 21 Hsieh, E. J., Gin, P., Gulmezian, M., Tran, U. C., Saiki, R., Marbois, B. N. and Clarke, C. F. (2007) *Saccharomyces cerevisiae* Coq9 polypeptide is a subunit of the mitochondrial coenzyme Q biosynthetic complex. *Arch. Biochem. Biophys.* 463, 19-26
- 22 Marbois, B., Gin, P., Faull, K. F., Poon, W. W., Lee, P. T., Strahan, J., Shepherd, J. N. and Clarke, C. F. (2005) Coq3 and Coq4 define a polypeptide complex in yeast mitochondria for the biosynthesis of coenzyme Q. *J. Biol. Chem.* 280, 20231-20238
- 23 Hagerman, R. A., Trotter, P. J. and Willis, R. A. (2002) The regulation of *COQ5* gene expression by energy source. *Free Radic Res.* 36, 485-490
- 24 Hagerman, R. A. and Willis, R. A. (2002) The yeast gene *COQ5* is differentially regulated by Mig1p, Rtg3p and Hap2p. *Biochim. Biophys. Acta.* 1578, 51-58
- 25 Huttemann, M., Lee, I., Samavati, L., Yu, H. and Doan, J. W. (2007) Regulation of mitochondrial oxidative phosphorylation through cell signaling. *Biochim. Biophys. Acta* 1773, 1701-1720
- 26 Acin-Perez, R., Salazar, E., Kamenetsky, M., Buck, J., Levin, L. R. and Manfredi, G. (2009) Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. *Cell Metab.* 9, 265-276
- 27 Chevtzoff, C., Vallortigara, J., Averet, N., Rigoulet, M. and Devin, A. (2005) The yeast cAMP protein kinase Tpk3p is involved in the regulation of mitochondrial enzymatic content during growth. *Biochim. Biophys. Acta.* 1706, 117-125
- 28 Dejean, L., Beauvoit, B., Alonso, A.-P., Bunoust, O., Guerin, B. and Rigoulet, M. (2002) cAMP-induced modulation of the growth yield of *Saccharomyces cerevisiae* during respiratory and respiro-fermentative metabolism. *Biochim. Biophys. Acta - Bioenergetics.* 1554, 159-169
- 29 Krause-Buchholz, U., Gey, U., Wunschmann, J., Becker, S. and Rodel, G. (2006) *YIL042c* and *YOR090c* encode the kinase and phosphatase of the *Saccharomyces cerevisiae* pyruvate dehydrogenase complex. *FEBS Lett.* 580, 2553-2560
- 30 Reinders, J. r., Wagner, K., Zahedi, R. P., Stojanovski, D., Eyrich, B., van der Laan, M., Rehling, P., Sickmann, A., Pfanner, N. and Meisinger, C. (2007) Profiling Phosphoproteins of Yeast Mitochondria Reveals a Role of Phosphorylation in Assembly of the ATP Synthase. *Mol. Cell. Proteom.* 6, 1896-1906
- 31 Xie, L. X., Hsieh, E. J., Watanabe, S., Allan, C. M., Chen, J. Y., Tran, U. C. and Clarke, C. F. (2011) Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast *coq8* mutants. *Biochim. Biophys. Acta.* In Press.

- 32 Lundblad, V. and Struhl, K. (2003) *Saccharomyces cerevisiae*. In Current Protocols in Molecular Biology (Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. and Struhl, K., eds.), John Wiley & Sons, Inc., New York
- 33 Tyagi, R., Lai, R. and Duggleby, R. G. (2004) A new approach to 'megaprimer' polymerase chain reaction mutagenesis without an intermediate gel purification step. BMC Biotech. 4, 2
- 34 Jimenez-Hidalgo, M., Santos-Ocana, C., Padilla, S., Villalba, J. M., Lopez-Lluch, G., Martin-Montalvo, A., Minor, R. K., Sinclair, D. A., de Cabo, R. and Navas, P. (2009) *NQRI* controls lifespan by regulating the promotion of respiratory metabolism in yeast. Aging Cell. 8, 140-151
- 35 Rodriguez, P., Mitton, B. and Kranias, E. G. (2005) Phosphorylation of glutathione-S-transferase by protein kinase C-alpha implications for affinity-tag purification. Biotechnol Lett. 27, 1869-1873
- 36 Jiang, Y., Li, Z., Schwarz, E. M., Lin, A., Guan, K., Ulevitch, R. J. and Han, J. (1997) Structure-function studies of p38 mitogen-activated protein kinase. Loop 12 influences substrate specificity and autophosphorylation, but not upstream kinase selection. J. Biol. Chem. 272, 11096-11102
- 37 Kulikova, T., Aldebert, P., Althorpe, N., Baker, W., Bates, K., Browne, P., van den Broek, A., Cochrane, G., Duggan, K., Eberhardt, R., Faruque, N., Garcia-Pastor, M., Harte, N., Kanz, C., Leinonen, R., Lin, Q., Lombard, V., Lopez, R., Mancuso, R., McHale, M., Nardone, F., Silventoinen, V., Stoehr, P., Stoesser, G., Tuli, M. A., Tzouvara, K., Vaughan, R., Wu, D., Zhu, W. and Apweiler, R. (2004) The EMBL Nucleotide Sequence Database. Nucleic Acids Res. 32, D27-30
- 38 Blom, N., Gammeltoft, S. and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J. Mol. Biol. 294, 1351-1362
- 39 Marbois, B. N. and Clarke, C. F. (1996) The *COQ7* gene encodes a protein in *Saccharomyces cerevisiae* necessary for ubiquinone biosynthesis. J. Biol. Chem. 271, 2995-3004
- 40 Leger, J., Kempf, M., Lee, G. and Brandt, R. (1997) Conversion of serine to aspartate imitates phosphorylation-induced changes in the structure and function of microtubule-associated protein tau. J. Biol. Chem. 272, 8441-8446
- 41 Milne, D. M., Palmer, R. H. and Meek, D. W. (1992) Mutation of the casein kinase II phosphorylation site abolishes the anti-proliferative activity of p53. Nucleic Acids Res. 20, 5565-5570
- 42 DeRisi, J. L., Iyer, V. R. and Brown, P. O. (1997) Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale. Science. 278, 680-686
- 43 Forsburg, S. L. and Guarente, L. (1989) Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes Dev. 3, 1166-1178
- 44 Fondrat, C. and Kalogeropoulos, A. (1994) Approaching the function of new genes by detection of their potential upstream activation sequences in *Saccharomyces cerevisiae*: application to chromosome III. Curr Genet. 25, 396-406
- 45 Boy-Marcotte, E., Perrot, M., Bussereau, F., Boucherie, H. and Jacquet, M. (1998) Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. J Bacteriol. 180, 1044-1052
- 46 Sippel, C. J., Goewert, R. R., Slachman, F. N. and Olson, R. E. (1983) The regulation of ubiquinone-6 biosynthesis by *Saccharomyces cerevisiae*. J. Biol. Chem. 258, 1057-1061

- 47 Poon, W. W., Marbois, B. N., Faul, K. F. and Clarke, C. F. (1995) 3-Hexaprenyl-4-hydrobenzoic acid forms a predominant intermediate pool in ubiquinone biosynthesis in *Saccharomyces cerevisiae*. *Archiv. Biochem. Biophys.* 320, 305-314
- 48 Thomson, M. (2002) Evidence of undiscovered cell regulatory mechanisms: phosphoproteins and protein kinases in mitochondria. *Cell Mol Life Sci.* 59, 213-219
- 49 Ohlmeier, S., Hiltunen, J. K. and Bergmann, U. (2010) Protein phosphorylation in mitochondria --a study on fermentative and respiratory growth of *Saccharomyces cerevisiae*. *Electrophoresis*. 31, 2869-2881
- 50 Ohlmeier, S., Kastaniotis, A. J., Hiltunen, J. K. and Bergmann, U. (2004) The yeast mitochondrial proteome, a study of fermentative and respiratory growth. *J. Biol. Chem.* 279, 3956-3979
- 51 Tomaska, L. (2000) Mitochondrial protein phosphorylation: lessons from yeasts. *Gene*. 255, 59-64
- 52 Orlova, M., Kanter, E., Krakovich, D. and Kuchin, S. (2006) Nitrogen availability and TOR regulate the Snf1 protein kinase in *Saccharomyces cerevisiae*. *Eukaryot Cell*. 5, 1831-1837
- 53 Slattery, M. G., Liko, D. and Heideman, W. (2008) Protein kinase A, TOR, and glucose transport control the response to nutrient repletion in *Saccharomyces cerevisiae*. *Eukaryot Cell*. 7, 358-367
- 54 Gey, U., Czupalla, C., Hoflack, B., Rodel, G. and Krause-Buchholz, U. (2008) Yeast pyruvate dehydrogenase complex is regulated by a concerted activity of two kinases and two phosphatases. *J. Biol. Chem.* 283, 9759-9767
- 55 Do, T. Q., Hsu, A. Y., Jonassen, T., Lee, P. T. and Clarke, C. F. (2001) A Defect in Coenzyme Q Biosynthesis Is Responsible for the Respiratory Deficiency in *Saccharomyces cerevisiae abc1* Mutants. *J. Biol. Chem.* 276, 18161-18168

Figure Legends

Figure 1. Reaction catalyzed by Coq7p. Coq7p is a hydroxylase linked extrinsically to the mitochondrial inner membrane converting the demethoxy-Q₆ (DMQ₆) into demethyl-Q₆.

Figure 2. In vitro phosphorylation of Coq7p versions. **(A)** The three predicted phospho-amino acids in Coq7p sequence have been modified by site-directed mutagenesis. SST protein corresponds to the wild-type Coq7p, AAA is a Coq7p version displaying 3 alanines instead of phospho-amino acids S20, S28 and T32, and DED corresponds to a Coq7p version where the phospho-amino acids were changed into two aspartic and one glutamic acid. Locations of one letter amino acid code point to the position of a modified amino acid starting from the S20 in the protein and finishing with the T32. Coq7p-AAA is permanently dephosphorylated and Coq7p-DED is permanently negatively charged. **(B)** GST-free proteins were obtained after digestion of Coq7p-SST-GST and Coq7p-AAA-GST with thrombin and used as substrate for *in vitro* phosphorylation with [γ -32P]ATP and PKA. Phosphorylated proteins were analyzed by SDS-PAGE followed by silver staining, anti-GST immunostaining and 32P detection by a radioactivity scanner (Typhoon 9410). GST corresponds to free GST added to include a positive phosphorylation control as well as the presence of GST linked to Coq7 proteins. AAA corresponds to Coq7p-AAA and SST to Coq7p-SST. The analysis was performed with purified proteins from the fractions 6, 7 and 8 from a previous size exclusion chromatography (Figure S1). **(C)** Previously phosphorylated Coq7-GST-SST (4 μ g), Coq7p-GST-AAA (4 μ g) and free GST (2 μ g) were bound to glutathione sepharose beads. Each sample was divided in two aliquots and one was treated with λ -phosphatase (5U) after removal from the original buffer. The bound radiolabeled phosphate was monitored in treated and non-treated samples in a scintillation counter. Data correspond to a representative experiment of a set of two, and it is represented as the average \pm SD. ** p<0.01, significant differences compared to the Coq7-GST-SST protein.

Figure 3. Phosphorylation of Coq7p in purified mitochondrial isolation. **(A)** Yeast *COQ7* mutant strains expressing either Coq7p-SST or Coq7p-AAA proteins fused to V5 epitope were cultured in SDc-Ura 2% galactose for 16 hours with shaking at 30°C. Wild-type Coq7p is named as Coq7p-SST and the permanently dephosphorylated Coq7p is named AAA. Mitochondria were permeabilized, incubated with [γ -32P]ATP and analyzed by SDS-PAGE, followed by SYPRO Ruby Red staining, anti-V5 immunostaining, 32P detection by a radioactivity scanner (Typhoon 9410) and also Pro-Q Diamond staining to detect phosphorylated proteins. **(B)** Similar amounts of mitochondria were subjected to immunoprecipitation with anti-V5 and 2-DE. 2-DE gels were visualized by silver staining and analyzed by Pro-Q Diamond staining to detect phosphorylated proteins and anti-V5 immunostaining. Data correspond to a representative experiment of a set of two. M: Molecular weight markers.

Figure 4. Coq7p phosphorylation is dependent on carbon source. Wild-type *COQ7* fused to V5 (Coq7p-SST-V5) was grown in SDc-Ura 2% galactose for 48 hours. Yeast were harvested, washed and resuspended in different culture media containing several carbon sources (glucose 0.5%, 2% y 10%, YPG and YPE) and incubated for 2 hours. Control: Washed cells without incubation. Purified mitochondria from these cultures were permeabilized and analyzed by SDS-PAGE followed by **(A)** Pro-Q Diamond staining to detect phosphorylated proteins and anti-V5 immunostaining. **(B)** Densitometry analysis of phosphorylated Coq7p-SST-V5/total Coq7p-SST-V5 ratio (ImageJ v1.41o). Data correspond to a representative experiment repeated two times. **(C)** Mitochondrial samples used in previous experiments were subjected to lipid extraction and quinone quantification. Samples were analyzed in triplicate and the experiment shown is representative of a set of two * p<0.05 or ** p<0.01, significant differences compared to Control

Figure 5. Coenzyme Q₆ determination in mitochondria from Coq7p versions. Cells from a *COQ7* mutant strain harboring the indicated plasmids were cultured in SDc-ura 2% glucose for 72 hours and subjected to mitochondrial purification, lipid extraction and quinone

quantification. Samples were analyzed in triplicate and the experiment was repeated at least three times. pRS316 is an empty single copy vector that was used to clone the wild-type version of *COQ7* controlled by its natural promoter and terminator (pNMQ7) and both mutated versions of *COQ7* obtained by site-directed mutagenesis from pNMQ7 as template, Coq7p-AAA (pL-AAA) and Coq7p-DED (pG-DED). pRS426 is an empty multicopy vector that was used to clone the wild-type version of *COQ7* controlled by its natural promoter and terminator (pmQ7). Data correspond to the BY4741 background. ND: Non detectable. The amount of CoQ₆ in other Coq7p versions is shown in the Table S3. * p<0.05, ** p<0.01, significant differences compared to pNMQ7 control

Figure 6. The evolution of coenzyme Q₆ and DMQ₆ ratio in Coq7p versions in glucose based medium. Cells from a *COQ7* mutant strain harboring several versions of Coq7p were cultured in SDc-ura 2% glucose for 24 hours and subjected to mitochondrial purification, lipid extraction and quinone quantification. Samples were analyzed in triplicate and the experiment shown is representative of a set of two. * p<0.05 or ** p<0.01, significant differences compared to the previous bar. (A) pNMQ7: Wild-type version, (B) Coq7p-AAA (pL-AAA) and (C) Coq7p-DED (pG-DED).

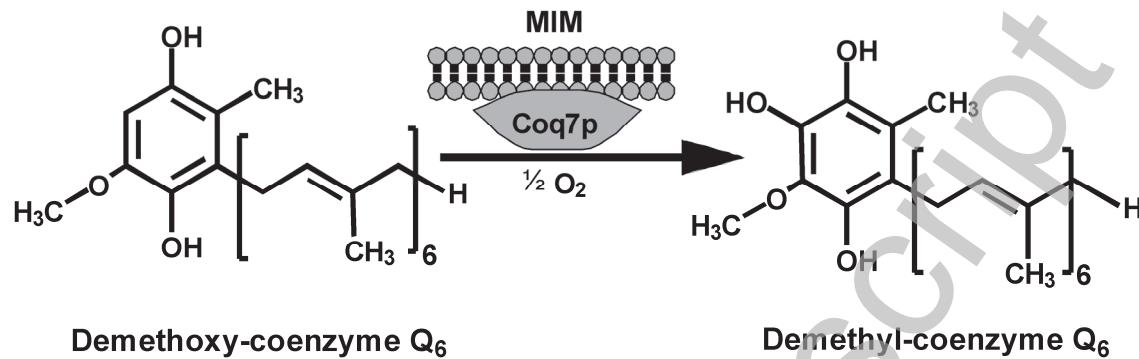
Figure 1

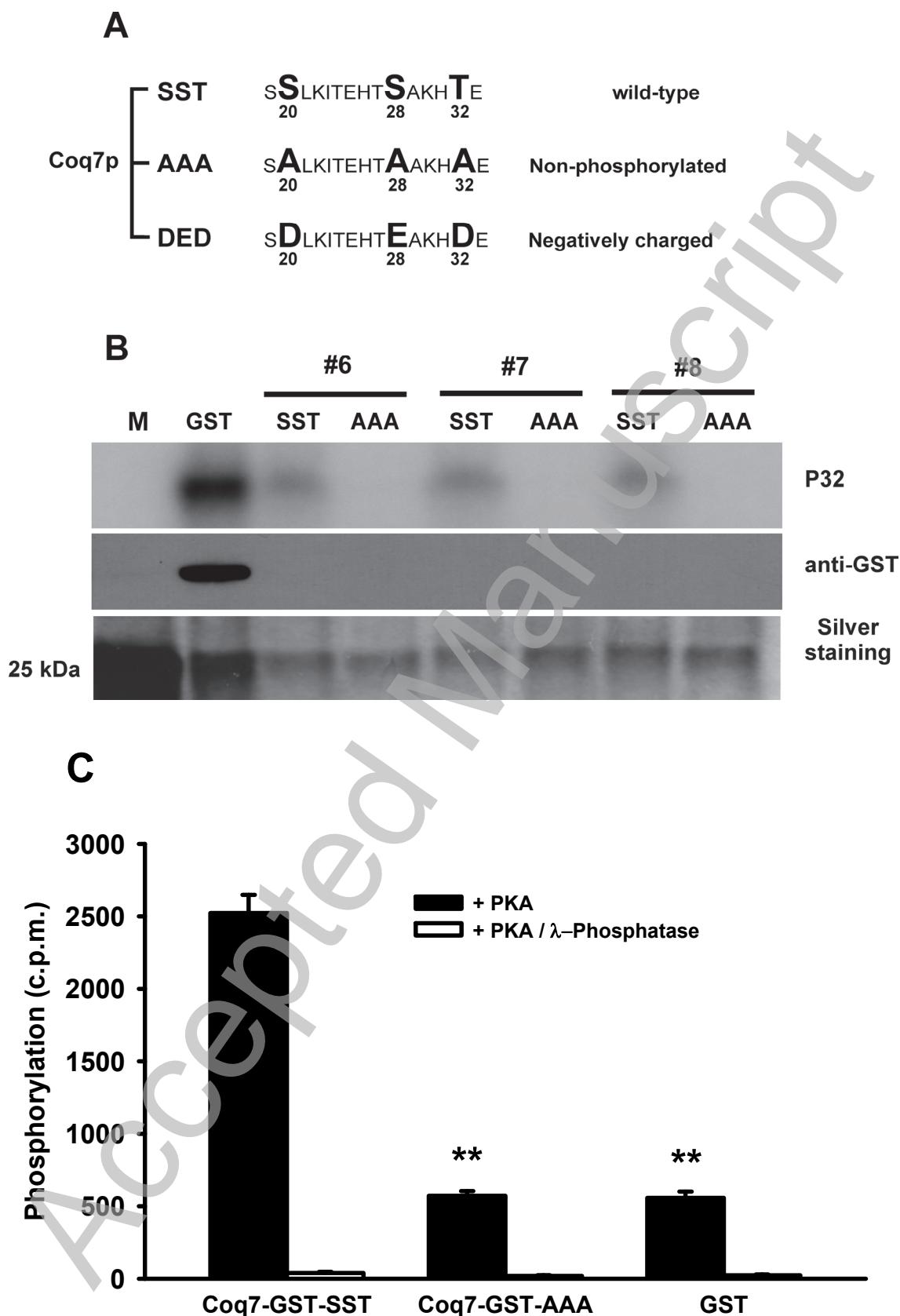
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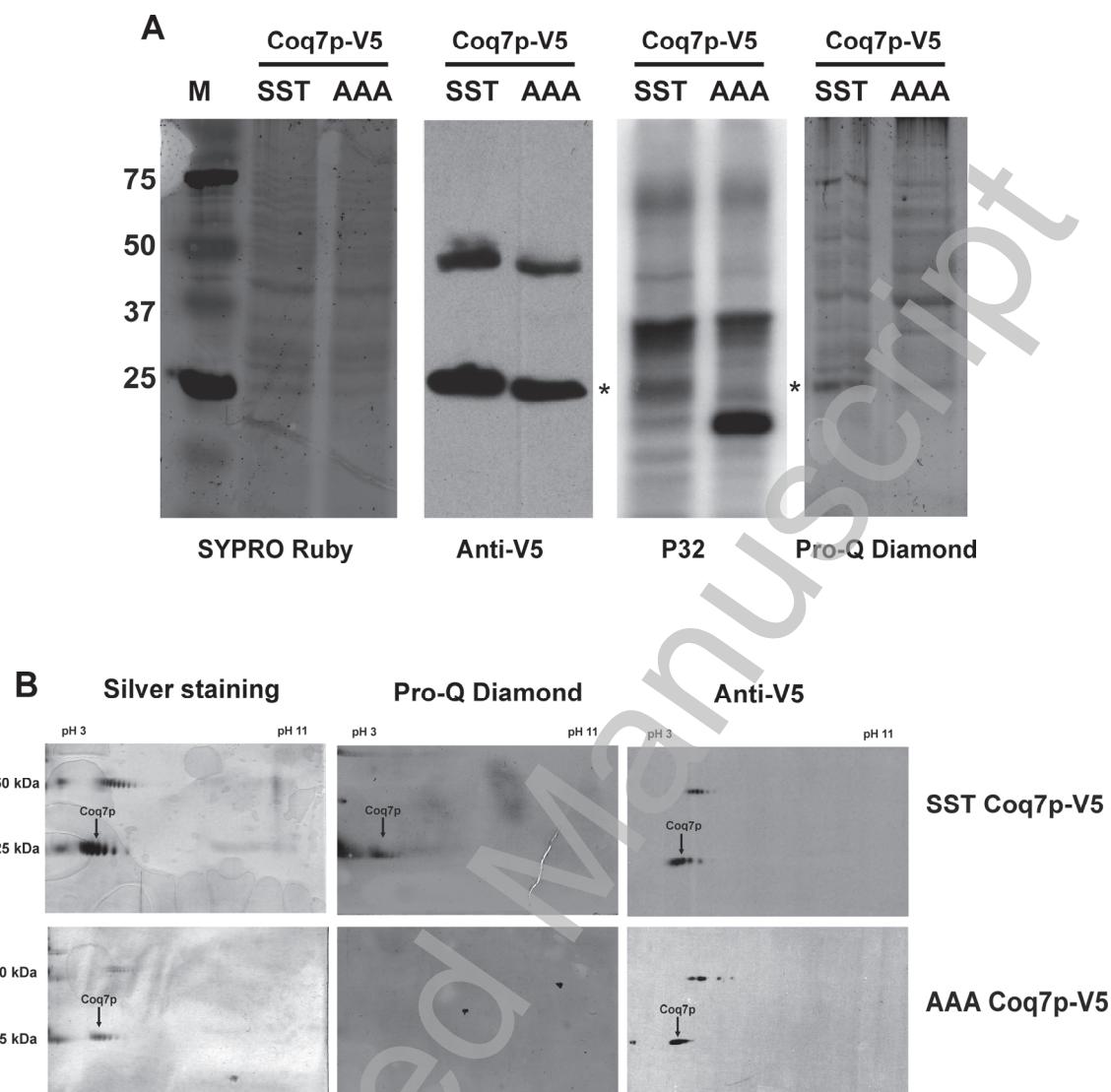
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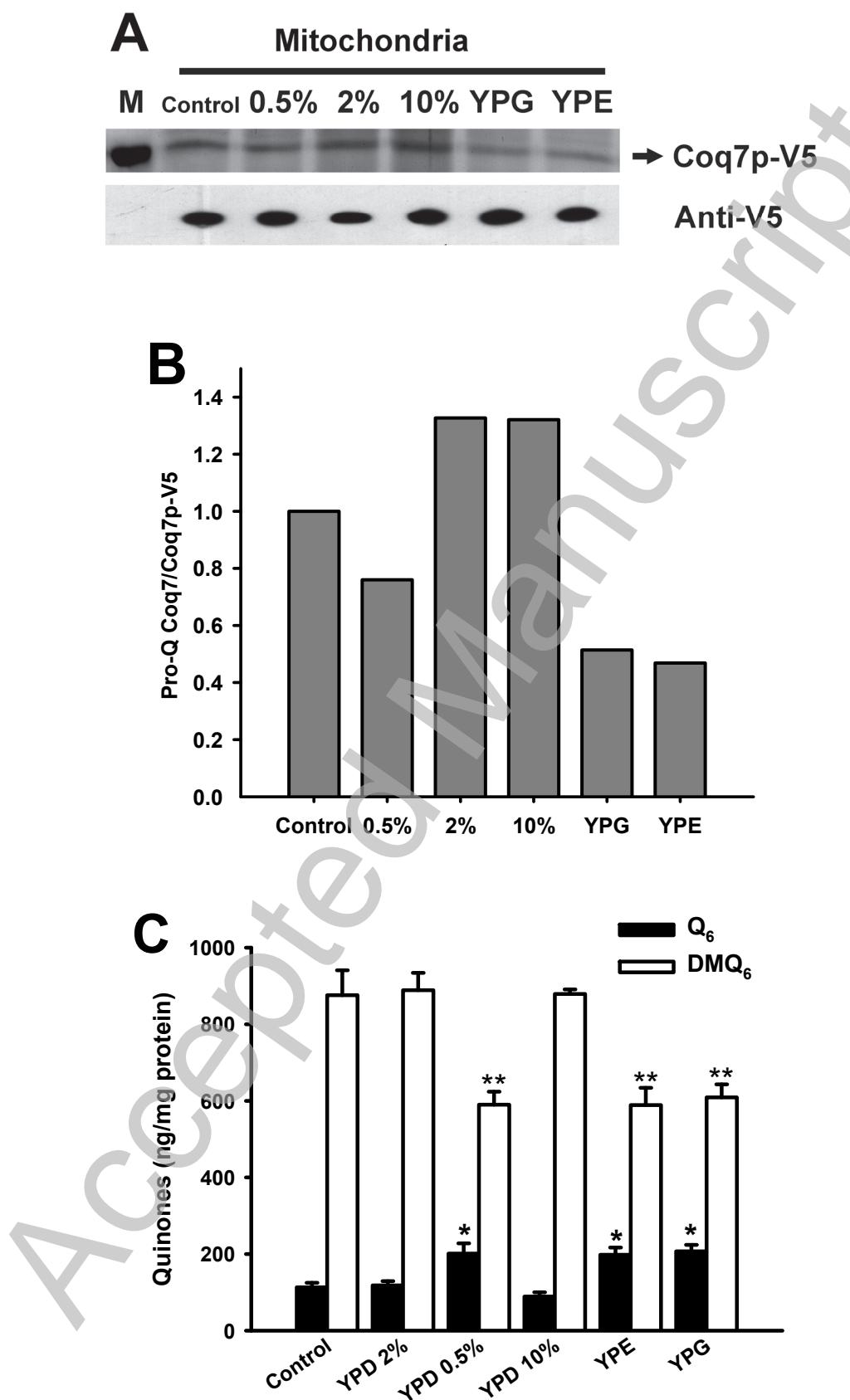
Figure 4

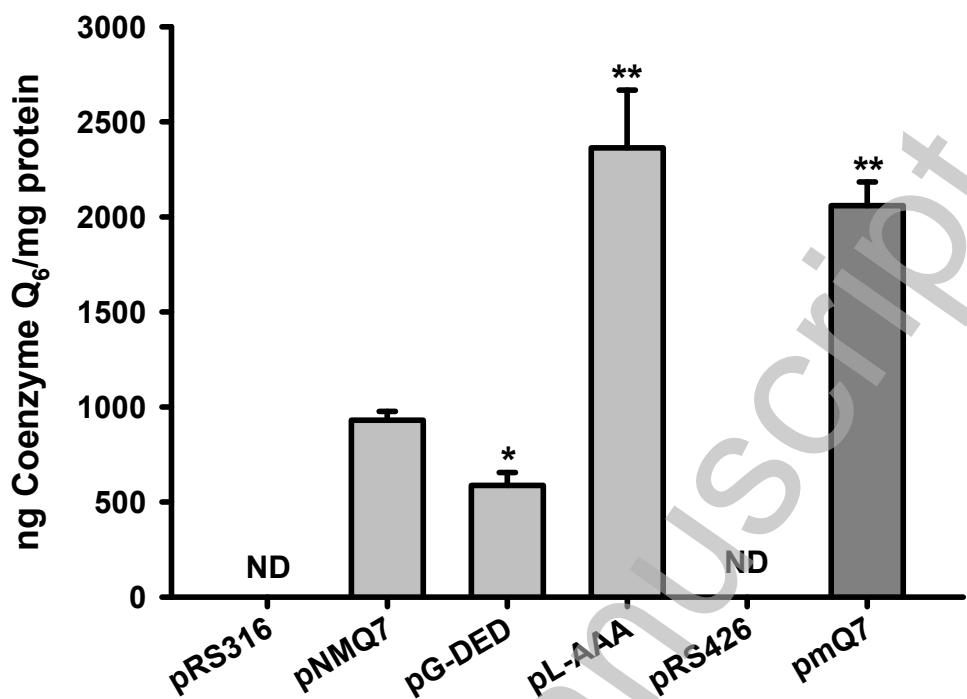
Figure 5

Figure 6