The role of Ubiquinone Synthesis Complex in *bc1* complex assembly of *Saccharomyces cerevisiae*

by

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Abstract

Mitochondria are dynamic organelles bounded by double-membranes found in eukaryotic cells carrying various critical cellular processes. Energy generation is arguably the most important of its functions. The Electron Transport Chain (ETC) is a series of protein complexes found in the inner mitochondrial membrane that possesses redox activities, by virtue of which they transfer electrons to the final electron acceptor, oxygen. This creates a proton motive force that in turn drives ATP production.

The *bc1* complex is the central component of the ETC chain and it functions in transporting electrons from Ubiquinone, a mobile electron carrier to Cytochrome c. It is made up of ten protein subunits, out of which only Cytochrome b (Cob) is encoded in the mitochondrial genome. Cob needs heme b as a co-factor and its translation is under a feed-back control that is activated in the absence of other *bc1* subunits. In addition to Cob, Cytochrome c1 (cyt1) and Reiske Iron-Sulphur protein (Rip1) are the other two catalytic redox active subunits. The other seven subunits support the structure of *bc1*. The mechanism in which the *bc1* complex subunits assemble is not fully defined. It is known to proceed in a step-wise fashion where intermediate sub-complexes of various *bc1* subunits progressively combine to form a fully functional enzyme. The goal of this study was to identify and define mechanisms of *bc1* assembly steps. The effect of deletion of Ubiquinone synthesis complex on the assembly of the *bc1* complex was investigated.

The Ubiquinone synthesis complex is a ten-protein subunit (Coq1-Coq10) complex that is needed for synthesizing ubiquinone, an anti-oxidant molecule that participates in ETC by donating electrons to *bc1*. Protein subunits of this complex are interdependent for stability. A *coq9* deletion strain was found to be defective in respiratory growth and *bc1* activity. The respiratory activities of this mutant could not be rescued by ubiquinone supplementation or COQ8 over-expression that generally stabilizes several Coq subunits indicating that the entire ubiquinone synthesis complex is necessary for stability of the *bc1* complex. Transient physical interactions between *bc1* complex and Coq complex are suggested by the co-migration of Qcr7 and Coq9 on sucrose density gradients. Basing on the data, a revised model for *bc1* assembly is proposed where the ubiquinone synthesis proteins interact with the early *bc1* assembly intermediate containing Cob and Qcr7. This interaction is a chaperoning function that stabilizes Qcr7 whose availability allows Cob translation. In the absence of a stabilizing Coq complex, Qcr7 is degraded allowing a feedback inhibition of Cob translation and stalling of *bc1* assembly. Therefore, this study has identified a novel role of Coq complex proteins in the *bc1* complex assembly that had not been described previously. Identification of this step can not only be helpful in understanding *bc1* disorder pathologies but it may also be useful in devising strategies for therapy by providing an alternative target for *bc1* disorders- the ubiquinone biosynthesis proteins.

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Chapter 1

Introduction and Review of Literature

1. Introduction

Energy generation is a process critical for the survival of any cell. In eukaryotes, energy generation occurs in double-membrane organelles called mitochondria, by the process of respiration. The inner membrane of mitochondria houses a series of five protein complexes (numbered I to V) collectively called the Electron Transport Chain (ETC) or the mitochondrial respiratory chain, which by transfer of electrons and concomitant production of a proton motive force, drives the synthesis of ATP/energy (process of oxidative phosphorylation/OXPHOS).

Each complex of the respiratory chain (I to V) is made up of several subunits. With the exception of Complex II, the respiratory complexes are made up of subunits that are encoded by both the mitochondrial and nuclear genes (Zeviani M., 2004). A delicate balance of information from the mitochondrial and nuclear genomes is therefore critical not only for the formation of a functional respiratory chain but also to adjust energy production to the energy demands of the organism (Poyton and McEwen, 1996). Accordingly, "Mutations in mitochondrial or nuclear DNA encoding subunits, components or regulators of the respiratory chain function can produce a wide range of OXPHOS diseases" (Zeviani and Di Donato, 2004).

Mitochondrial disorders comprise a group of progressive metabolic and neurological disorders that result from defects in the mitochondria. In the U.S., 1 out of every 4000 newborns is found to develop a mitochondrial disorder by the age of 10. Many of these disorders are known to occur due to defects in the assembly of one or more of the five respiratory protein complexes There are no complete cures for mitochondrial diseases, and the available treatments are rather

ineffective. An understanding of the assembly mechanisms of these complexes is therefore, critical for the development of effective treatments for such disorders.

Saccharomyces cerevisiae has been used extensively to study ETC assembly pathways as it harbors homologs of mammalian mitochondrial proteins, and employs fermentation for energy generation in the event of defective ETC complex assembly. Therefore, it is an amenable model to study respiratory mutations. Although, significantly large amounts of information on the assembly mechanisms of most of these complexes have been made available using this model, there is still a lack of information on the specifics of the *bc1* assembly pathway.

2. Mitochondrial disorders:

Mitochondrial disorders comprise a group of syndromes and clinical manifestations which may be both genetic and non-genetic, arising due to defects in assembly or functioning of the respiratory chain. Several disorders implying defects in various respiratory complexes have been identified and studied till date (Zeviani and Di Donato, 2004). These defects may arise due to mutations in either the nuclear or mitochondrial genes. They may range from localised tissue defects, such as the Bjornstad syndrome (twisted hair shafts and sensorineural hearing loss) (to fatal systemic diseases such as MNGIE syndrome (myo-neuro-gastrointestinal encephalopathy) (Selvaag, E., 2000, Taanman JW, et al., 2009).

Some of the disorders involving ETC complexes are noted below:

Complex implicated	*Genes mutated*	*Disease/Syndrome*
Complex I	NDUFS1	Leigh Syndrome, Complex I deficiency (Pronicki, M., et al, 2008)
Complex II	SDHA	Leigh Syndrome (Horvath, R. et al., 2006)
Complex III	UQCRB	Hypokalaemia, Lactic acidosis
Complex III	BCS1L	Gracile Syndrome (Visapaa, 2002), congenital cataract, ComplexIII-deficient encephalomyopathy, Renal tubulopathy (Lonlay, et al, 2001)
Complex IV	SCO1	COX^- hepathopathy and ketoacidotic coma (Valnot, I., et al, 2000)
Complex IV	COX15	COX^- hypertrophic cardiomyopathy (Antonicka, et al, 2003)
Complex V	ATP12	ComplexV-deficiency encephalopathy (Zeviani and Di Donato, 2004)

Respiratory complex disorders have no known cures. Lack of suitable animal models and rarity and heterogeneity of the diseases have contributed to the lack of development of proper treatments. Present treatments involve supportive measures such as improved and supplemental diet, treatment of seizures and other complications, correction of lactic acidosis, etc., to improve the quality of life of the patients (Parikh, S., 2009).

The development of yeast (*Saccharomyces cerevisiae)*, as a model to study respiratory complexes, is an important step towards the understanding of the pathologies of respiratory complex disorders (Tzagoloff A.,1995, Berry et al., 2000 and Zara et al., 2004). As yeast has several homologs for human ETC complex proteins, specific human disease mutations can be introduced in the yeast genes to understand their effects. Moreover, since yeast can also survive in the absence of respiration by employing fermentation, such respiratory yeast mutants remain viable for detailed studies. Blue Native PAGE (BN-PAGE), an electrophoretic technique that employs non-ionic detergents has been extensively used to study respiratory protein complex assembly pathways, as it allows these complexes to retain their native conformations (Schagger and Pfeiffer, 2000). As a result, in mutations of respiratory proteins, sub-assembly complexes have been identified that indicate the sequence in which individual protein subunits may come together (Berden et al., 1988, Crivellone et al., 1988, Grivell L. A., 1989 and Zara, et al., 2004).

Yeast models have been extensively used to study Complex III assembly. Various assembly factors such as Bcs1p, Cbp3p and Cbp4p have been identified in yeast and their corresponding human homologs have been discovered by similarity searches (Wu and Tzagoloff, 1984, Tzagoloff et al, 1992, Cruciat et al., 1999). Studies on the *bc1* complex are however, still in its infancy. The understanding of the *bc1* complex assembly mechanism using a yeast model would be pivotal in understanding Complex III disease pathologies.

3. *bc1* /Complex III/ ubiquinol-cytochrome c oxidoreductase Structure and Function:

The *bc1* complex/Complex III in human is the central most complex of the ETC, where it is embedded in the inner mitochondrial membrane. It functions in energy generation by accepting electrons from a lipophilic molecule, Ubiquinone, and transferring it to another protein cytochrome c, during which it contributes to an electrochemical gradient across the inner mitochondrial membrane by pumping protons from the mitochondrial matrix to the inter-membrane space.

The yeast *bc1* complex is made up of ten-subunits (11 in human), nine of which are nuclear encoded, while only one, Cob, which forms the central core of the complex, is encoded by a mitochondrial gene. These subunits are either inserted or bound to the inner mitochondrial membrane (Tzagoloff A.,1995, Berry et al., 2000, and Smith et al., 2004). Three of the *bc1* subunits – cytochrome b (cyt b/Cob), cytochrome c1 (cyt1) and Reiske Iron-Sulphur protein (Rip/ISP) additionally have Iron-containing prosthetic groups: heme b, heme c and an Iron-Sulphur cluster respectively, which form redox catalytic sites for participation in electron transfer. These subunits are therefore, also referred to as the catalytic subunits. The other seven subunits, core 1 (Qcr1/COR1), core 2 (Qcr2/COR2), Qcr6p, Qcr7p, Qcr8p, Qcr9p and Qcr10p, lack such co-factors and their functions are yet to be identified (Zara et al., 2009). Gene inactivation studies though have shown that their presence is necessary for the functional *bc1* complex to form (Crivellone et al., 1988).

In addition to these subunits, six post-translational assembly factors for the *bc1* complex, viz., Cbp3p, Cbp4p, Cbp6p, Bca1, Bcs1 and Mzm1 have also been reported (Wu and Tzagoloff, 1989, Crivellone, 1994, Cruciat et al., 1999, Mathieu et al, 2011, Gruschke et al, 2011, Smith et al., 2012). Bca1, Cbp3p-Cbp6p complex and Cbp4p are known to function in stabilizing the early intermediates of the *bc1* complex whereas Bcs1 and Mzm1 helps in stabilization and incorporation of Rip1 (Cruciat et al., 1999, Kronekova and Rodel, 2005, Conte et al., 2011, Cui et al, 2012, Mathieu et al., 2011, Gruschke et al., 2012).

4. Chaperones and assembly factors:

In general, molecular chaperones are a class of proteins that help other proteins to fold/ unfold in proper conformations or assemble in molecular complexes. Respiratory complexes also need the assistance of chaperone proteins to assemble. Additionally, several factors that help in translational regulation or post-translational modifications of proteins into mature forms are essential for the formation of a fully functional respiratory complex. As an example, Complex IV/Cytochrome oxidase requires the activities of about 20 different proteins for the synthesis and translation of its subunits (Barrientos et al., 2002; Herrmann and Funes, 2005; Fontanesi et al, 2006, Mick et al, 2011). For the *bc1* complex, some chaperone proteins and translational factors have been identified so far, out of which the functions of a few still remain obscure. With several steps of the *bc1* assembly mechanism yet undefined, there is a possibility of the identification of novel assembly factors and their associated functions.

4.1 Bcs1:

The Bcs1p protein is a chaperone of the *bc1* complex which recruits Rip to the *bc1* complex. Within the mitochondria, it is found to be anchored to the inner membrane. It falls under the class of AAA family of proteins (ATPases associated with diverse cellular activities), that bind to ATP and drive ATP-dependent dissociation and unfolding/ folding of nucleic acids and proteins. Like the other members of the AAA family, it has three structural domains: the N-terminal domain which is required for its targetting and import into the mitochondria, the Bcs1p-specific domain whose exact function is unknown and the C-terminal domain which specifically binds to ATP and is conserved among the AAA family of proteins (Nouet et al., 2009).

Bcs1p's role in *bc1* assembly was first identified by Tzagoloff et.al in 1992. BCS1 deletion strains showed respiratory activity deficiency and the levels of Rip1 were found to be significantly low in such strains. Cruciat et al., (1999) analyzed the respiratory complexes by BN-PAGE and further confirmed its chaperone activity. In their study, BCS1Δ strains were unable to form the respiratory *bc1* complex, and the *bc1* assembly halted at the pre-Rip addition step. Although indicating a *bc1* assembly role for Bcs1, these studies failed to detect any direct interaction between Rip1 and Bcs1p.

To date, several mutations in the human homolog Bcs1L have been identified and most have been found to be involved in clinical complications in humans. BCS1L was initially found to be mutated in patients suffering with renal tubulopathy, encephalopathy and liver failure (Lonlay et al., 2001). It was later also implicated in GRACILE (Growth Retardation, Amino aciduria,

Cholestasis, Iron overload, Lactic acidosis, and Early death) syndrome, a recessively inherited lethal disease characterized by fetal growth retardation, lactic acidosis, aminoaciduria, cholestasis, and abnormalities in iron metabolism (Visapaa et al., 2002). In addition, point mutations in BCS1L were also found to lead to a rare inherited disease, the Bjornstad syndrome, marked by sensorineural hearing loss and baldness (Hinson et al., 2007). Although presenting different clinical manifestations, all BCS1 mutations show significant complex III deficiency.

Since yeast and human homologs have high sequence similarity, many studies have tried to understand the mechanism behind disease conditions by introducing human mutations in yeast. Nouet et al. (2010) tried to analyze the function of mutant amino acid residues in the context of its structure. Their study demonstrated that 2^{nd} / 3^{rd} domain junction amino acids are critical for Bcs1 activity and stability. The study also proposed the presence of non-*bc1* complex partners for this chaperone. Conte et al., (2011) recreated three different human mutations in yeast BCS1 and found that two could cause respiratory incompetency in yeast. Further in these mutants, Bcs1p was found as a *bc1* assembly arresting, non-interacting 400 kDa complex.

Since BCS1L mutations are responsible for a majority of *bc1* disorders, and since its mechanism of action remains undefined, it is important to design experiments which could give further insights on both disease pathology and *bc1* complex assembly.

4.2 Mzm1: *M*itochondrial *z*inc *m*aintenance protein is another recently identified bc1 assembly factor. It was first linked to the maintenance of labile pools of Zn found in the mitochondrial matrix (Atkinson, 2010). Later it was confirmed that the stability of the Zn pools is not solely

dependent on Mzm1 but rather is based on the availability of the other bc1 subunits, such as Qcr7 and Qcr9. However, in *mzm1Δ* yeast cells, bc1 assembly was shown to be stalled at the late core assembly intermediate step. Further, these cells were shown to have decreased stability of Rip1, which was further exacerbated at 37˚C. This defect could be suppressed by the over-expression of Rip1 (Atkinson, 2011). Typically, un-inserted Rip1is unstable especially so at higher temperatures, as is also seen in this mutant. Therefore it is postulated that mzm1 may function in the insertion of Rip in the mitochondrial inner membrane either by stabilizing it or by presenting it to Bcs1.

LYRM7, or MZM1L for Mzm-like protein that bears amino acid sequence homology to yeast Mzm1 was recently identified and characterized in human cells and was shown to be involved in bc1 assembly (E. Sanchez et al, 2013). Expression of the human homolog was shown to be able to suppress the mzm1Δ defect in yeast cells. LYRM7 was shown to physically interact and comigrate with UQCRFS1, a cleavage product of the Rip1 protein that is additionally found in the human *bc1* complex. Expression of the human homolog was shown to be able to suppress the mzm1Δ defect in yeast cells. However in contrast to Mzm1 in yeast, elevated expression of LYRM7 in human cells resulted in the retention and accumulation of UQCRFS1 in the mitochondrial matrix impairing the maturation of bc1. Therefore, stoichiometric ratios of LYRM7 and its interacting partner are critical for a functional *bc1* complex.

4.3 Cbp3 and Cbp6:

Cbp3 and Cbp6 are proteins that act as a complex for the efficient translation of Cob mRNA. Cbp3 is a 39kDa membrane protein whose deletion leads to the loss of steady state levels of apocytochrome b, Qcr7 and Qcr8 subunits (Wu and Tzagoloff, 1989). Cbp6 is a 18kDa protein

whose absence leads to respiratory deficiency and concomitant loss of cytochrome b (Dieckmann and Tzagoloff, 1985). It has been recently shown that Cbp3-Cbp6 complex bind to the ribosomal exit tunnel and interact with the newly translated Cob (Gruschke et al, 2011). It remains bound to Cob till the addition of the early *bc1* subunits Qcr7 and Qcr8. This therefore, is not only necessary for efficient translation of Cob but also introduces a regulatory feedback loop where the assembly progression of *bc1* is tied to cytochrome b synthesis (Gruschke et al, 2012).

4.4 Bca1: Many of the assembly factor encoding genes of respiratory complexes belong to a group of puf-3 dependent genes whose mRNA contains a Puf-3 binding motif. Bca1 is a Puf3-dependent mitochondrial inner membrane protein with an IMS facing soluble C- terminal domain. It was identified in a transcriptome screen by Mathieu et al (2011). Its deletion leads to inability to grow on lactate containing non-fermentable media, decreased heme b levels, decreased steady state-levels of Cyt b and Rip1 subunits and diminished *bc1* supercomplexes. Further, in *bca1Δrip1Δ* cells , pre-Complex III, a stable 500kDa complex that accumulates when late assembly subunits such as Rip1are absent, is diminished. In wild type cells, immuno-precipitation assays fail to pull down any *bc1* subunits and 2D gels detect low molecular weight streaks of Bca1. However, 600kDa oligomers of Bca1 accumulate in rip1Δ cells. These results lead to conclusions that Bca1 may interact transiently with the *bc1* subnunits at its early assembly steps, prior to the insertion of Rip1.

5. The *bc1* assembly pathway:

The *bc1* assembly mechanism is a little explored and a not well understood area (Zara et al., 2009). Rapid strides though are presently being made in this field. Most of the initial models of *bc1* assembly were proposed basing on yeast deletion studies, where a single or a pair of *bc1* subunit genes were deleted and its effect was observed on the *bc1* complex structure. (Berden et al., 1988; Crivellone et al., 1988 and Grivell L. A., 1989). These and recent studies (Zara et al., 2004 and Zara et al., 2007) have suggested the sequential assembly of the functional *bc1* complex, with the help of intermediate sub-complexes.

Blue native Polyacrylamide gel electrophoresis (BN-PAGE), followed by 2D electrophoresis and immunodecoration helped to confirm the existence of both the central core- Qcr7-Qcr8-Cob and Qcr1-Qcr2 sub-complexes in various individual experiments. (Schagger and Pfieffer, 2000, Dudkina et al., 2005, McKenzie et al., 2007 and Zara et al., 2007).

The Qcr7-Qcr8-Cob subcomplex was the earliest stable *bc1* intermediate described by various groups (Berden et al., 1988, Crivellone et al., 1988, Grivell L. A., 1989 and Zara et al., 2009). It was indicated to form the central core of the *bc1* complex embedded in the mitochondrial inner membrane (Hunte et al., 2002). The stability of this sub-complex was found to be dependent on the presence of the three participating subunits. In the absence of any one of the three subunits this sub-complex is undetectable, indicating that proteins turnover or fail to assemble in the absence of the interacting partners. Similarly, a second sub-complex of Qcr1 and Qcr2 was

found consistently in various yeast deletion strains, lacking one or more of the supernumerary subunits. (Berden et al., 1988, Crivellone et al., 1988, Grivell L. A.,1989 and Zara et al., 2009).

Qcr9, Qcr10 and Rip were found to be involved in the late steps of *bc1* assembly (Brandt et al., 1994, Berden et al., 1988, Crivellone et al., 1988 and Grivell L. A., 1989). The deletion of these subunits did not result in degradation of the above-mentioned subcomplexes. However, Rip deficiency leads to respiratory defect in yeast, while Qcr10 deficient cells retain nearly 60% of the wild type *bc1* activity (Brandt et al., 1994). The exact interactions between these two subunits are yet to be understood. Nonetheless, later studies identified Bcs1p, an assembly chaperone, which helps in recruiting Rip to the *bc1* complex (Tzagoloff et al., 1992 and Cruciat et al., 1999). Further, an interaction between Rip and Qcr9p was also indicated, in a study dealing with ΔQCR1, ΔQCR2 and ΔCYT1, where both subunits were found to form a 44kDa complex (Zara et al., 2007). Since the size of this complex is almost twice the combined sizes of Rip and Qcr9p, it suggests the presence of additional assembly factors.

Recently, Zara et al. (2007) demonstrated the presence of a larger stable 500kDa complex in ΔQCR9 yeast strains. 2D PAGE and immunodecoration helped to determine the composition of this complex to be one made of Cob, cyt1, Qcr1, Qcr2, Qcr6p, Qcr7p, Qcr8p, Qcr9p. Rip was also detected in the mitochondria but as a non-interacting 35kDa complex. A similar 500kDa complex was also demonstrated previously in ΔISP and ΔBCS1, but the exact composition could not be determined (Cruciat et al., 1999). Conte et al. (2011) confirmed that this 500 kDa complex is a stable productive intermediate of the *bc1* complex and not a *bc1* degradation product.

In wild type yeast cells, the *bc1* complex forms supracomplexes with cytochrome oxidase. BN-PAGE analysis of wild type mitochondria detects *bc1* in a 670, 850 and 1000 kDa complex; the larger two of which include one and two molecules of cytochrome oxidase. Qcr6p is found in 670 and 1000 kDa complexes and not in the 850 kDa complex. It was also detected in the 500 kDa complex found in ΔBCS1 and ΔISP strains (Conte et al., 2011). Further the presence of the assembly factor Bcs1p, was also detected in both the 500 kDa in mutants and the 670 kDa in wild type cells (Zara et al., 2007, Conte et al., 2011).

Basing on different results from these studies, a putative model for *bc1* assembly pathway has been proposed (Zara et al., 2009, Gruscke et al, 2012). Cob seeds bc1 assembly at the mitochodrial inner membrane. It is translated and bound to Cbp3-Cbp6 complex at the ribosomal tunnel exit. After release from the ribosome, Cob-Cbp3-Cbp6 along with Cbp4 form Intermediate I. This setup awaits the addition of Qcr7 and Qcr8, that releases Cbp3 and Cbp6, this in turn makes Cbp3-Cbp6 available for further rounds of Cob translation. Qcr7, Qcr8, Cob and Cbp4 mark Intermediate II of the *bc1* complex. Qcr1 and Qcr2 are added next which releases Cbp4 forming Intermediate III. Qcr6 and Cyt1 sub-complex joins Intermediate III to form a stable 500 kDa subcomplex/Intermediate IV. Qcr9p, Qcr10p and Rip are added in the final step that is facilitated by Mzm1 and Bcs1 to form the final functional *bc1*. The present model (Fig. 2) as it stands, does not answer questions such as: what are the factors that facilitate Qcr7/Qcr8, Qcr1/Qcr2 and Qcr6/Cyt1 sub-complex assembly prior to its loading in *bc1*? At what step and in what manner does Cob recruit heme and if there are dedicated chaperones for heme loading in *bc1*. These and other such questions should be directing the future course of *bc1* assembly

studies, since they would address deeper understanding of respiratory complex biogenesis as well as help to understand mitochondrial disease mechanisms.

6. Ubiquinone synthesis complex:

Ubiquinone (UQ) or Coenzyme Q (Q) is an important lipid component of the electron transport chain; it serves as a link in the transfer of electrons between Complex II and Complex III of ETC. In a recent study it was indicated that each *bc1* monomer has 3 tightly bound UQ molecules (Bartoschek et al., 2001). In performing its redox role, UQ cycles between oxidized and reduced states (UQ/UQH_2). Incompletely oxidized UQ, semiquinone, cause oxidative damage to the cell and therefore the *bc1* complex' role of oxidizing UQH_2 is critical for this aspect.

UQ synthesis is found to occur in the matrix side of the mitochondrial inner membrane (Hseih et al., 2007, Johnson et al., 2005). UQ biosynthesis requires the activities of 9 different polypeptides: Coq1, Coq2, Coq3, Coq4, Coq5, Coq6, Coq7, Coq8 and Coq9. Coq10, a new gene is also found to play a role in UQ synthesis, but possibly as a chaperone, since its deficiency causes decreased respiratory activity while the levels of UQ are not affected (Barros et al., 2005). Yeast Coq mutants are non-respiring (unable to grow on respiratory medium that contains glycerol or ethanol) while on fermentative medium they form petite (small) white colonies.

Several studies have indicated that there is a strong interdependence of the Coq polypeptides in terms of structure and function. For eg., COQ9 null mutants show a dramatic decrease in the

products of COQ3 and COQ5 genes (Poon et al., 1999, Belogrudov et al., 2001). Additionally, yeast cells with mutations in any gene from COQ3 to COQ9 accumulate the same biochemical intermediate of UQ, 3-hexaprenyl-4-hydroxybenzoic acid (HHB) (Poon et al., 1997, Johnson et al., 2005). Further, BN-PAGE analyses of wild-type yeast mitochondrial proteins have shown that Coq2, Coq3, Coq4, Coq7 and Coq9 migrate together in a high molecular weight complex (Tran et al., 2006).

Since UQ is the substrate of the *bc1* complex, its role in stabilizing the *bc1* complex cannot be ruled out. Various studies with yeast COQ mutants have suggested a similar role for UQ and/or the Coq complex. Cytochrome c1 levels were remarkably diminished in UQ deficient cells, which returned to normal when UQ was added exogenously to the cells. (Santos-Ocana et al., 1998). In a similar study, cyt b levels were also found to be significantly reduced in UQ deficient cells (Johnson et al., 2005). Additionally, Coq8 was initially postulated to be a chaperone of the *bc1* complex. Deletion of Coq8 was shown to drastically decrease *bc1* activity and halve Cytochrome oxidase activities. However, no change was seen in the steady state levels of *bc1* subunits or the assembly of the mature complex (Brasseur et al, 1997). Additional studies are therefore needed to determine the exact role of UQ/Coq complex in assembling or stabilizing the *bc1* complex.

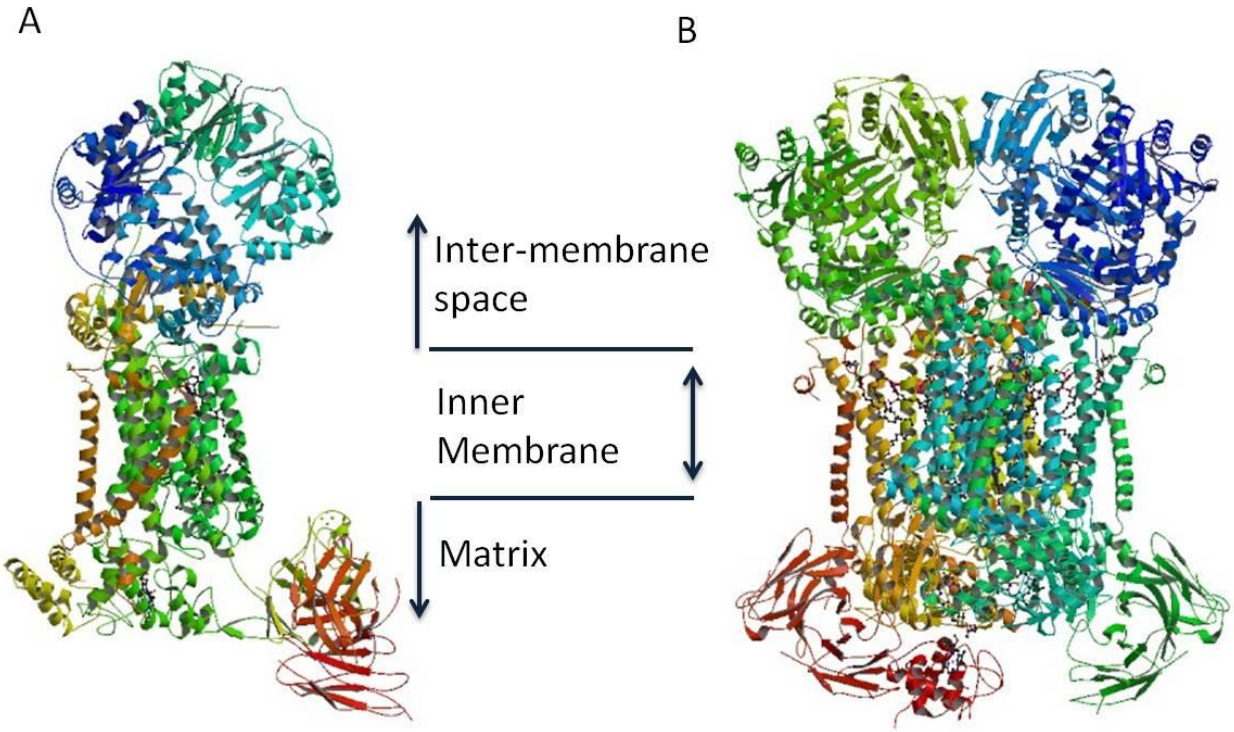

Figure 1: Model of *bc1* structure. A) A monomer of *bc1* complex that is found in the inner mitochondrial membrane. Cob is represented in bright green with 8 membrane-spanning helices, Qcr1 in red, Qcr2 in green on matrix side interacting with Qcr1, Qcr7 in olive green on the matrix side, Qcr8 in gold, Qcr6 in green on the IMS side, Qcr9 in dark brown across membrane, , Cytochrome c1 in blue, Rip1 in purple and Qcr10 in cyan. B) A *bc1* dimer representing a catalytically active complex.

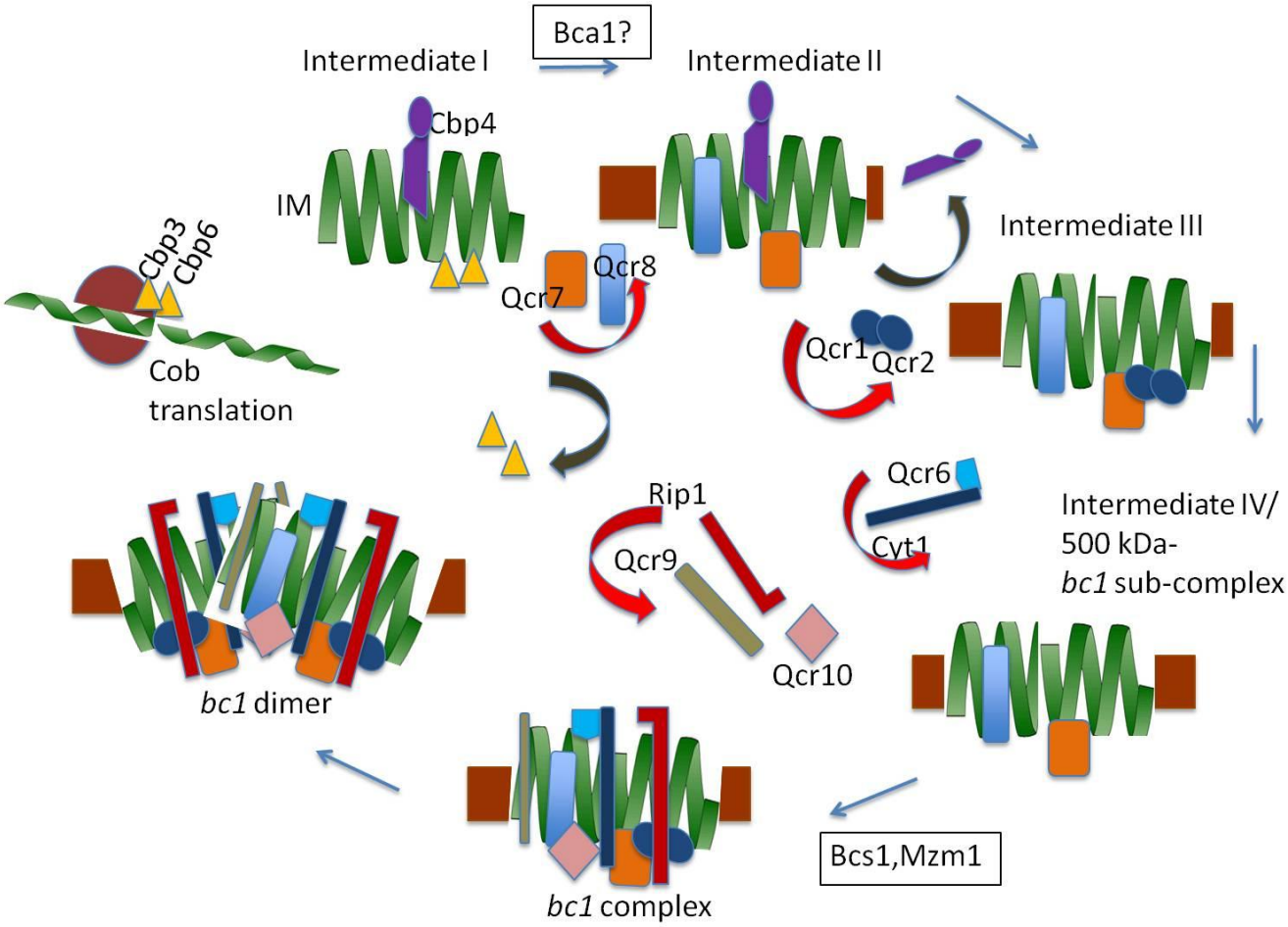

Figure 2: Schematic of *bc1* complex assembly. Cob is the only mitochondrially-encoded *bc1* subunit. It is inserted in the inner membrane with the continuous interaction of post-translational factors Cbp3-Cbp6 (first bound to Cob at ribosomal exit tunnel) that along with Cbp4 forms Intermediate I. The addition of Qcr7 and Qcr8 forms Intermediate II and leads to removal of these factors, while Cbp4 remains bound. Intermediate III is formed upon insertion of Qcr1-Qcr2 and removal of Cbp4. Cytochrome c1 and Qcr6 are added next to form the 500kDa sub-complex. Qcr9 is added next and the combined actions of Bcs1 and Mzm1 inserts Rip1. Qcr10 is the last subunit that is added in the assembly pathway. Dimerization of *bc1* leads to the formation of a functional complex. The exact step where a second set of subunits are added in the monomer is still unknown.

The red curved arrows indicate insertion of specific subunits in the complex at the indicated steps. Grey curved arrows indicate removal of a specific assembly factor/chaperone. Square boxes indicate known chaperones of *bc1* assembly pathway. However, the exact role of Bca1 is undefined.

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Chapter 2

Ubiqunone synthesis complex deficiency leads to Qcr7 turnover and *bc1* heme defect

Abstract

The *bc1* complex is the central component of the energy-generating mitochondrial electron transport chain. It is made up of 10 protein subunits, three of which require catalytic cofactors that include heme B, heme C and an Fe:S cluster. It has been demonstrated using various *bc1* deletion mutants that the assembly of the *bc1* complex occurs through a series of stages that involve the formation of sub-complex assembly intermediates. However, the assembly pathway of the *bc1* complex is not yet fully defined. The mechanism by which heme cofactor is inserted in Cytochrome b, the only mitochondrially-encoded subunit that seeds *bc1* assembly is still a mystery. Additionally, it is speculated that unidentified factors/chaperones may be involved in stabilizing the *bc1* intermediates prior to their insertion.

In the present study we demonstrate that in the absence of a Coq complex protein – Coq9, the *bc1* activity and oxygen consumption is reduced. This decreased activity cannot be reversed by the supplementation with a Ubiquinone analog, Q2. Moreover, high-pressure liquid chromatography measurements indicate a drastic reduction in heme levels of the coq9 deletion mutants. The heme levels could not be reversed by the addition of heme precursors. There was no accumulation of heme intermediates such as porphyrin and the molecules such as ergosterol that need heme-containing enzymes for biosynthesis showed a normal profile indicating that heme biosynthesis machinery is intact in our mutant. However, the heme defect seemed to correlate with decreased Qcr7 stability and *bc1* assembly. The over-expression of Qcr7 does not rescue the depleted heme levels indicating that an intact Coq complex that is destabilized in any Coq subunit deletion is necessary for *bc1* stability. Further, Qcr7 instability and heme depletion

implicates the role of Coq complex in the early *bc1* assembly steps. This study therefore, demonstrates for the first time a possible role for the Coq complex in *bc1* assembly.

1. Introduction

The ubiquinol-cytochrome c reductase (*bc1* complex/Complex III) is an important component of the mitochondrial respiratory chain, a series of protein complexes required for energy generation in eukaryotes. In *Saccharomyces cerevisiae,* it is made of nine nuclear and one mitochondrially-encoded subunit (Zara et al., 2004). Three subunits- Cob, Cyt1 and Rip1 are catalytic while the rest (Qcr1, Qcr2, Qcr6, Qcr7, Qcr8, Qcr9, Qcr10) are believed to provide structural stability (Zara et al., 2004). While Cob, the heme b containing redox subunit seeds the *bc1* core; other supernumerary subunits along with Cyt1 and Rip1 assemble around it to form a functional complex. The assembly of *bc1* complex is facilitated by various assembly factors, having translational and post-translational roles. The complete assembly of *bc1* occurs in a modular fashion progressing through a series of assembly intermediates.

Cbs1 and Cbs2 in an as yet ill-defined manner, act as translational activators of Cob mRNA (Rodel, 1986). In addition, Cbp3 and Cbp6 bind to the ribosomal tunnel exit and interact with the newly synthesized Cob, thereby releasing the protein and making it available for the additional factor - Cbp4 to bind (Gruschke et al, 2011). Cbp4, Cbp3, Cbp6 and Cob thereby form the first *bc1* intermediate. Cbp3-Cbp6 have been postulated to also participate in a feedback loop that blocks further synthesis of new Cob by being unavailable, in the absence of the subunits next in line in *bc1* assembly (Gruschke et al, 2011). The subsequent addition of Qcr7 and Qcr8 releases Cbp3-Cbp6 forming intermediate II, containing Cbp4, Cob, Qcr7 and Qcr8. Cbp4 is released with the addition of Cor1 and Cor2 forming intermediate III. Qcr6 and Cyt1 addition forms the 500 kDa stable sub-complex containing Cob, Qcr7, Qcr8, Cor1, Cor2, Qcr6, and Cyt1 (Zara et

al, 2007). Rip1, Qcr9 and Qcr10 are finally added to complete the assembly. Here, Mzm1 helps in stabilizing the Fe:S cluster of Rip1 before its incorporation by Bcs1 in the inner membrane (Atkinson 2011; Cruciat 1999). Recently Bca1, another factor discovered by a transcriptome screen, has been shown to be involved in *bc1* assembly (Mathieu, 2011). The exact intermediate it affects is debatable and is only observed in Δbcs1 and Δrip1 mutants in a 600kDa complex or as low molecular weight complexes in a wild type cell (Mathieu, 2011).

It is speculated that several additional factors needed for *bc1* assembly are as yet un-identified. Additionally, details of the mechanism of sub-complex assemblies are also yet to be defined. One important question is how cofactor insertion occurs in the catalytic subunits and what regulates the balance between insertion and assembly progression.

In the present study, the affect of Coq complex proteins on the stability of *bc1* subunits is analyzed. It is found that the deletion of Coq9 destabilizes the Qcr7 subunit of the *bc1* complex and greatly perturbs the levels of heme cofactor in a *coq9* mutant. We propose that the stability of Coq complex is critical for maintaining *bc1* heme levels and stabilizing *bc1* complex.

2. Materials and Methods

2.1 Yeast strains and plasmids used:

Cells: S288C (ATCC 201388: MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, Qcr7::TAP) from Open Biosystems. Additionally, a *coq9* deletion was constructed in a S288C-Qcr7::TAP strain.

Plasmids used: pRS326 (met25-coq9myc-cyc1), pRS426 (met25-qcr7myc-cyc1)

2.2 Yeast growth media: Yeast cells were cultured in liquid media containing 1% Yeast Extract, 2% Peptone and 2% Dextrose (YPD) for extraction of mitochondria and other assays. For analysis of respiratory growth, cells were normalized at OD600 and serially diluted before plating on solid media containing 1% Yeast Extract, 2% Peptone and 2% Lactate -Glycerol (YPLG). 6μM Q_2 and 1mM Vanillic Acid were used as additives in the media when needed. Synthetic media containing 2% dextrose (SCD) and lacking relevant amino acids were used for selection of yeast transformants. $FeCl_3$ (100mg/L) and Aminolevulinic acid (ALA- 250μM final) were used to drive heme synthesis exogenously whenever needed.

2.3 DNA (Genomic and Plasmid) extraction, PCR amplification and detection:

Genomic DNA was obtained from the desired yeast cells by using the 5 Prime DNA extraction kit following the manufacturer's protocols. Similarly Plasmid DNA extraction was carried out using Qiagen's miniprep kit. DNA quantification (A260) was done using ND1000 v3.7.1 software and the Nanodrop spectrophotometer. PCR amplifications were performed using the Eppendorf mastercycler personal machine. Primer annealing temperatures and extension times

were calculated and optimized basing on the primer melting temperatures and length of the desired amplicon respectively.

DNA electrophoresis was carried out using 1% agarose gels in 1X TAE buffer (40 mM Tris Acetate, 1mM EDTA, pH 8.2-8.5). DNA samples were electrophoresed at 100 V for 45 minutes. A 10kb DNA ladder from New England Biolabs (NEB), was used as a standard for DNA size measurements. For the detection of the bands, Ethidium Bromide was added at a concentration of 12.5µg/L of 1% agarose. An AlphaEase imager was used to visualize DNA bands.

2.4 Yeast transformation: Yeast cells (50ml) were grown overnight to OD600 of 1.0 in YPD liquid media. The cells were washed twice with ice-cold sterile water and once with chilled 1M Sorbitol by centrifugation (3500rpm for 5 mins at 4˚C). The cells were then suspended in 2ml of 1M Sorbitol and 65 µl of cell suspension was electroporated at 1700 V with 2-2.5 µg of the vector using an Eppendorf eporator. The electroporated cells were immediately resuspended in 1 ml of 1M Sorbitol and allowed to recover for 30 mins to 1 hour at 30˚C before plating on the appropriate selection plates.

2.5 Oxygen consumption assay: Desired yeast cells (3ml) were grown overnight in YPD or appropriate synthetic liquid medium to OD600=1.00. Cells were washed twice with miliQ water and finally resuspended in 3 ml water. A biological oxygen monitor (YSI) was used to record oxygen consumption readings of cell suspensions at an interval of every 30 seconds for a total of 3 minutes. The difference of the final-initial reading was divided by the O.D of the cells to calculate values that depicted rates of oxygen consumption.

2.6 Generation of Qcr7::TAP *coq9Δ* mutants: The yeast gene knockout cassette (Kan^r gene) was amplified from a COQ9 deletion strain of the commercial yeast deletion collection using primers appended with homologous regions surrounding 100-200bp upstream and downstream regions of the COQ9 gene, forward primer: 5'-CCATTCACC TATACAGTAGTATAC-3' and reverse 5'-CAGCAGCTTCCACTGGCCCAGG -3'.

The 1.4 kb amplicon was transformed into Qcr7-TAP (Open Biosystems) strain by electroporation and plated on YPD+G418 (.3mg/ml) plates. Genomic DNA from transformants were analysed by amplifying with the primers followed by size comparison. Positive clones resulted in 1.4 kb amplicons while false positives gave approximately 1000 kb amplicons, corresponding to the original COQ9 open reading frame.

2.7 Cloning and expression of Qcr7-myc: Qcr7 gene was amplified from wild type cells by using primers: Forward 5'-GCGGTGCGGGATCCATGCCACAGTCTTTTACG-3' and reverse, 5'-CCCAAAGCTCGAGTCACAGGTCTTCTTCAGAGATCAGTTTCTG TTCCAGGTCTTCTTCAGAGATCAGTTTCTGTTCTTTGGAGACCTCTATGTTG-3'. The forward primer contained sites for BamHI and the reverse primer had sites for XhoI restriction endonucleases. Both primers encoded homology to regions upstream and downstream of the Qcr7 gene. Additionally, the reverse primer had three myc sequence repeats. After amplification the amplicon and pRS426 vector containing met25 promoter and cyc1 terminator were cut with restriction endonuclease BamHI and XhoI. This was followed by ligation of the amplicon to the vector with T4 DNA Ligase and transformation into *E .coli* and plating on LB-Ampicillin plates.

Positive colonies were tested by restriction drop out of Qcr7-myc gene from the vector. Positive plasmids were sequenced for confirmation and transformed in wild type and Qcr7::TAP *coq9Δ* cells.

2.8 Mitochondrial Protein isolation and measurement: 1 Liter overnight cultures grown in liquid YPD at 30˚C in a shaking incubator were used to obtain mitochondrial proteins. Following centrifugation of the cultures at 3500 rpm for 5 minutes the cells were treated with a suspension of lyticase (Sigma) in 1.2 M Sorbitol (1000 units/gm cells) and incubated for 2-4 hours at 30˚C. The cell suspensions were then centrifuged at 3500 rpm for 10 minutes. The pellet was then vortexed three times with glass beads for a duration of a minute each. Following this, the mixtures were centrifuged at 1500 rpm for 5 minutes to remove the broken cell membranes and the supernatant was centrifuged at 15000 rpm for 10 minutes to obtain crude mitochondrial pellets. The pellets were then resuspended in 10 ml of 1.2 M Sorbitol and 10 ml of 22% Nycodenz in 1.2 M Sorbitol was overlaid before spinning at 12000 rpm for 50 minutes. The supernatant was finally centrifuged at 12000 rpm for 30 minutes to obtain purified mitochondrial proteins. Protein concentrations were measured by Bradford's assay. 2 µl of the isolated mitochondrial proteins were mixed with 998µl of Bradford's reagent (Amresco) followed by incubation for five minutes at room temperature. The OD_{595} of the mixture was measured using a Shimadzu UV 2450 UV-Visible spectrophotometer and the concentrations were calculated using the values generated from UVProbe software. Varying BSA concentrations were used to generate the standard curve.

2.9 SDS-Polyacrylamide Gel Electrophoresis and Western blot analysis: Appropriate concentrations (25, 30 or 40µg) of proteins were denatured by boiling in 1X Laemmli buffer (0.1% 2-Mercaptoethanol, 0.0005% Bromophenol blue, 10% Glycerol, 2% SDS , and 63 mM Tris-HCl (pH 6.8)) (Laemmli, 1970) followed by separation on polyacrylamide gels (10%). Gels were run at 150 V for 2 hours and electro-blotted onto nitrocellulose membranes for 1.5 hours at 150V. Subsequently, membranes were blocked with 5% BSA in PBS for 1 hour and probed overnight with appropriate primary antibodies (anti-TAP (Genscript), anti-myc (Abcam) or anti-porin (Abcam) antibodies) reconstituted in 1X PBS followed by probing (1 hr) with appropriate fluorescent Cy3 or Cy5 secondary antibodies (GE-Amersham) reconstituted in 1X PBS. Visualization of proteins was done using a LAS4100 imager.

3. Results and Discussion

3.1 Respiratory Growth Phenotype of *Δcoq9*

Coq9 is a component of the ubiquinone synthesis complex. As discussed previously, a loss of any of the proteins of this complex leads to turnover of the other subunits, and thereby ubiquinone synthesis is hindered in such mutants. A *Δcoq9* mutation in the S288C yeast background was generated homologous recombination with a $G418^r$ cassette. The mutant showed inability to grow on glycerol containing fermentative medium (Fig1a.). This could be explained to be as a result of the obstruction of the respiratory chain (ETC) activity, as ubiquinone participates in donating electrons to the *bc1* complex. This phenotype is also in line with the previous published reports of coq deletion mutants that exhibit inability to grow on non-

fermentative media (Hseih et al, 2007). However, as expected, respiratory growth is regained by complementation with Coq9-myc, expressed from a high copy vector, pRS326, also confirming the accuracy of gene knockouts.

In order to identify the possibility of a defect in the components of the ETC apart from ubiquinone synthesis, two approaches were followed:

1. Oxygen consumption was measured for the mutant in the presence and absence of exogenous Ubiquinone. Oxygen consumption levels were expected to rise to wild type levels if the mutant harbored only a Ubiquinone defect. Contrastingly, *Δcoq9* with supplemented UQ did not show an improvement in oxygen consumption (Fig. 1c). This necessitated the need for assaying the respiratory enzyme activities to detect possible defects. Cytochrome oxidase activities were about 50% of the wild type cells. Interestingly, SDH-bc1 activity was remarkably low in *Δcoq9* (Fig.1b). Since the exact enzymatic function of Coq9 has not yet been defined, it was hypothesized that Coq9 may have a dual role of being a ubiquinone synthesis complex member that also affects *bc1* independent of the presence of ubiquinone. It is interesting to note that a similar result was obtained in a *COQ8* deletion mutant in a previous study (Brasseur et al, 1997). The assembly of *bc1* complex was unaffected in such mutants; however, the electron transfer kinetics of the complex and Quinol binding was impaired. Moreover, Ubiquinone supplementation partially rescued the respiratory deficiency in *in vitro* tests.
2. Mitochondria extracted from mutant cells were used to test heme levels, as visual inspection of proteins showed decreased pigmentation (Fig. 2a). HPLC analysis demonstrated a drastic decrease in heme levels (Fig. 2a). A direct correlation between

low *bc1* activity and heme levels could be made, as heme is an essential cofactor of the *bc1* catalytic subunits (Cob and Cyt1).

3.2 Qcr7- an early *bc1* intermediate subunit is unstable in *Δcoq9*

To test the effect of *COQ9* deletion on *bc1* subunits, the steady state levels of *bc1* early assembly subunit- Qcr7 was analyzed. The *COQ9* deletion was generated in a Qcr7-TAP background and western blots were probed with anti-TAP antibody. Results indicate that Qcr7 is unstable in absence of Coq9 (Fig. 3a). Complementation with Coq9-myc vector regained wild type Qcr7 levels. This demonstrates that the absence of Coq9 disrupts *bc1* stability by acting on early *bc1* assembly intermediates. Qcr7-Qcr8 are the earliest structural subunits that seed *bc1* assembly after the incorporation of Cob in the inner membrane (Zara, 2009, Gruschke, 2012) . It has been previously shown that Qcr7-Qcr8 are unaffected by absence of Cob translation. Therefore, it is possible that Coq9 affects Qcr7 directly by chaperoning the Qcr7-Qcr8 intermediate or by regulating Qcr7 at the translational level.

3.3 Qcr7 translation is unaffected by Coq9 mutation

To test whether Coq9 regulates the translation of Qcr7, QCR7-myc was cloned and expressed from a regulated promoter (*MET25*) of a high copy vector in wild type cells. Western blots revealed that Qcr7 was expressed normally from this construct (Fig. 3b), suggesting that the affect of Coq9 is post-translational. Further, expression of this construct in the deletion seems to exert a stabilizing effect on the endogenously expressed Qcr7-TAP that would otherwise be turned over (Fig. 3b). However, this stabilization is not sufficient for rescuing heme levels (Fig. 3d) or oxygen consumption in the mutant (Fig. 3e). This suggests that coq complex/Coq9 may

have a heme chaperoning role in *bc1* assembly, which is followed by the attachment of supernumerary subunits like Qcr7. Additionally, it is important to note that Qcr7 has a conserved Puf3 binding motif as seen in RNA-protein interaction screens (Freeberg, 2013). Bca1, an assembly factor for *bc1,* was identified based on its Puf3 dependent expression (Mathieu, 2011). It is therefore likely that Qcr7 is an assembly factor that is needed in early steps of *bc1* assembly, which is also subsequently retained in the complex. The stability of Qcr7 is in turn dependent on the presence of the Coq complex that is perturbed by the absence of Coq9.

3.4 Heme as a marker for *bc1* complex defect

It can be hypothesized that the low levels of heme in *Δcoq9* is a result of three possibilities:

1. Turnover of heme in the absence of Qcr7:

   If Qcr7 functions as a heme-stabilizing chaperone, supplementation of excess heme may overcome the requirement for Qcr7 binding. A similar outcome could be expected if on the other hand, the only role of Coq complex in *bc1* assembly is to act as a chaperone of heme.

2. Inhibition of heme synthesis :

   If there is a heme biosynthesis defect then the other proteins needing heme would also be defective. Alternatively, a porphyrin peak, would be detected when testing for heme via HPLC.

3. Inhibition of Cob translation:

   This supports the model of Gruschke et al, 2012, that if early *bc1* intermediates are not available, Cbp3-Cbp6 are not free for Cob translation. Therefore, the resulting absence of Cob leads to the absence of the heme cofactor.

The first hypothesis was tested by incorporating heme biosynthesis precursors iron and amino-levulinic acid into the growth medium. It can be predicted that the presence of precursors would increase the levels of heme available in the cell and increased flux of heme may bypass the need for Qcr7 to act as a chaperone feeding heme into the *bc1* complex. Additionally, presence of heme may bypass the need for Coq9 if it is involved in heme synthesis steps.
Data indicates that growth in the presence of precursors does not lead to the production or accumulation of heme (Fig. 2b). The levels of heme remain nearly the same as that of the untreated mutant, i.e., about 30% of the wild type strain. This rules out the possibility that excess heme may rescue respiratory growth in *Δcoq9*.

Secondly, none of the coq null mutants indicate an accumulation of heme synthesis intermediates such as porphyrin by HPLC analysis (Fig. 4b). This indicates that heme synthesis pathway is unaffected in the mutant. The probability of Coq9 affecting heme synthesis is eliminated as other heme-requiring enzymes such as those involved in ergosterol synthesis were stable (Fig. 4a). Additionally, the dramatic decrease in cellular heme in *COQ9* mutants implies that the *bc1* may be the major sink of heme in the mitochondria.

Therefore, the most plausible explanation for the heme defect in our mutant based on this and previous works (Gruschke et al, 2012) is the absence of Cob to receive heme. Cob translation and availability for receiving heme and seeding *bc1* complex assembly depends on the presence of early subunits such as Qcr7. Qcr7 is translated independent of the Coq complex. It is possible that the Coq complex chaperones heme before feeding it into Cob that allows for Qcr7 addition.

The absence of the Coq complex leads to rapid turnover of heme and degradation of the Qcr7 subunit via a protease. Qcr7 turnover stalls Cob in a Cbp3-Cbp6-Cob complex thereby inhibiting further Cob translation which is supported by the result that supplementation with heme precursors or overexpression of Qcr7 fails to rescue *bc1* assembly. Alternatively, a dedicated chaperone inserts heme while Coq complex stablizes Qcr7-Qcr8 sucomplex. The absence of Coq complex fails to stabilize and insert Qcr7-Qcr8 that leads to a feedback loop that stalls Cob translation. However, it remains to be tested if Coq/Qcr7-Qcr8 complex or heme-Coq complex could be isolated in future experiments.

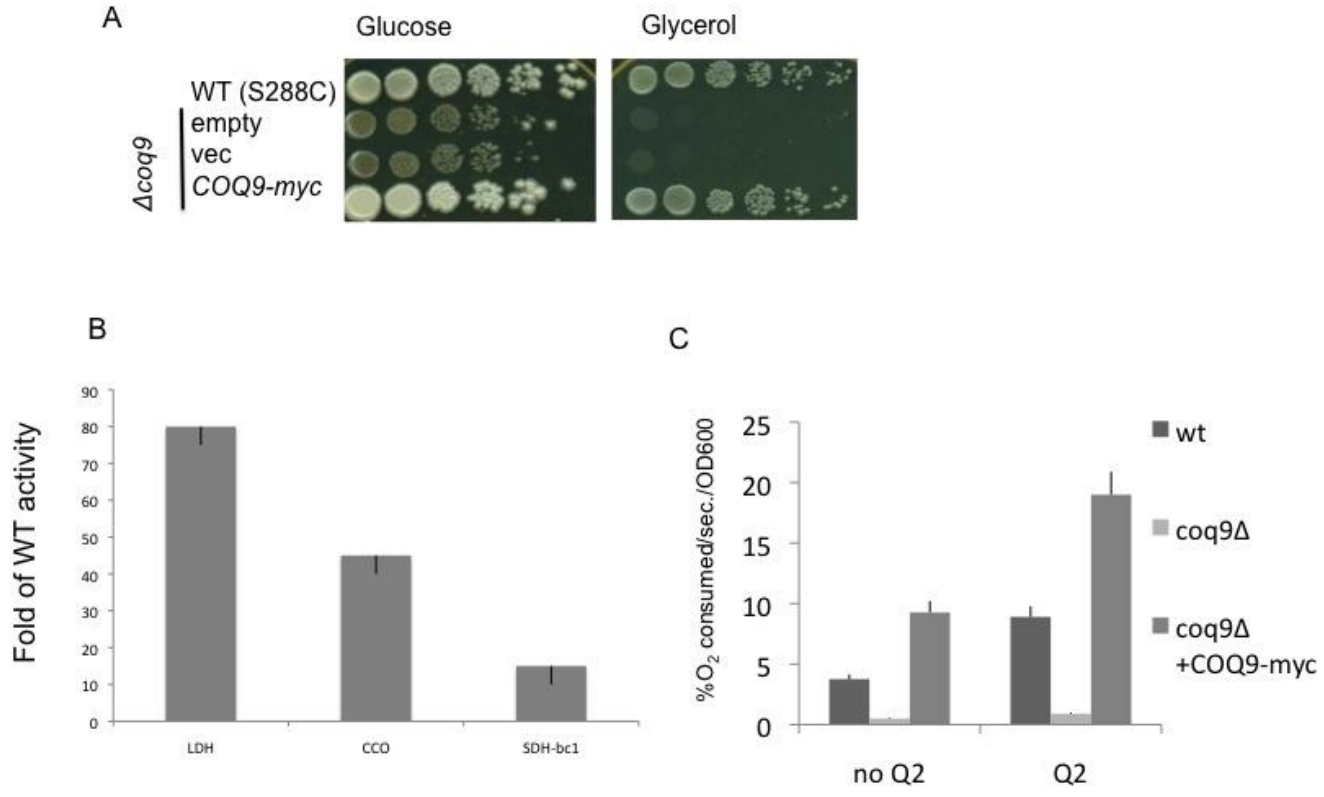

Figure1. *coq9* null mutant shows respiratory defect. A) Growth phenotype of *Δcoq9* cells on fermentative and respiratory media (glucose and glycerol). Wild-type (WT) and deletion mutant (*Δcoq9)* with no vector, an empty vector and vector expressing *COQ9-myc* were spotted on solid media plates at equivalent cell densities and serially diluted 10-fold five times. Plates were incubated at 30°C. Drop tests indicate that growth of *Δcoq9* cells is severely retarded on glycerol-containing media. B) Lactate dehydrogenase(LDH), Cytochrome c oxidae(CCO) and Succinate dehydrogenase-bc1 activities were assay spectrophotometrically. *Δcoq9* cells have secerely reduced *bc1* activity while other enzymes are comparable to wild type cells. C) Effect of exogenous supplementation of Q2 on oxygen consumption and mitochondrial heme. Equal cell amounts obtained from WT, *Δcoq9* and *Δcoq9+COQ9-myc* liquid glucose cultures were tested for oxygen consumption in the absence and presence of 6μM Q2(a Q6 analog) using an oxygen probe. Supplementation with Ubiquinone does not increase oxygen consumption in *Δcoq9.* Presence of the polypeptide is necessary for respiration.
Data are presented as a mean ± SD of three independent experiments. The statistical result for O_2 consumption by Student's *t*-test was: *coq9*Δ vs. WT (no Q2) and *coq9*Δ vs. WT(Q2), $P<0.05$ and *coq9*Δ (no Q2) vs. *coq9*Δ (Q2), $P>0.05$.

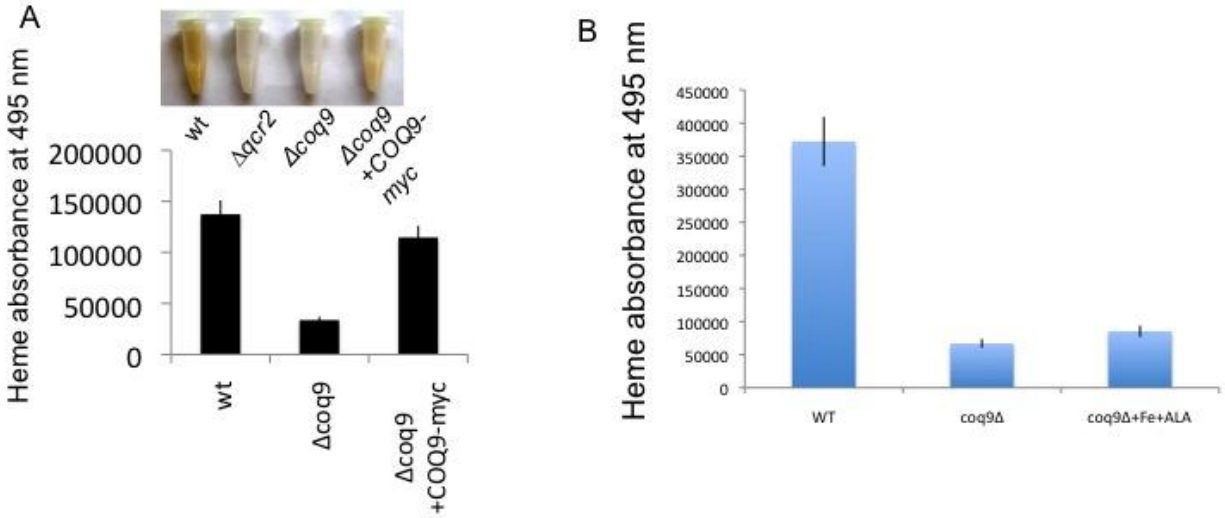

Figure 2. *Δcoq9* has decreased heme concentrations, which is not rescued by supplementation with heme biosynthesis precursors. A) Heme absorbance measured by HPLC from equal concentrations of total mitochondrial proteins extracted from WT, *Δcoq9* and *Δcoq9+COQ9-myc* cells. Student t-test shows P-value for *Δcoq9* vs. WT and *Δcoq9* vs. *Δcoq9*+COQ9myc, <0.05. The inset shows the relatively pale color of mitochondrial mixture obtained from *Δcoq9* that resembles the *bc1* complex mutant, *Δqcr2.* B) Supplementation with heme precursors (Fe and ALA) does not rescue wild type heme levels in *Δcoq9. P*-value for WT vs *Δcoq9* and WT vs. *Δcoq9*+Fe+ALA is <0.05, and for *Δcoq9* vs. *Δcoq9*+FE+ALA is > 0.05.

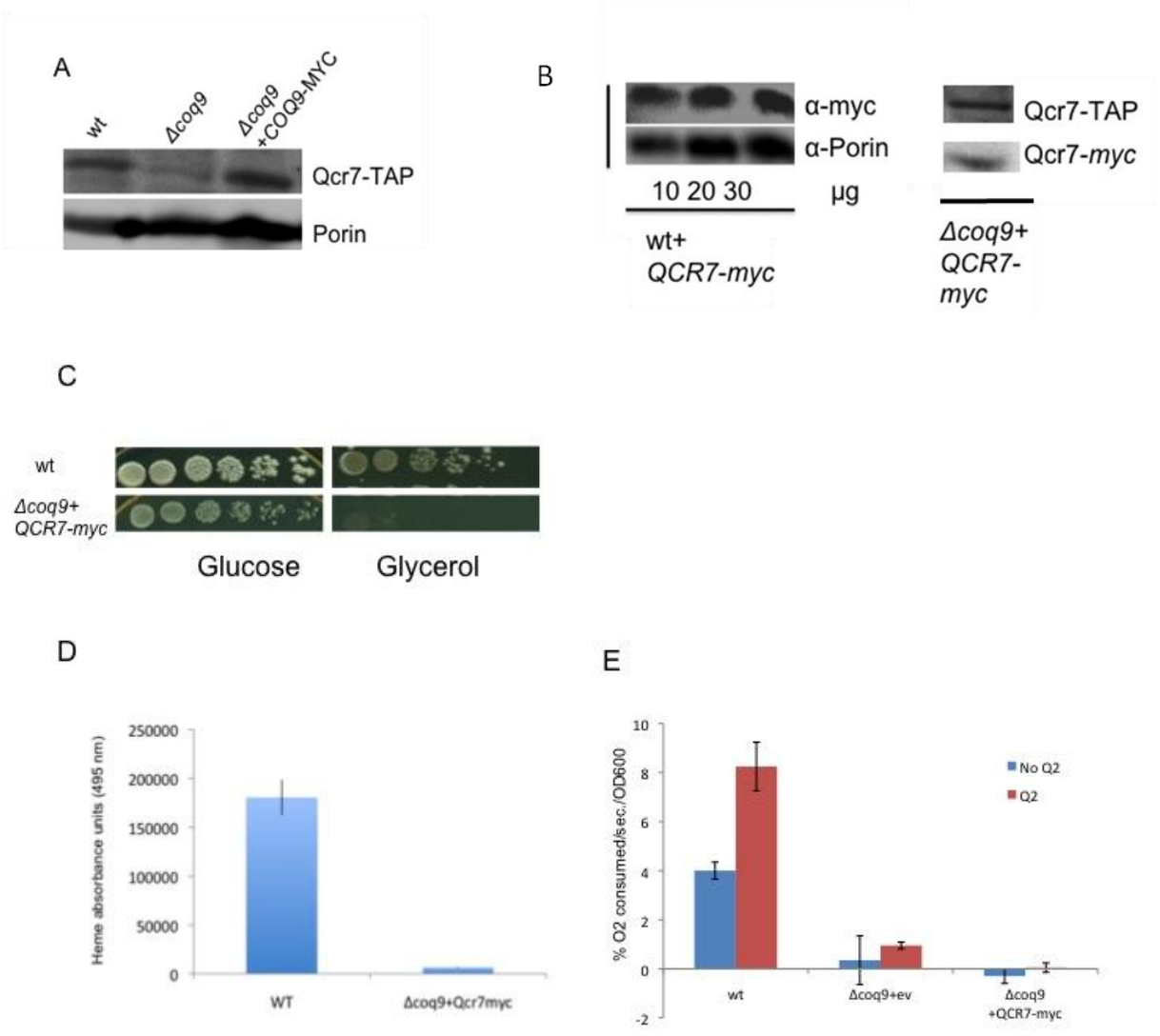

Figure 3. Stability of Qcr7 and the effect of its overexpression in *Δcoq9.* A) Western blot of Qcr7-TAP from WT, *Δcoq9* and *Δcoq9*+*COQ9*-myc cells using anti-TAP antibody. Qcr7 is unstable in *Δcoq9* but is stabilized by complementation with *COQ9-myc.*Porin blot was used as control for equal protein loads. B) Upper panel shows a western blot of three concentrations, 10, 20, 30 μg of mitochondrial protein isolated from WT cells transformed with a vector over-expressing Qcr7-myc. Anti-myc antibody was used to detect Qcr7-*myc*. Lower panel shows a western blot of Qcr7 in a *Δcoq9* cell probed with anti-TAP and anti-myc antibodies. The exogenous expression of Qcr7-myc stabilizes endogenously expressed Qcr7-TAP. C) Respiratory growth phenotype of *Δcoq9* on glycerol media upon overexpression of Qcr7-myc. D) Heme absorbance measured by HPLC indicates no increase in the mutant over-expressing *Qcr7-myc*. The *P*-value of WT vs. *Δcoq9*+Qcr7myc is <0.05. E) Oxygen consumption does not increase in the presence of over-expressed Qcr7 and excess Ubiquinone in *Δcoq9. P*-value by student t-test for WT vs. *Δcoq9*+Qcr7myc (no Q2) and WT vs *Δcoq9*+Qcr7myc (Q2)is <0.05 and P-value for *Δcoq9*+ev vs. *Δcoq9*+Qcr7myc (in both conditions-no Q2 and Q2) is >0.05.

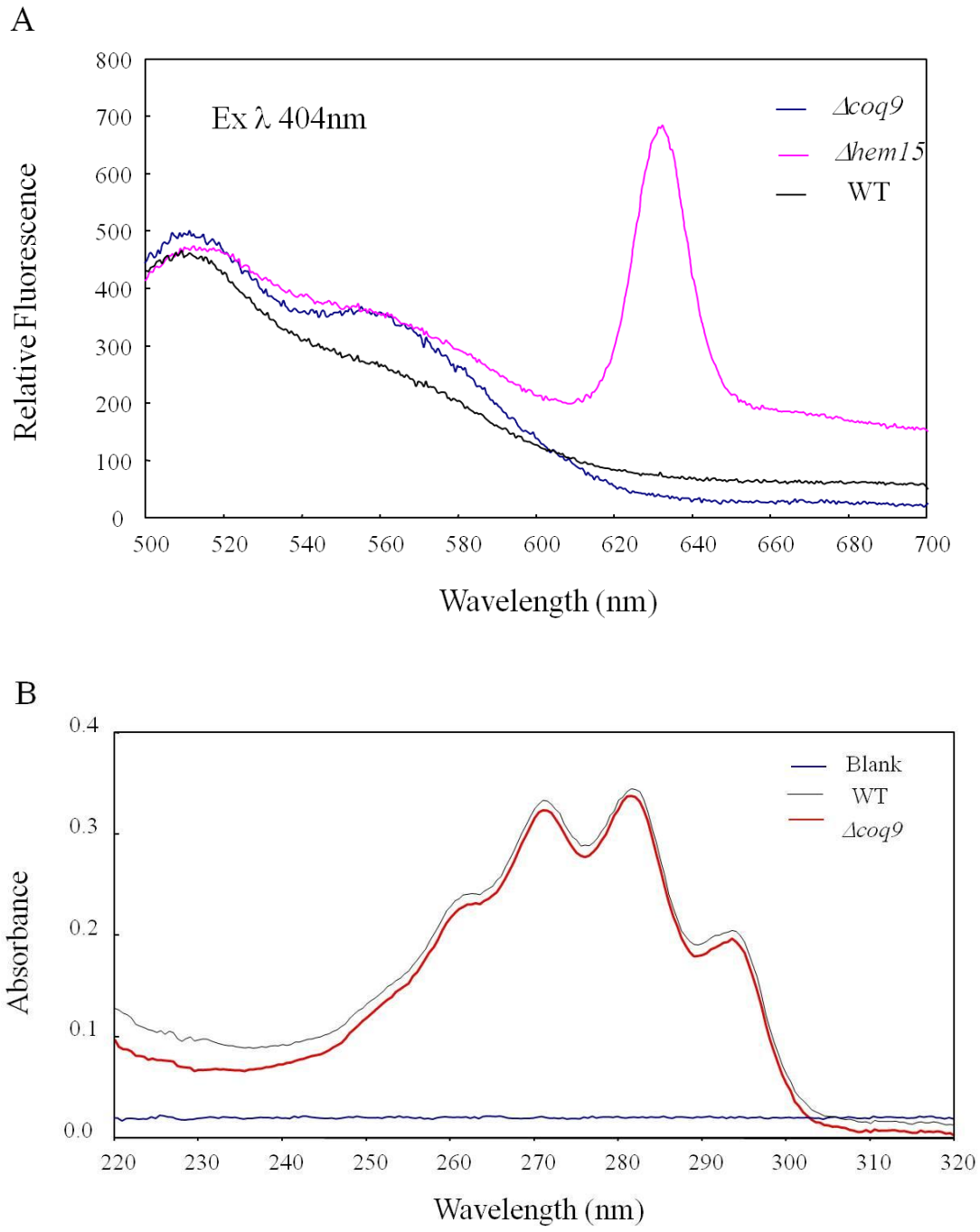

Figure 4. Heme biosynthesis pathway is unaffected in *Δcoq9*.A) Porphyrin levels- a heme intermediate that accumulates in heme biosynthesis mutants was measured in the mutant. *Δcoq9* does not accumulate porphyrin while *Δhem15* shows a buildup.
B) Ergosterol and dihydroergosterol levels of wild type and *Δcoq9* were measured. The levels of these proteins are normal in the mutant.

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Chapter 3

Ubiquinone synthesis complex interacts with *bc1* subunits at an early assembly step

Abstract

Ubiquinone is a substrate of the *bc1* complex of the electron transport chain, which is responsible for producing energy in eukaryotes. However, the interactions between the Ubiquinone synthesis proteins (Coq1-10) that form a complex at the mitochondrial inner membrane, and the *bc1* complex subunits have not been characterized. In the previous experiments (Chapter 1), we have shown that the deletion of Coq proteins lead to inability of the *bc1* complex to assemble completely. Moreover, we have shown that Coq9 deletion leads to defective heme levels and Qcr7 protein instability, both of which are critical for early *bc1* assembly steps.

In the present study, the direct physical interactions between the *bc1* complex subunit- Qcr7 and Coq9 were studied. Firstly, TAP purification of *bc1* proteins from coq9 deletion indicated decreased stability of the various *bc1* proteins, confirming our previous results. Secondly, migration of Coq9-myc and Qcr7-TAP on sucrose density gradient fractions indicated the presence of a 400 kDa complex that may be an interface for the physical contacts between Coq complex and *bc1*. These results were further supported by immunoprecipitation experiments that in some attempts were successful in isolating an interaction between Coq9 and Qcr7.

With the data generated, the model of *bc1* assembly could be revised by adding a Coq complex component in *bc1* assembly pathway. We propose, that in the early steps of *bc1* assembly, the Coq complex stabilizes the *bc1* intermediates Qcr7-Qcr8 prior to their addition in the inner membrane. The absence of a stable Coq complex therefore, leads to stalling of *bc1* complex assembly beyond the Cob insertion step.

1 Introduction

Ubiquinone (UQ) is a lipophilic molecule that shuttles electrons between *bc1* and Cytochrome c. Therefore, its role is critical for the functioning of ETC. It is synthesized at the inner membrane of the mitochondria by the concerted activities of Coq enzymes, on the precursor Hydroxy-benzoic Acid (HBA). Each Coq protein therefore, catalyzes a specific chemical reaction in the conversion of HBA to UQ. It has been shown previously that the stability of Coq proteins are dependent upon each other (Hseih et al, 2007). Therefore, any *coq* gene deletion leads to the same intermediate, 3-hexaprenyl-4-hydroxybenzoic acid (HHB) being accumulated (Poon et al., 1997, Johnson et al., 2005). Experiments involving feeding of alternative precursors such as para-aminobenzoic acid and vanillic acid along with over-expression of the kinase, *COQ8*, that stabilizes many of the Coq proteins (except Coq9), have been pivotal in identifying the exact chemical reaction that each Coq protein catalyzes due to the accumulation of diagnostic intermediates (Xie et al., 2012).

A majority of the Coq proteins are present in a large complex associated with the inner mitochondrial membrane (Gin and Clarke, 2005, Hseih et al, 2007). Previously, Rip1 has been shown to co-migrate with this high molecular weight complex of Coq proteins (Hseih et al, 2007) indicating potential interactions between *bc1* and Coq members. Further, *COQ8* was initially identified as a suppressor of Cob translational defect (Bousquet,1991). It was later also proposed to function as a chaperone for the *bc1* complex (Brasseur, 1997). However, accumulation of a prenylated intermediate and the rescue of respiration by UQ supplementation in *coq8* null mutants lead to it being assigned a function in UQ biosynthesis (Do et al, 2001). Here, we attempt to identify physical interactions between Coq9 and Qcr7, as previously the

deletion of *COQ9* was shown to cause Qcr7 instability. Tandem affinity purification, co-migration on sucrose gradients and immunoprecipitation data indicate transient physical interactions between Coq proteins and *bc1* complex.

2 Materials and Methods

2.1 Yeast strains and plasmids used:

Saccharomyces cerevisiae cells were derived from S288C background strain (ATCC 201388: MATa, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) obtained from Open Biosystems. Strains included Qcr7::TAP, Qcr7::TAP *coq9Δ,* Cor2::TAP, Cor2TAP::*coq9Δ*. Additionally, *coq9Δ* cells transformed with S288C *COQ9-MYC* vector were also used.

2.2 Yeast growth media: Yeast cells were cultured in liquid media containing 1% Yeast Extract, 2% Peptone and 2% Dextrose (YPD) for extraction of mitochondria and other assays. For analysis of respiratory growth, cells were normalized at OD600 and serially diluted before plating on solid media containing 1% Yeast Extract, 2% Peptone and 2% Lactate -Glycerol (YPLG).

2.3 DNA (Genomic and Plasmid) extraction, PCR amplification and detection:

Genomic DNA was obtained from the desired yeast cells by using the 5 Prime DNA extraction kit following the manufacturer's protocols. Similarly Plasmid DNA extraction was carried out using Qiagen's miniprep kit. DNA quantification (A260) was done using ND1000 v3.7.1 software and the Nanodrop spectrophotometer. PCR amplifications were performed using the

Eppendorf mastercycler personal machine. Primer annealing temperatures and extension times were calculated and optimized basing on the primer melting temperatures and length of the desired amplicon respectively.

DNA electrophoresis was carried out using 1% agarose gels in 1X TAE buffer (40 mM Tris Acetate, 1mM EDTA, pH 8.2-8.5). DNA samples were electrophoresed at 100 V for 45 minutes. A 10kb DNA ladder from New England Biolabs (NEB), was used as a standard for DNA size measurements. For the detection of the bands, Ethidium Bromide was added at a concentration of 12.5µg/L of 1% agarose. An AlphaEase imager was used to visualize DNA bands.

2.4 Generation of Qcr2::TAP *coq9Δ* mutants: The yeast gene knockout cassette (Kan^r gene) was amplified from a *COQ9* deletion strain from the commercial yeast deletion collection using primers appended with homologous regions surrounding 100-200bp upstream and downstream regions of *COQ9* gene, forward primer: 5'-CCATTCACC TATACAGTAGTATAC-3' and reverse 5'-CAGCAGCTTCCACTGGCCCAGG -3'.

The 1.4 kb amplicon was transformed into Qcr7-TAP (Open Biosystems) strain by electroporation and plated on YPD+G418 (0.3 mg/ml) plates. Genomic DNA from transformants were analyzed by amplifying with the above primers and size comparison. Positive clones resulted in 1.4 kb amplicons while false positives gave approximately 1000 kb amplicons, corresponding to original *COQ9* open reading frame.

2.5 Yeast transformation: Yeast cells (50ml) were grown overnight to OD600 of 1.0 in YPD liquid media. The cells were washed twice with ice-cold sterile water and once with chilled 1M Sorbitol by centrifugation (3500 rpm for 5 mins at 4˚C). The cells were then suspended in 2ml of

1M Sorbitol and 65 µl of cell suspension was electroporated at 1700 V with 2-2.5 µg of the vector using an Eppendorf eporator. The electroporated cells were immediately resuspended in 1 ml of 1M Sorbitol and allowed to recover for 30 mins to 1 hour at 30˚C before plating on the appropriate selection plates.

2.6 Mitochondrial Protein isolation and measurement: 1 Liter overnight cultures grown in liquid YPD at 30˚C in a shaking incubator were used to obtain mitochondrial proteins. Following centrifugation of the cultures at 3500 rpm for 5 minutes the cells were treated with a suspension of lyticase (Sigma) in 1.2 M Sorbitol (1000 units/gm cells) and incubated for 2-4 hours at 30˚C. The cell suspensions were then centrifuged at 3500 rpm for 10 minutes. The pellet was then vortexed three times with glass beads for 1 minute each. Following this, the mixtures were centrifuged at 1500 rpm for 5 minutes to remove the broken cell membranes and the supernatant was centrifuged at 15000 rpm for 10 minutes to obtain crude mitochondrial pellets. The pellets were then resuspended in 10 ml of 1.2 M Sorbitol and 10 ml of 22% Nycodenz in 1.2 M Sorbitol was overlaid before spinning at 12000 rpm for 50 minutes. The supernatant was finally centrifuged at 12000 rpm for 30 minutes to obtain purified mitochondrial proteins. Protein concentrations were measured by Bradford's assay. 2 µl of the isolated mitochondrial proteins were mixed with 998µl of Bradford's reagent (Amresco) followed by incubation for five minutes at room temperature. The OD_{595} of the mixture was measured using a Shimadzu UV 2450 UV-Visible spectrophotometer and the concentrations were calculated using the values generated from UVProbe software. Varying BSA concentrations were used to generate the standard curve.

2.7 SDS-Polyacrylamide Gel Electrophoresis and Western blot analysis: Appropriate concentrations (25, 30 or 40µg) of proteins were denatured by boiling in 1X Laemmli buffer (0.1% 2-Mercaptoethanol, 0.0005% Bromophenol blue, 10% Glycerol, 2% SDS , and 63 mM Tris-HCl (pH 6.8)) (Laemmli, 1970) followed by separation on polyacrylamide gels (10%). Gels were run at 150 V for 2 hours and electro-blotted onto nitrocellulose membranes for 1.5 hours at 150V. Subsequently, membranes were blocked with 5% BSA in PBS for 1 hour and probed overnight with appropriate primary antibodies antibodies (anti-TAP (Genscript), anti-myc (Abcam) or anti-porin (Abcam) antibodies) reconstituted in 1X PBS followed by probing (1 hr) with appropriate fluorescent Cy3 or Cy5 secondary antibodies (GE-Amersham) reconstituted in 1X PBS. Visualization of proteins was done using a LAS4100 imager.

2.8 *bc1* complex/Coq complex purification: *bc1*/Coq complex subunits were pulled down using the Tandem affinity purification protocol developed by Atkinson et al (2011). Briefly, mitochondria from Cor2::TAP, Cor2TAP::coq9Δ strains (200 µg) were lysed in 450 µl of IPP150 buffer (10 mM Tris, pH 7.4, 150 mM $NaCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF], with 1% digitonin) on ice for 10 min. Lysates were clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. IgG magnetic beads (50 µl; NEB S1431S) were rinsed three times in IPP150 buffer (with 0.5% digitonin). Beads were added to each lysate and incubated overnight at 4°C with rotation. Beads were precipitated magnetically and rinsed two times in tobacco etch virus (TEV) protease buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 0.1 M dithiothreitol [DTT], and 0.5% digitonin). TEV protease (100 units; Invitrogen) was added to 1 ml TEV buffer, and 500 µl was added to beads and incubated for 1 h at room temperature. As the Cor2::TAP strain remained associated with the IgG beads after TEV treatment, IgG beads were

precipitated and rinsed three times in 0.5 ml calmodulin binding buffer (10 mM Tris, pH 7.4, 150 mM $NaCl_2$, 4 mM $CaCl_2$, 1 mM magnesium acetate, 1 mM imidazole, 10 mM 2-mercaptoethanol, and 0.5% digitonin). Calmodulin beads (100 µl) were rinsed in 0.5 ml binding buffer three times, resuspended in an 0.5-ml final volume, and added directly to precipitated IgG beads. The mixed beads were incubated at 4°C with rotation for 4 h. IgG beads were removed from the calmodulin bead bed magnetically. Calmodulin beads were rinsed three times in 0.5 ml buffer, with precipitation between rinses with brief spins. Precipitated beads were boiled in 0.1 ml Laemmli buffer and loaded into duplicate lanes of Bis-Tris 12% NuPAGE gels (Invitrogen) and run in the manufacturer's recommended morpholineethanesulfonic acid (MES) SDS running buffer. Gels were divided for parallel immunoblot analysis and Sypro ruby staining (Invitrogen) as recommended by the manufacturer. For mass spectrometry, indicated Sypro ruby-stained bands were excised from the gel for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

2.9 Sucrose density gradient centrifugation: A discontinuous sucrose density gradient was prepared by layering successive decreasing sucrose densities solutions (15-35%) upon one another in a 5ml Sorvall centrifuge tube. Volumes used were as follows: 15% -1ml, 20% -1ml, 25% -1ml, 27.5% - .75ml, 30% - .5m and 35% - .25ml. 250 µg of mitochondrial samples (*QCR7::TAP coq9Δ + COQ9-myc*) were lysed in 450 µl of IPP150 buffer (10 mM Tris, pH 7.4, 150 mM $NaCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF], with 1% digitonin) on ice for 30 min. Lysates were clarified by centrifugation at 10,000 × *g* for 20 min at 4°C. The supernatant was layered on the sucrose gradient and ran for 16 hours at 37,500 rpm, at 4°C in a Sorvall hanging bucket ultracentrifuge. Post-centrifugation, 20 fractions of 200 µl each were collected

and saved at 4˚C for western analysis. Samples were analyzed for the presence of Qcr7-TAP and Coq9-myc and the banding patterns were matched according to fraction numbers to identify co-migration of these subunits.

2.10 Co-Immunoprecipitation of Coq9-myc and Qcr7-TAP

Protein magnetic beads were washed thrice in PBS (pH 7.4) and incubated with mouse anti-myc antibodies for 3 hours at room temperature. The beads were washed again in PBS thrice. Fractions 11-13 of the sucrose gradients were pooled and incubated overnight with the prepared beads at 4˚C. The beads were washed thrice with PBS and eluted by boiling for 5 minutes with 50µl of 1% SDS . This was repeated to collect a total of two eluates. The samples were run on 10% polyacrylamide gels followed by western blotting and probing with anti-TAP antibodies.

3. Results and Discussion

3.1 Tandem affinity purification reveals new *bc1* interacting proteins

The instability of the Qcr7 subunit warranted the investigation of the stability of the other early assembling subunits. Qcr2 (or Cor2) subunit of the *bc1* complex is one of the early assembling *bc1* subunits that forms a stable sub-complex with Qcr1 (Cor1) (Zara et al., 2004). The stability of Qcr2 in a *COQ9* deletion was tested in a Qcr2-TAP yeast strain. Western blots revealed that unlike Qcr7, the stability and steady-state levels of Qcr2 remain unchanged in the mutant (Fig. 1a). Therefore, in contrast to the previous data (Chapter 2), the absence of Coq9 and other Coq proteins does not affect the stability of Qcr2. This indicates that the assembly of the Qcr1-Qcr2-

Cyt1 sub-complex is either independent of Cob translation and insertion in the membrane. This may also imply that this assembly step precedes the Cob translation and Qcr7 attachment. Tandem affinity purification technique was employed to exploit the stability of Qcr2 to trap the subunits of the *bc1* complex that may try to assemble in normal and/or aberrant sub-complexes. Mitochondrial samples from wild type and *COQ9* deletion cells harboring Qcr2-TAP were compared after affinity purification by denaturing protein electrophoresis and sypro-staining. The protein bands could not be identified even after multiple rounds of mass spectrometry, therefore, bands were identified by comparing with the results from Atkinson et al. (2011). Bands corresponding to Cob and Cyt1 (~40kD and ~34kD) were depleted in the mutant cells (Fig. 1b) correlating with our previous heme deficiency data. Moreover, most of the *bc1* subunits seemed to be unstable in our mutant, reinforcing the idea that an early assembly step is affected in the absence of Coq9, which prevents the assembly and stability of the late assembling *bc1* members.

3.2 Co-migration and immuno-precipitation suggest transient interactions between *bc1* and Coq complex

Co-immunoprecipitation of *bc1* subunits with Coq complex subunits was performed in order to identify physical interactions between them. Specifically, mitochondrial proteins from strain containing Qcr7-Tap and Coq9-myc were analyzed. Coq9-myc was pulled down with anti-myc antibody and the precipitate was eluted and probed with anti-Tap antibody. Several experimental repeats generated a single band identifiable with anti-Tap antibody. However, co-elution of Qcr7-Tap was not completely reproducible, indicating the presence of a potentially weak physical interaction between Qcr7 and Coq9 subunits.

In a second approach to identify Qcr7-Coq9 interactions, sucrose density gradient migration patterns of mitochondrial proteins from cells harboring Qcr7-Tap and Coq9-myc were analyzed. Initially, patterns of fractionation of native marker complexes containing a mix of Thyroglobulin (669kDa), Ferritin (440kDa), catalase (232 kDa) and Lactate dehydrogenase (140kDa). Samples were treated with a mild detergent (0.5% digitonin), prior to separation on a sucrose gradient. Twenty individual fractions were Coomassie-stained and scores assigned to each band basing on the relative intensity. Peak plots and gels demonstrated that the sucrose gradients were able to isolate bands of various sizes into distinct fractions (Fig. 2a, 2b). Heavier molecules concentrated in the higher sucrose concentrations. Further gels were able to effectively fractionate bands in the range of 150- 700 kDa, the sizes expected for the *bc1* complex bands. The trial run helped to establish optimal detergent and sucrose concentrations for separating desired protein samples.

Subsequently, mitochondrial fractions co-expressing Qcr7-Tap and Coq9-myc were fractionated after solubilization in 0.5% digitonin. Digitonin does not break the mitochondrial super-complexes, thereby it was expected that any interactions at higher molecular forms that may exist between Coq complex and *bc1* complex will be retained. Twenty fractions obtained from the gradient were probed with both α-myc and α-Tap antibodies to identify fractions that contained both Qcr7 and Coq9. Fractions 11, 12 and 13 consistently demonstrated the co-migration and presence of Coq9-myc and Qcr7-TAP (Fig 3a). These fractions were enriched in proteins of about 440 kDa in our native marker run and previous studies had shown that dodecyl maltoside accumulate *bc1* complex subunits in these fractions (Taylor et al., 2003). Our treatment may have relieved super-complex conformations but yet interestingly, it had

demonstrated co-migration of Coq9 with Qcr7. Moreover, Coq9 bands were also detected in much higher molecular weight retaining fractions consistent with the published data that Coq subunits interact in a high molecular weight complex (Tran et al., 2006). It cannot be ruled out that Coq9 stays in contact with *bc1* that subsequently forms a *bc1*-Cytochrome oxidase supercomplex (Cruciat et al, 2000). Previous studies have shown cytochrome oxidase complex subunits separating in fractions 14 and 15 (Hanson et al., 2001). However, Qcr7 is not detected in the heavier fractions that could be attributed to the buried epitope site in the *bc1* dimer/ cytochrome oxidase supercomplex.

Since immunoprecipitation detection of Qcr7-Coq9 had failed previously, the enriched fractions from sucrose gradients that were positive for Qcr7 and Coq9 (f 11-13) were pooled and used for co-immunoprecipitation. Analysis demonstrated the presence of the Qcr7-TAP subunit in eluates obtained by capturing Coq9 on anti-myc beads (Fig. 3b). This was however, not consistently reproducible. This demonstrated that transient interactions may be present between Qcr7 and Coq9 that could be detected in vitro by using samples enriched for the desired proteins.

Lastly, although co-migration presents an interaction between the Coq complex and Qcr7, it does not detect at what exact step the *bc*1 complex members make initial contact with Coq complex. Higher detergent concentrations may identify the lowest molecular weight complexes that include *bc1* and Coq complex members.

3.3 A putative model of Coq complex-*bc1* interaction

Our previous results and present data indicates a role for Coq complex in the assembly and stability of *bc1* complex. Basing on this information a putative model for *bc1* complex interaction with Coq complex can be proposed (Fig. 4). Cob needs heme cofactor for catalytic

function. It is translated and bound to Cbp3-Cbp6 that are relieved upon binding to the subunits Qcr7 and Qcr8 (Gruschke, 2012). On the other hand, Coq complex interacts with Qcr7 post-translationally and stabilize Qcr7/Qcr7-Qcr8 complex. It cannot be stated definitively if heme insertion occurs via an unidentified dedicated chaperone for Cob or if Coq complex acts as a chaperone. Once heme is inserted, the concomitant addition of Qcr7-Qcr8 releases Cbp3-Cbp6 for further rounds of Cob translation and heme insertion. Deletion of individual Coq subunits destabilize the Coq complex and obstruct the stabilizing role of Coq for Qcr7. This disruption leads to halt of Cob translation and heme insertion, therefore Coq mutants are respiratory and heme deficient. The Coq complex may interact via Coq9 and remain in proximity with the *bc1* complex that may enhance ubiquinone interaction with Qcr8; Qcr8 is a ubiquinone binding subunit of *bc1* complex. In this manner, the efficiency of regulation of *bc1* complex is enhanced and ubiquinone feeding in *bc1* complex is made easier.

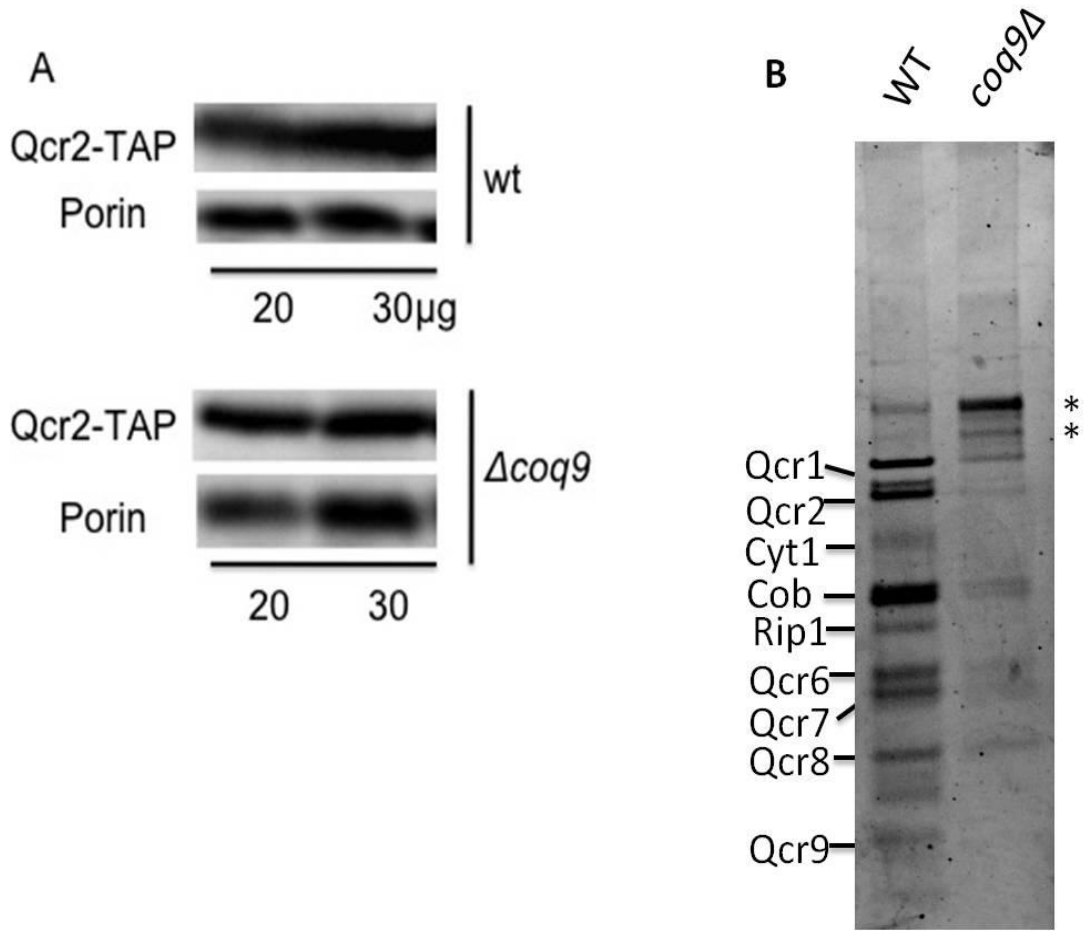

Figure 1. Effect of *COQ9* deletion on *bc1* subunits. A) Western blot of Qcr2-TAP using 20 and 30µg of mitochondrial protein mixtures from WT cell containing a TAP-tagged Qcr2 and *coq9* deletion in the same background. Qcr2-TAP is stably expressed in *Δcoq9*. B) Purified *bc1* complex obtained by pull down of Qcr2-TAP from mitochondrial proteins of WT and *Δcoq9* was run on polyacrylamide gel followed by sypro-staining. Cob and cyt1 along with other subunits are depleted in *Δcoq9* and protein bands marked with asterisk give an enhanced signal in the mutant.

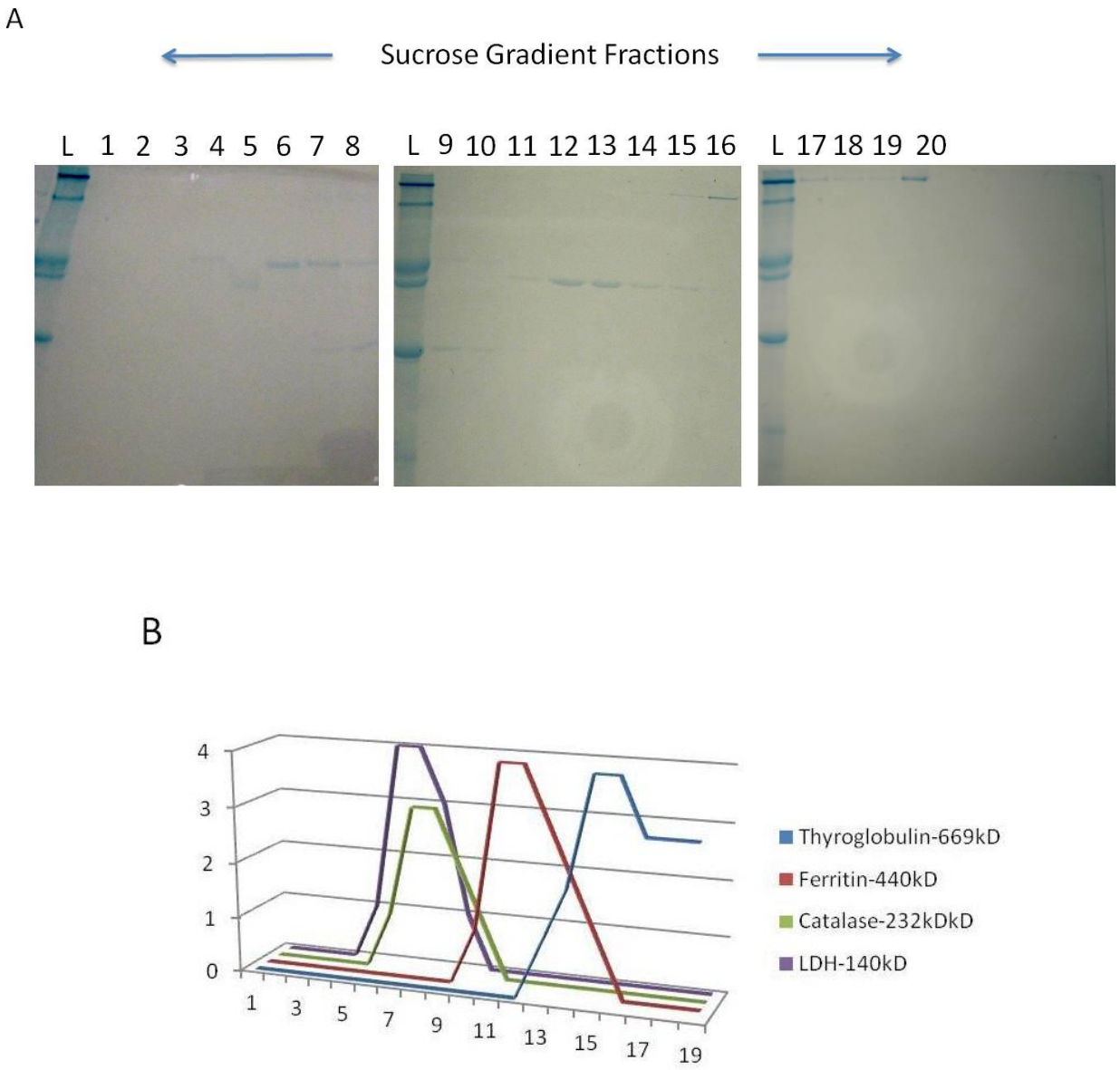

Figure 2. Sucrose gradient fractionation of marker proteins. A) Native marker samples containing a mix of proteins Thyroglobulin, Ferritin, Catalase and Lactate Dehydrogenase were run on a gradient of Sucrose solutions (15-35%). Twenty fractions were collected and run on 10% polyacrylamide gels followed by coomassie staining of isolated proteins. Individual proteins can be seen to fractionate distinctly in various fractions. L denotes the native marker mix. B) Bands were scored for relative intensity in the range of 0-4 and the values were plotted to identify peaks of individual proteins. All four proteins form individual and distinct peaks were they separate on the gradient. The heaviest band peaks at the heaviest and lower fractions on the sucrose column.

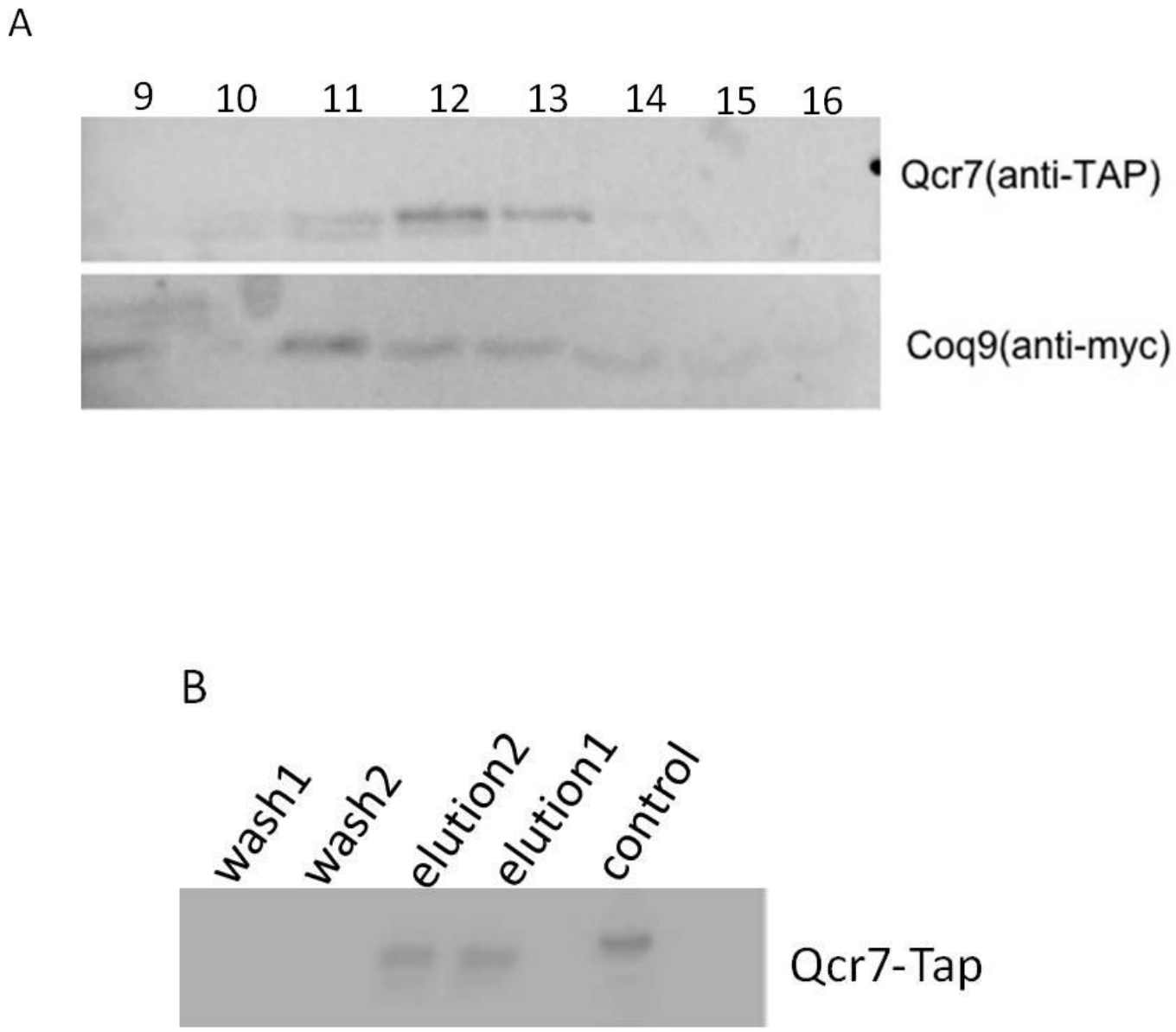

Figure 3. Physical interactions between Coq and *bc1* complexes. A) Sucrose fractions of mitochondria from strains expressing Qcr7-TAP and Coq9-myc proteins show co-migration in fractions 11-13. Coq9-myc is also detected in higher fractions. B) Co-immunoprecipitation of Qcr7-TAP with Coq9-myc captured on anti-myc beads.

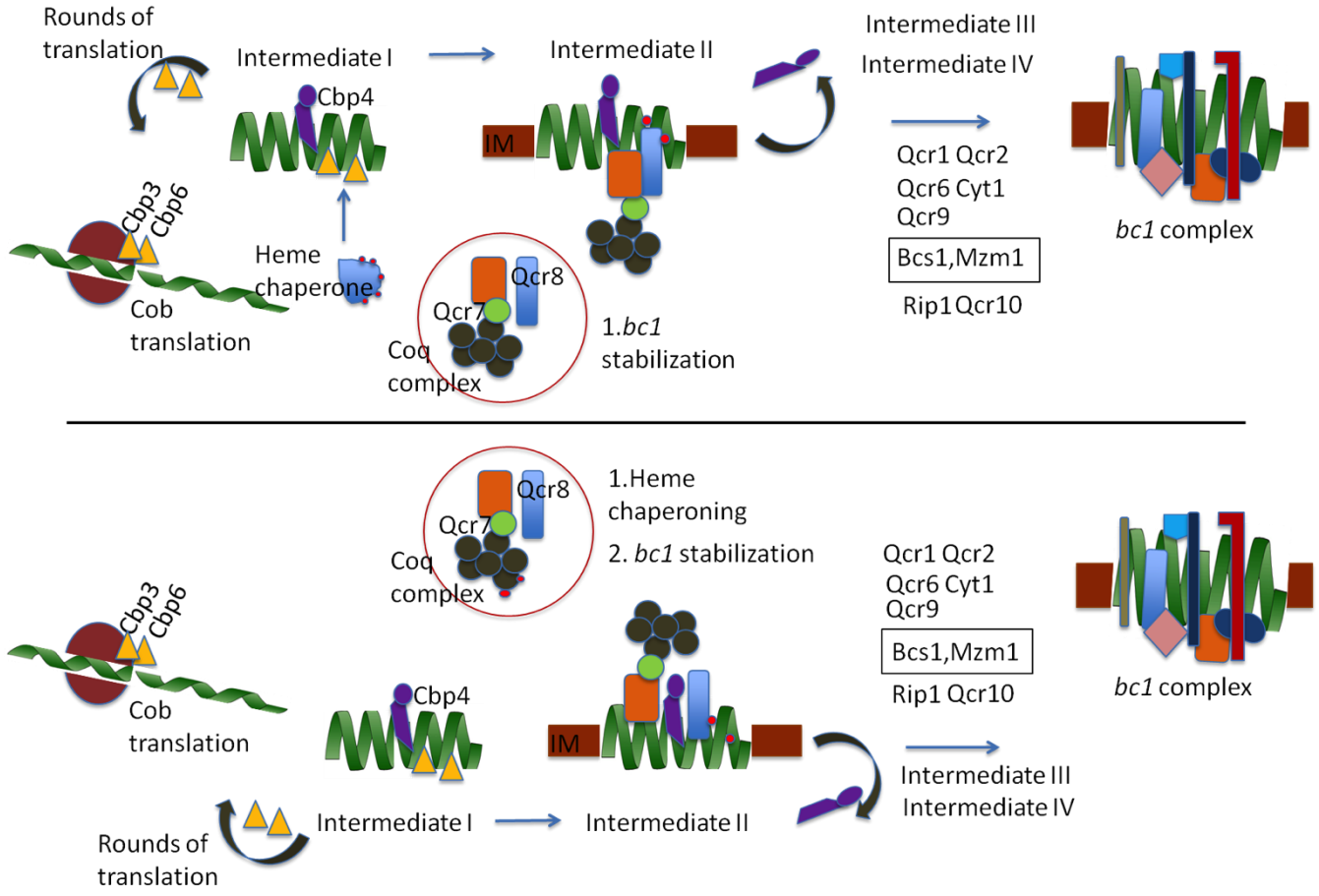

Figure 4. A proposed model for Coq complex interaction with *bc1* complex assembly. Coq complex (Coq9 serves as a point of contact depicted in green) interacts with *bc1* via Qcr7 at an early assembly step. The upper panel indicates heme chaperoning by a dedicated unknown heme chaperone that inserts heme, enabling Coq complex to insert Qcr7-Qcr8 in the membrane. The lower panel shows the putative heme binding role of Coq complex as well as *bc1* stabilization.

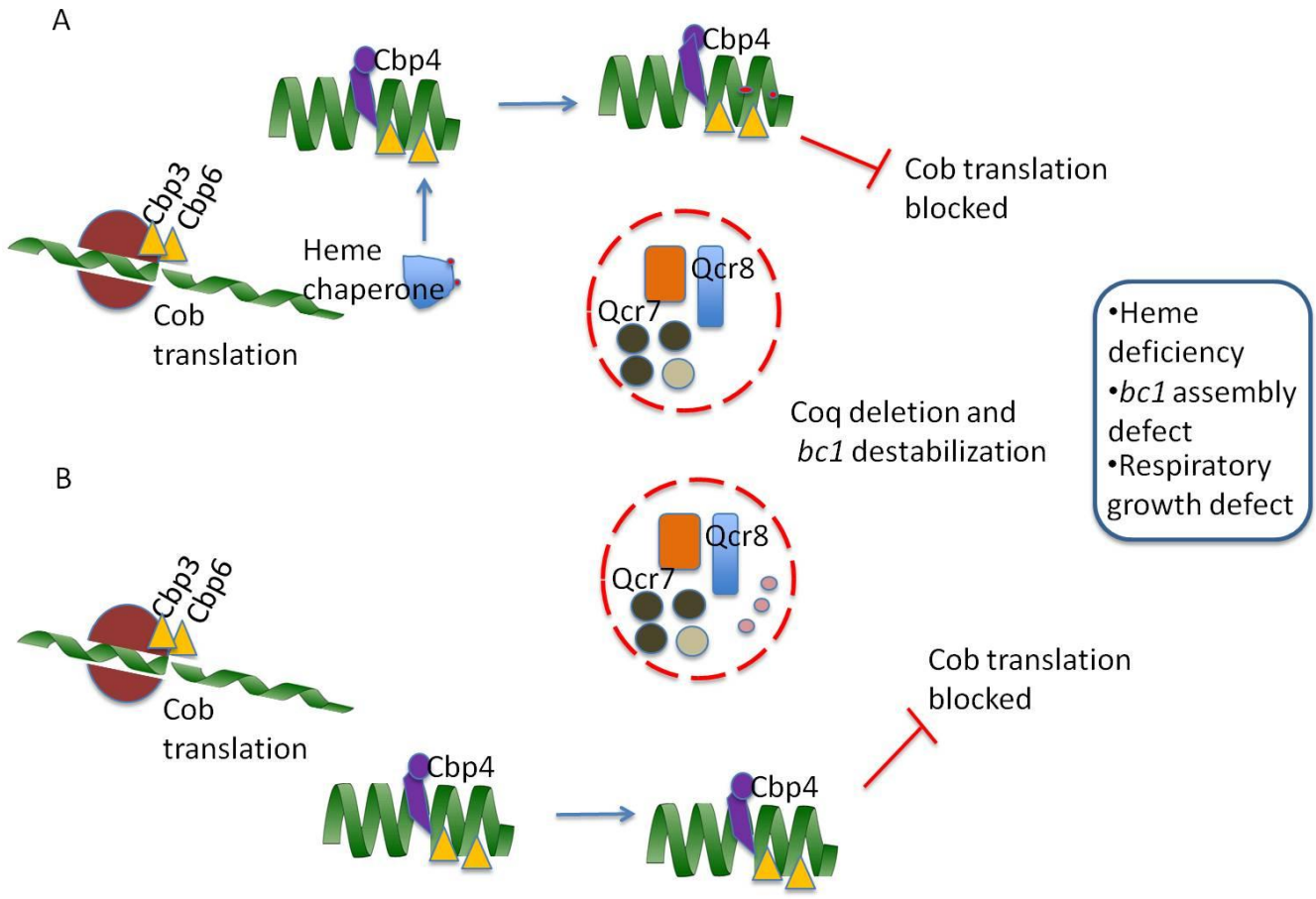

Figure 5. Effect of Coq complex deletion on the bc1 complex. A) Schematic of *bc1* destabilization when Coq interaction is lost. Qcr7 is degraded and cannot be assembled in the absence of Coq members (deletion). Failure of insertion does not allow Cbp3-Cbp6 to be available for Cob exit from ribosome blocking Cob synthesis.
B) Coq complex is not available for heme chaperoning and Qcr7 stabilization, resulting in degradation and blockage of Cob translation. The end result is a heme defect and *bc1* assembly failure in both cases.

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Chapter 4

Oxidative stress is the likely cause of the galactose growth defect of *coq* mutants

Abstract

Galactose is a non-conventional carbon source for *Saccharomyces cerevisiae* that is metabolized by the enzymes of the Leloir pathway. Genes encoding the Leloir enzymes are tightly regulated and are repressed by the presence of glucose. Although, the metabolism of galactose in yeast has been defined in terms of enzymes involved, there seems to be a lack of complete understanding of its regulatory components. In humans, defects in galactose metabolism leads to galactosemia, a condition that can be mimicked in yeast by deleting *GAL7* or *GAL10* genes. On the other hand, lithium can also induce galactose toxicity in wild type cells. Such defects are attributed to the accumulation of galactose-1-phosphate, an intermediate of the Leloir pathway. By-passing the step that is blocked in such defects, or discovering alternative mechanisms that can channel upstream intermediates away from accumulating the toxic galactose-1-phosphate, will be helpful in devising therapies for galactosemic individuals as well as make yeast efficient in converting galactose-rich feedstocks.

Ubquinone synthesis genes have so far never been implicated in galactose metabolism or its regulation. However, in the present study, Ubiquinone synthesis complex gene deletions were detected to be defective in some facet of galactose metabolism by their inability to grown on galactose-containing medium. Therefore, the main objective of this study was to identify the mechanism that was affected in these mutants and discover extragenic suppressors of this defect. Growth of the cells under various conditions indicated that ubiquinone alone, was sufficient to rescue the galactose growth defect, eliminating the possibility of a direct role of Coq proteins in galactose metabolism. Genomic library screening in a *COQ2* deletion, identified a suppressor plasmid containing *HSP10* and an uncharacterized open reading frame (*YOR020W*) that could

reverse the defect. Although Hsp10 has been implicated in the rescue of galactose growth in the suppressor screens, the induction of heat shock protein response alone is an unlikely cause for growth on galactose, as shown by lack of growth at higher temperatures. Further, presence of lithium in the medium does not induce galactose toxicity in our mutants, demonstrating that in *COQ* mutants lacking ubiquinone, oxidative stress is the probable cause for inability to utilize galactose. The function of *YOR020W* in galactose metabolism should be the focus of future experiments, as it shows similarities with the nucleotide exchanging receptors of higher eukaryotes.

1 Introduction

Galactose is an alternative carbon source that can be utilized by yeast in the absence of glucose. It is metabolized by a group of enzymes of the Leloir pathway that converts it into metabolically useful glucose 6-phosphate (Frey, 1996). Glucose 6-phosphate can then be used for glycolysis and energy generation. The Leloir enzymes (encoded by GAL genes) include galactose mutarotase (Gal10p), galactokinase (Gal1p), galactose-1-phosphate uridyl transferase (Gal7p), UDP-galactose-4-epimerase (Gal10p) and a phosphoglucomutase (Pgm1p & Pgm2p) (Sellick et al, 2008). In *Saccharomyces cerevisiae* unlike other organisms, the mutarotase and epimerase activities are contained within a single enzyme (Majumdar et al., 2004). Briefly, galactomutarotase converts β-D-galactose into α-D-galactose which in turn is converted by galactokinase to galactose-1-phosphate. Galactose-1-phosphate uridyl transferase then transfers the uridyl monophosphate group from UDP-glucose to galactose-1-phosphate, forming UDP-galactose and glucose-1-phosphate. Next, UDP-galactose-4-epimerase converts UDP-galactose to UDP-glucose. Finally, glucose-1-phosphate is converted to glucose-6-phosphate by phosphoglucomutase. Transcription of the Leloir enzymes is under strict regulation. In the presence of glucose, the Leloir enzymes are repressed by the transcription factor Mig1p. However, when the concentrations of glucose decrease, a group of 3 proteins Gal4p, Gal 80p and Gal3p activate *GAL* genes (Plat and Reece, 1998).

In humans, defects in the gene encoding galactose-1-phosphate uridyl transferase (*GAL7*) causes classical galactosemia that is marked by increased galactose-1-phosphate accumulation in cells. Galactokinase (*GAL1*) and UDP-galactose-4-epimerase (*GAL10*) gene defects causes type II and type III galctosemia respectively (Berry, 2012). The clinical manifestations of galactosemia

include liver dysfunction, cataract formation and mental retardation. In all cases, galactosemia requires dietary changes and clinical intervention (Berry, 2012).

In *Saccharomyces cerevisiae*, deletion of *GAL7* and *GAL10* leads to galactosemia-like phenotype, marked by inability to grow on galactose-containing medium. A similar phenotype can be induced in wild type cells by exposure to Lithium, as it inhibits phosphoglucomutase, a key enzyme of galactose metabolism (Bro, et al, 2003). However, the deletion of *GAL4*, the transcriptional activator of *GAL* genes or *GAL1*, the gene encoding galactokinase reverses this phenotype, suggesting that accumulation of galactose-1-phosphate is toxic for cells (Masuda et al., 2008). Further it has been shown that the complete bypass of the Leloir pathway in the absence of any of its components is better tolerated by the cell. Over-expression of aldose reductase (*GRE3*) leads to conversion of galactose to galactitol which is better tolerated by the cell. This also effectively overcomes lithium-induced galactose toxicity (Masuda et al., 2008). Screening for suppressors of galactose toxicity may therefore not only provide further insights into metabolic regulation mechanisms but can also provide strategies for treatment and management of galactosemia. Further, understanding of the regulation of galactose metabolism also has commercial implications. Yeast cells can be engineered to effectively utilize feedstocks that have increasing galactose concentrations, thereby increasing yields of biotechnological products and decreasing costs of production.

In this study, the galactose growth phenotypes of various *coq* deletion strains were characterized. Interestingly, such mutants show inability to grow on galactose containing medium. Attempts are made to identify suppressors of this defect, and propose a role for ubiquinone and identify mechanism of galactose tolerance in suppressors.

2 Materials and Methods

2.1 Yeast strains, plasmids and culture conditions:

Saccharomyces cerevisiae wild-type strain BY4741 (*MAT*a, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0,* and *GAL2*$^{+}$) was used as the control strain. Strains deleted in *gal7, coq2, coq3, coq7, coq9* and *coq10* were obtained from MATa deletion library (Open Biosystems). Cells were grown at 30 °C in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose) and in synthetic (SC) medium with 2% galactose (0.67% yeast nitrogen base, 2% glucose and appropriate auxotrophic supplements, such as 0.003% uracil, leucine, histidine, methionine, lysine, adenine and tryptophan). When needed, lithium was added to the medium to a final concentration of 40 mM. Q2, a ubiquinone analog (6 µM) or Vanillic acid (10mM) was supplemented to SC medium as required.

A yeast genomic library was constructed from a BY4741 *coa1Δ* strain in a pRS423 plasmid. pRS423 was used for cloning and expression of *HSP10* and *YOR020W* genes.

2.2 Isolation of Suppressors of galactose phenotype by genomic library screening

coq2Δ cells were transformed with a high copy yeast genomic library, yielding about 1000 colonies on SC-His plates. Colonies were replica plated on SC Gal medium and incubated for 4 days at 30°C. Thirty one (S1 –S31) suppressors were then isolated from plates and sub-cultured in selective minimal medium (SC-His). Suppressor plasmids were isolated from yeast cells using the EZNA minikit I following the protocol of bacterial plasmid DNA purification. A total of ten plasmids selected randomly were amplified in *E. coli* by transformation. *coq2Δ* was re-transformed with suppressor plasmids and plated on selective medium (SC-His) followed by

replica plating on SC Gal plates to remove false positives. A total of 4 plasmids were obtained. Plasmids (100ng) were restricted with EcoRV for 2 hours at 37°C followed by electrophoresis at 150V for 2 hours. Three distinct banding patterns were observed.

Three suppressor plasmids were sent for sequencing (operon). Only one plasmid contained two complete gene sequences. This was used to subsequently transform *coq2, coq3, coq7, coq9 and coq10.* Transformants were compared for growth on glucose (2%), and galactose (2%) containing medium. 6 μM ubiquinone/and or vanillic acid was supplemented in the medium when required.

2.3 Cloning of *HSP10* and *YOR020W* genes

HSP10 and *YOR020W* were amplified from wild type genomic DNA using the following primers respectively: HSP10 Forward : 5'GGCCGCGAGCTCGGTCATTAACGG-3'

Reverse: 5'-CCAAGGCTCGAGCACATAGTGTCC-3'

YOR020w Forward : 5'-GGCCGCGAGCTCCCAATGAAGAG-3'

Reverse : 5'-CCAAGGCTCGAGCATTGCCGTTTTGCT-3'

Primers were used to introduce SacI and XhoI sites in the upstream and downstream regions of the gene respectively. The amplicons were ligated to pRS426 vector using the SacI and XhoI generated cohesive ends. The generated plasmids were transformed into *coq2Δ* yeast cells and transformants were selected on SC minus uracil plates.

2.4 Induction of heat shock protein response

Dilutions of untransformed *coq* mutant cells were plated on 2% galactose solid medium and incubated at 37°C for 4 days followed by inspection for growth.

2.5 Protein structure modelling and motif search

BlastP search were done for identification of Yor020w homologs. I-TASSER and Swiss model platforms were used for automated secondary structure predictions. PRINTS database was used for motif searches.

3. Results and Discussion

3.1 Oxidative defect in *coq* mutants causes a galactose uptake defect

Mutants of *GAL* genes are unable to metabolize galactose because of the accumulation of the toxic compound, galactose-1-phosphate (Masuda et al, 2008). Mutants of the *COQ* genes have been shown to have a galactose growth defect but its cause is unknown. In order to understand the relation between ubiquinone, its biosynthesis machinery and galactose utilization we tested various *coq* mutants for growth on galactose-containing medium. The *coq* mutants are defective in ubiquinone biosynthesis, therefore rescue of the galactose phenotype was tested after supplementation with ubiquinone. Q_2 , an analog of yeast Ubiquinone (Q_6) was added to the galactose medium and the mutants were grown at 30°C for 3-4 days. Interestingly, this rescued the growth of the mutants (Fig.1).

Vanillic acid acts as an artificial alternate precursor for ubiquinone biosynthesis; it bypasses the initial steps of ubiquinone biosynthesis that require the action of several Coq proteins. However, its addition does not rescue the galactose growth defect in our experiments (Fig 1.). This is due to the absence of a stable Coq complex that is needed for ubiquinone biosynthesis. Ubiquinone is an anti-oxidant that scavenges reactive oxygen within the cell. It also participates in the ETC by

donating electrons to the *bc1* complex. Therefore, this data indicates that the likely cause for the phenotype is an inability to survive oxidative stress in the presence of galactose. Galactose may induce a further burst in the ROS levels or may add up to the environmental stress.

3.2 Lithium does not induce galactose toxicity in *coq* mutants

GAL mutants are deficient in the galactose metabolism enzymes and therefore accumulate galactose-1-phosphate that is toxic to the cell. However, in the absence of a mutation, lithium can also induce galactose toxicity as it inhibits phosphoglucomutase. The *coq* deletion strains *coq2Δ*, *coq3Δ*, *coq7Δ*, *coq9Δ*, and *coq10Δ* were grown on media containing galactose (2%) supplemented with glucose (0.5%), as on plain galactose medium the strains are completely inviable. The *coq* mutants were resistant to lithium (Fig. 2) indicating that the growth defect in these mutants are not as a result of galactose toxicity but as a result of inability to utilize galactose.

3.3 Whole genome library screening reveals a plasmid containing *HSP10* and an uncharacterized protein *YOR020W* as a suppressor of galactose uptake defect

To further characterize the mechanism of mutant response to galactose exposure, a whole genome library was screened for identification of multi-copy suppressors of galactose phenotype in a *coq2Δ* cell. A suppressor plasmid carrying *HSP10* and *YOR020W* Open Reading frames (Fig. 3a), was isolated that rescued the galactose growth defect of *coq2Δ*, *coq3Δ* , and *coq9Δ* reproducibly (Fig. 3b and 3c). The known functions of the isolated genes were analyzed to make a correlation between the phenotype and the suppressor.

The first candidate, *HSP10* encodes for a protein in the family of heat shock proteins. These proteins respond to stress signals of the cell and they also mediate protein folding and sorting in the cell. Hsp10 is a mitochondrial matrix co-chaperone that works in folding, assembly as well as sorting of some proteins. It has been demonstrated to be a regulator of the main mitochondrial protein folding chaperone Hsp60 and can be isolated as a Hsp10-Hsp60 complex (Hohfeld and Hartl, 1994, Rospert et al, 1993). Rip1 is also the target of the sorting activity of Hsp10. Since our mutants are deficient in galactose growth and recover on ubiquinone supplementation, it could be hypothesized that the stress-response heat shock proteins may circumvent the oxidative stress in the absence of ubiquinone and presence of galactose. In a simpler approach to test this, the growth response of untransformed mutants was observed by inducing the endogenous heat shock proteins by exposure to par-optimal growth temperature (37^{o}C). In this case, this failed to rescue any growth on galactose medium (Fig. 4). This result warrants cloning of individual genes from the suppressor plasmid to attribute the phenotype rescue to a single gene, which will be addressed in the future.

3.4 *YOR020W* homology search and protein modeling

YOR020W was a potential candidate that rescues galactose defect in our mutants. *YOR020W* encodes for a 90 amino acid long, 9.6 kDa uncharacterized protein of unknown function. It was localized to the mitochondrial compartment basing on high-throughput approaches that involved multidimensional LC-MS/MS, 1D-SDS-PAGE combined with nano-LC-MS/MS and 2D-PAGE with subsequent MALDI-mass fingerprinting (Reinders et al, 2006).

The absence of information warranted modeling of the protein structure to predict it's function. Swiss-model and I-TASSER modeling platforms were used to derive the 3D model of Yor020p

with a C score of - ( ).The obtained model depicted two anti-parallel helices and three coils in the protein (Fig 5a). Further, it is predicted to be a membrane protein.

BlastP with Protein Databank indicated homology with Atp9 of *Saccharomyces cerevisiae* and Atp19 of *Saccharomyces arbaricola* as well as *Candida orthopsilosis* (Fig 5a). Both proteins are part of the ATP synthase complex that drive ATP production in yeast. The role of both the subunits are obscure. Atp19 has no known homologs in other eukaryotes and is found specifically bound to yeast ATP synthase dimer (Arnold et al, 1998).

Motif searches identified Na+/K+ ATPase as well as rhodopsin-like G protein-coupled receptor superfamily signatures (Fig. 5b). Since these proteins are involved in nucleotide exchange/binding functions (ADP/ATP and GDP/GTP), it can be hypothesized that Yor020w functions as a nucleotide binding protein in a yet unknown pathway or it may be an unidentified ATP synthase subunit. Further analysis such as immunoprecipitation with ATP synthase subunits or ATP/ADP binding assays may be needed to confirm its role.

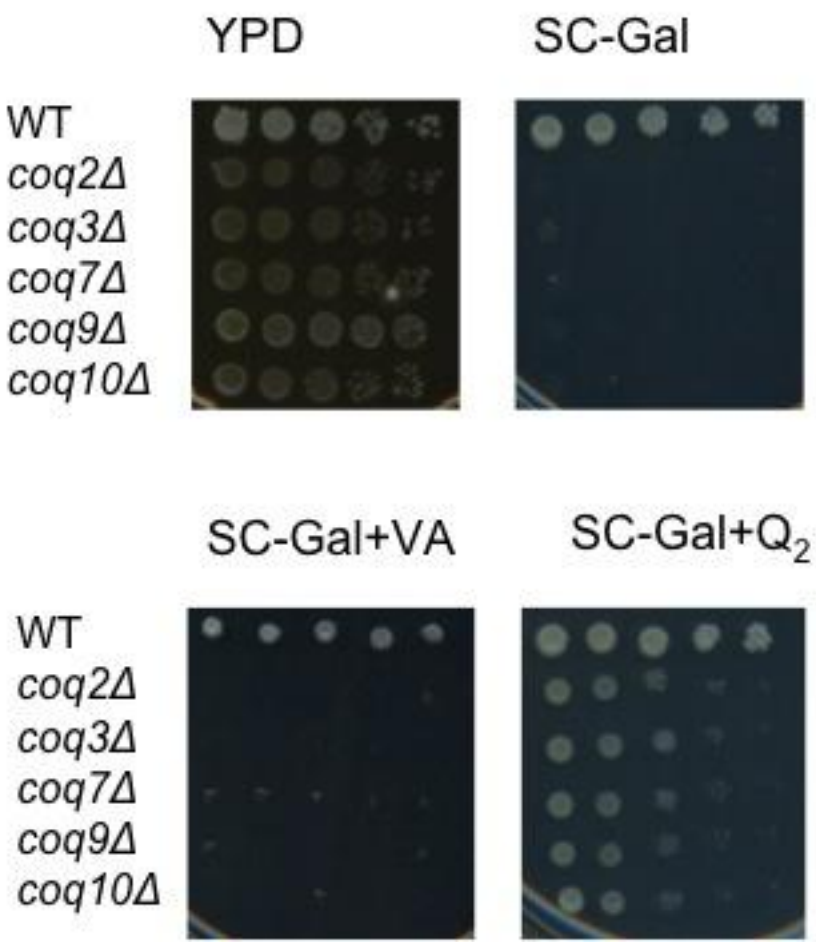

Figure 1. Exogenous supplementation of Ubiquinone rescues Galactose uptake in c*oq* deletion mutants. The upper panel shows the phenotype of *coq* mutants on glucose and galactose containing medium. Mutants are unable to utilize galactose as a carbon source. Lower panel shows galactose utilization by mutants upon supplementation with Ubiquinone. Vanillic acid an aromatic alternative precursor of ubiquinone shows no effect on mutants. Cells were grown at 30°C for all conditions tested.

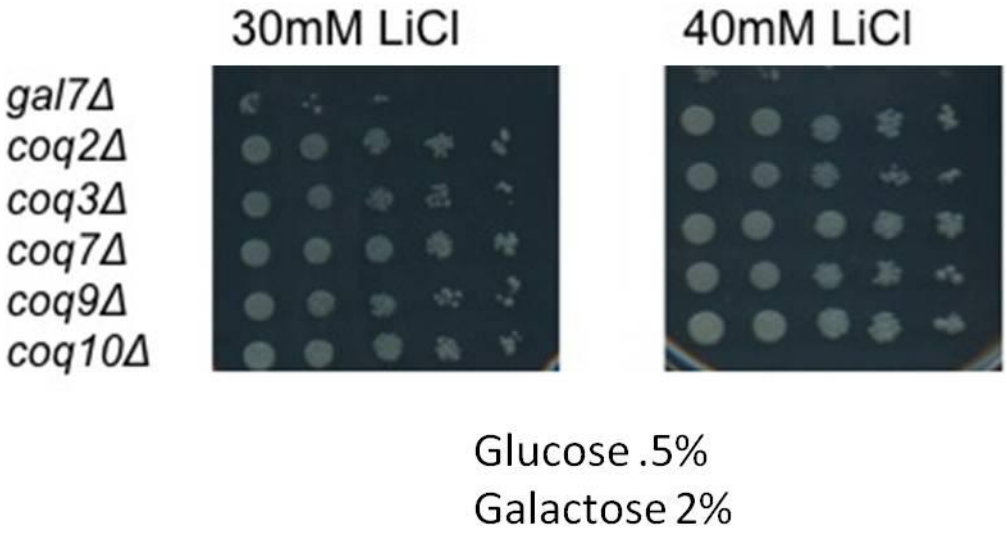

Figure 2. Lithium does not induce galactose toxicity in coq deletion mutants. *coq* mutants were grown on SC galactose medium supplemented with glucose to favor initial growth. Cells were grown at 30˚C for 4 days.

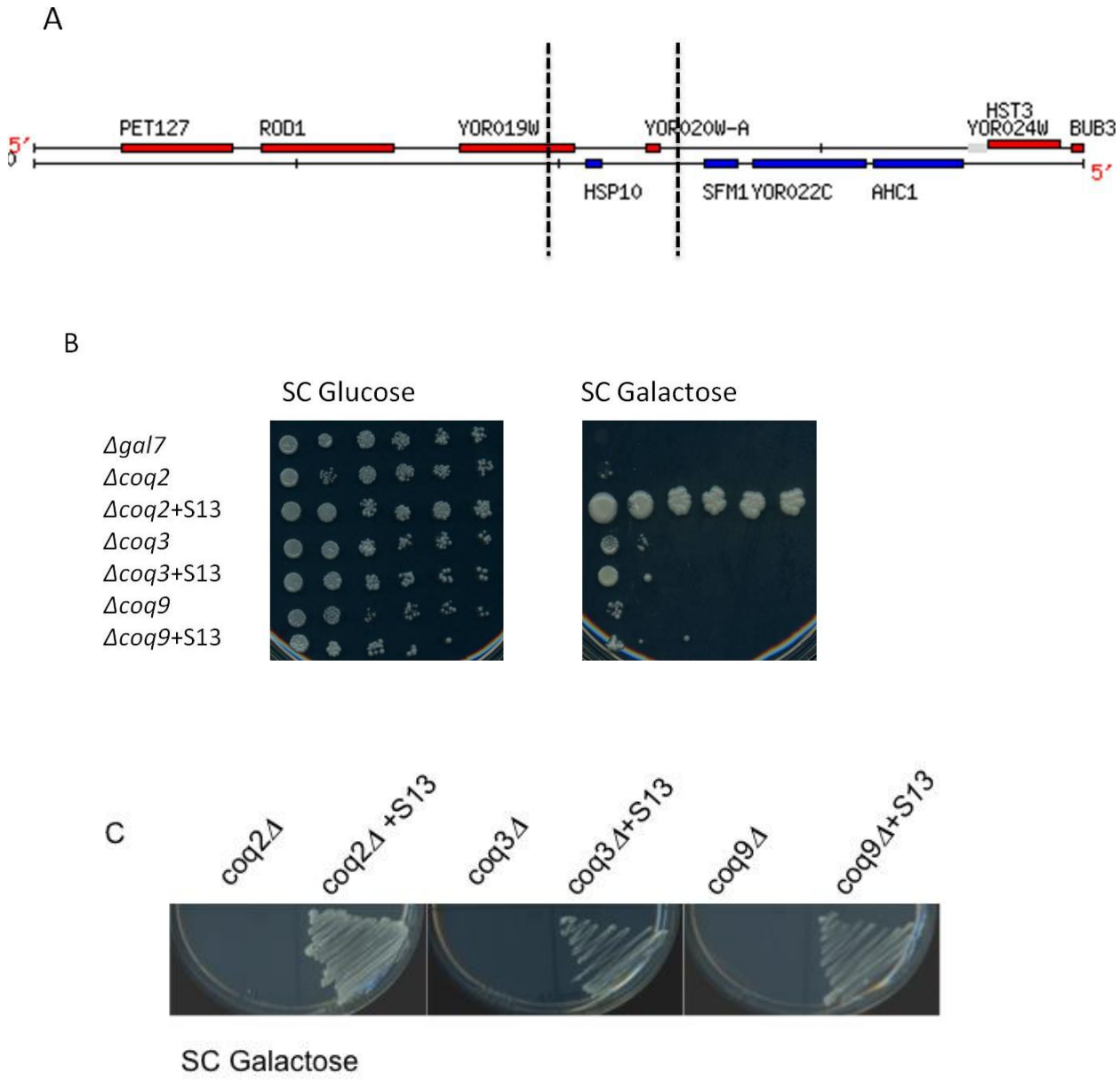

Figure 3. Multi-copy suppression of galactose phenotype. BY4743 *coq2Δ* was transformed with a genomic library and replica plated on galactose medium, followed by isolation of the suppressor plasmid from rescued colonies. A) Sequencing of suppressor plasmid (S13) demonstrated the presence of two genes- *HSP10* and *YOR020W*. The ideogram shows the margins of the sequenced fragment with dashed lines on the yeast chromosome. B) The suppressor showed a strong rescue of *coq2Δ* while other mutants were weakly suppressed and were sensitive to pre-culture conditions including agitation. C) Rescue of *coq2Δ*, *coq3Δ*, and *coq9Δ* by S13 on galactose medium without agitation.

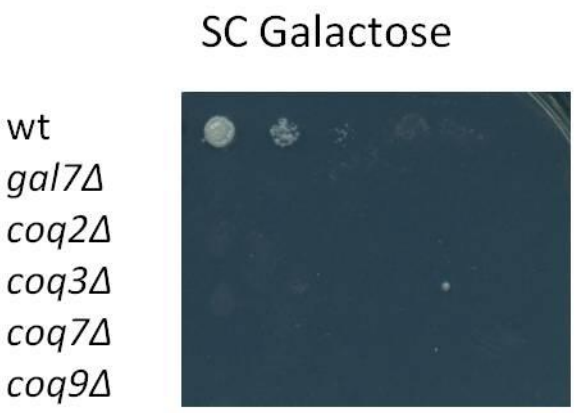

Figure 4. Induction of heat shock response. Untransformed *coq* deletion cells were grown on Galactose containing medium (2%) at 37°C for 4 days to induce a heat shock response. None of the mutants exhibit a reversal of the galactose growth defect.

A

Yor020w	MGAAYKVFGKTVQPHVLAIST
Atp9 *Saccharomyces cerevisiae*	MGAAY GK + PH LAI T
Yor020w	MGAAYKVFGKTVQPHVLAIST--FIATAAVASYF-TTKPKT
Atp19p *Saccharomyces arboricola*	MGAAY +FGK + PH LAI T ++ V + F + KPKT
Yor020w	MGAAYKVFGKTVQPHVLAISTF--IATAAVASYFTTKP
Atp19h *Candida orthopsilosis*	MGAAY +FGKTVQPH LA++T + + +T KP

B

Prints Name:
GPCRRHODOPSN1
Description:
Rhodopsin-like GPCR superfamily signature

Position	Sequence	Score
8..32	FGkTVQPHVLAISTFIaTAAVASYF	1018

Prints Name:
GPCRRHODOPSN4
Description:
Rhodopsin-like GPCR superfamily signature

Position	Sequence	Score
13..34	QPHVLAISTFIATAAVASYFTT	1056
11..32	TVQPHVLAIsTFIATAAVASYF	1053
15..36	HVLAISTFIaTAAVASYFTTkp	1010
17..38	LAISTFIATaAVASyFTTKPkT	1004

C

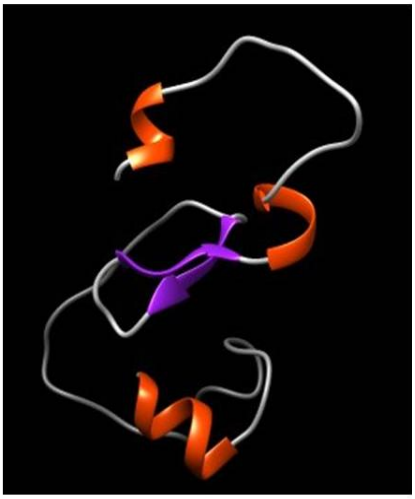

Figure 5. Homology search and protein stucture prediction. A) Yor020 shows conserved sequences found in ATP synthase subunits of related yeast strains. B) Motif searches indicate that Yor020 carries signatures of Rhodopsin-ike G protein coupled receptor superfamily and Na^{+}/K^{+} transporters. C) A model of Yor020 protein that show two anti-parallel alpha helices and three coiled motifs.

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