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The Energetic State of Mitochondria Modulates Complex III Biogenesis through the ATP-Dependent Activity of Bcs1

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SUMMARY

Our understanding of the mechanisms involved in mitochondrial biogenesis has continuously expanded during the last decades, yet little is known about how they are modulated to optimize the functioning of mitochondria. Here, we show that mutations in the ATP binding domain of Bcs1, a chaperone involved in the assembly of complex III, can be rescued by mutations that decrease the ATP hydrolytic activity of the ATP synthase. Our results reveal a Bcs1-mediated control loop in which the biogenesis of complex III is modulated by the energy-transducing activity of mitochondria. Although ATP is well known as a regulator of a number of cellular activities, we show here that ATP can be also used to modulate the biogenesis of an enzyme by controlling a specific chaperone involved in its assembly. Our study further highlights the intramitochondrial adenine nucleotide pool as a potential target for the treatment of Bcs1-based disorders.

INTRODUCTION

Mitochondrial oxidative phosphorylation (OXPHOS), which provides most of the ATP in animal cells, relies upon five multisubunit complexes (I–V) embedded within the inner membrane of mitochondria. The respiratory complexes (I–IV) transfer electrons to the final acceptor, oxygen. This transfer is coupled to proton translocation across the inner membrane, and the resulting transmembrane proton gradient is used by the ATP synthase (complex V) to synthesize ATP from ADP and inorganic phosphate. Due to its dual genetic origin, nuclear and mitochondrial, the biogenesis of the OXPHOS system is an intricate process involving numerous factors that execute highly specific functions ranging from the synthesis of the individual subunits to their assembly into the respiratory complexes. In addition, the respiratory complexes are organized into supramolecular structures or "supercomplexes," also called respirasomes, containing complexes I, III, and IV in higher eukaryotes and complexes III and IV in yeast (Schägger and Pfeiffer, 2000; Cruciat et al., 2000; Heinemeyer et al., 2007; Dudkina et al., 2011).

Complex III has a central position in the respiratory chain, allowing ubiquinol oxidation and cytochrome *c* reduction. It is an important site of proton translocation and production of reactive oxygen species. Complex III consists of 11 or 10 different subunits in mammals and yeast, respectively, three of which are catalytic: cytochrome b (Cytb), cytochrome *c1* (Cyt1) and the Rieske-FeS protein Rip1 (Iwata et al., 1998; Hunte et al., 2000). In all eukaryotes, Cytb is encoded by the mitochondrial DNA, whereas the other complex III subunits have a nuclear origin. The complex is assembled through a dynamic modular pathway, starting with an early core containing Cytb and the subunits Qcr7 and Qcr8 and finishing with the incorporation of Rip1 (Figure 1A; for reviews, see Zara et al., 2009; Smith et al., 2012). Two proteins, Mzm1 and Bcs1, are required during the late stages of complex III assembly in yeast. Mzm1 appears to stabilize Rip1 (Cui et al., 2012). Deficiencies of Bcs1 lead to the accumulation of an inactive pre-complex III (pre-III) lacking Rip1 (Nobrega et al., 1992; Cruciat et al., 1999; 2000; Conte et al., 2011). Bcs1 mediates the translocation of Rip1 from the matrix to the intermembrane space, and the release of Rip1 depends on the hydrolysis of Bcs1-bound ATP (Wagener et al., 2011).

Bcs1 is detected in a high-molecular-weight complex that is anchored to the inner membrane and protruding into the matrix. An internal signal within the N-terminal domain targets Bcs1 to mitochondria (Fölsch et al., 1996). Bcs1 contains a Bcs1-specific domain and a highly conserved AAA region typical of the AAA-protein family (ATPase associated with diverse cellular activities; Figure 1B). This region contains the Walker A and B motifs of P loop ATPases involved in ATP binding and hydrolysis as well as a number of additional conserved structural elements such as the SRH (second region of homology). AAA proteins drive ATP-dependent dissociation, unfolding, or folding of nucleic acids and proteins (for review, see Hanson and Whiteheart, 2005). In mitochondria, the AAA proteins play a central role in the biogenesis and quality control of proteins (Gerdes et al., 2012).

Mutations in the human gene *BCS1L* (*BCS1*-like) are the most frequent nuclear mutations resulting in complex III-related

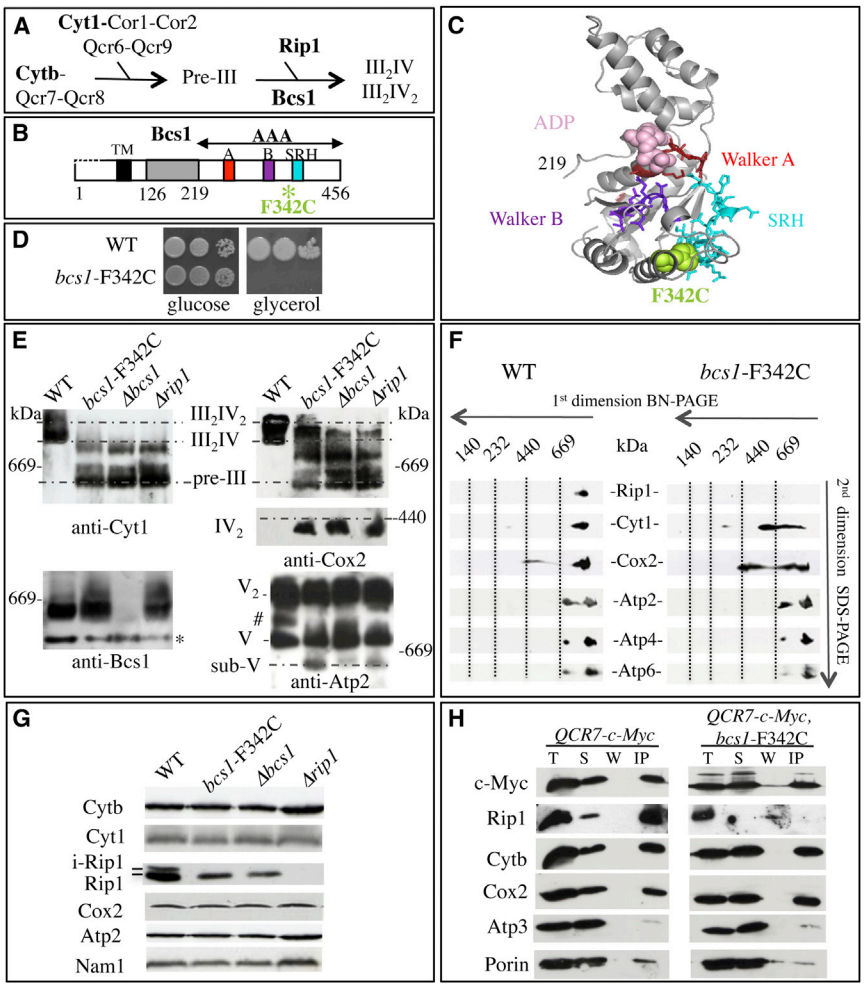

Figure 1. The *bcs1*-F342C Mutation Located in the AAA Domain of Bcs1 Affects the Assembly of Complex III and of Supercomplexes III/IV

(A) Schematic representation of the modular assembly pathway of complex III. The three catalytic subunits, Cytb, Cyt1, and Rip1, as well as Bcs1 are in bold.

(B) Schematic representation of the Bcs1 protein. Positions of the transmembrane domain (TM), Bcs1-specific domain (gray), and the AAA domain with the positions of Walker A (red), B (purple), and SRH (blue) motifs and the amino acid F342 (green, *) are indicated. See also Figure S1.

(C) Theoretical structural model of the AAA domain of the yeast Bcs1 (amino acids 219–456; Nouet et al., 2009). The nucleotide (ADP), the main conserved motifs of AAA proteins, and the residue F342 are indicated. The figure was generated with the PyMOL v1.3 software.

(D) Dilution series of cells from WT and *bcs1*-F342C were spotted on fermentable (glucose) and respiratory (glycerol) media and incubated for 4 days at 28°C.

(E) Mitochondrial complexes were analyzed by BN-PAGE and immunoblotted with antibodies against Cyt1, Cox2, Bcs1, and Atp2. Positions of the supercomplexes III_2 + IV_2 and III_2 + IV, the 500 kDa precomplex III (pre-III), dimers of complex IV (IV_2), dimers (V_2) and monomers (V) of ATP synthase, as well as the positions of the low-amount band (#) and the band sub-V, are indicated. *, nonspecific band revealed by the anti-Bcs1 antibody. Positions of the protein molecular mass markers (669 and 440 kDa) are indicated. See also Figure S2.

(F) Mitochondrial complexes from WT and *bcs1*-F342C were analyzed by a first-dimension BN-PAGE followed by a second-dimension SDS-PAGE and then immunoblotted with antibodies against Rip1, Cyt1, Cox2, Atp2, Atp4, and Atp6. Positions of the protein molecular mass markers are indicated.

(G) Mitochondrial proteins purified from WT, *bcs1*-F342C, *Δbcs1*, and *Δrip1* strains were analyzed by SDS-PAGE and immunoblotted with antibodies against Cytb, Cyt1, Rip1, Cox2, Atp2, and Nam1 as loading control. In our mitochondrial preparations from WT, the intermediate (i-Rip1) and mature forms of Rip1 were detected with Rip1 antibodies.

(H) Mitochondrial proteins purified from the *QCR7-c-Myc* and *QCR7-c-Myc bcs1*-F342C strains were subjected to coimmunoprecipitation experiments. The fractions were analyzed by SDS-PAGE and immunoblotted with antibodies against Rip1, Cytb, Cox2, Atp3, and porin as negative control. T, total; S, supernatant; W, washing; IP, immunoprecipitate.

pathologies; very different clinical phenotypes are associated with these mutations, ranging from the mild Bjornstad syndrome to the lethal GRACILE syndrome (e.g., de Lonlay et al., 2001; Visapää et al., 2002; De Meirleir et al., 2003; Hinson et al., 2007; Fernandez Vizarra et al., 2007; Morán et al., 2010; Kotarsky et al., 2010; Levéen et al., 2011). An extensive mutational study of yeast Bcs1 has revealed the importance of the residues located at the junction between the Bcs1-specific and the AAA domains for the activity and stability of the protein (Nouet et al., 2009). Interestingly, several human pathogenic mutations are located at this junction.

In this paper, we report the identification of extragenic compensatory mutations of respiratory-deficient *bcs1* mutations located in the ATP binding domain of the yeast protein, among which one is the equivalent of a mutation found in a human patient. Remarkably, the compensatory mutations preferentially target the mitochondrial ATP synthase and lead to a strong decrease in the mitochondrial ATP hydrolytic activity while maintaining a sufficient level of ATP synthesis. We further show that increasing the ATP concentration in an in vitro assay also compensates for the Bcs1 deficiency. Based on these findings, we propose a model in which the ATP dependency of the protein Bcs1 is not just a requirement for its chaperon activity but also a way to couple the rate of complex III biogenesis to the energy-transducing activity of mitochondria.

RESULTS

Characterization of the *bcs1-F342C* Mutant

Previously, we isolated a yeast mutant with the single amino acid substitution F342C that modifies a highly conserved region of the AAA domain of Bcs1 (Nouet et al., 2009; Figure S1 available online). According to the theoretical three-dimensional (3D) model of the yeast Bcs1 protein (Figure 1C), the residue

F342 is located in the vicinity of the conserved SRH motif (see also Discussion). The *bcs1*-F342C mutant was unable to grow on respiratory substrates (Figure 1D), and it did not affect the steady-state level and oligomerization of Bcs1 (Figure 1E), suggesting that it probably decreased the activity of the protein.

As the OXPHOS complexes are organized into supramolecular structures, we have analyzed the effect of the *bcs1*-F342C mutation on supercomplexes III/IV and on ATP synthase oligomers. Under the blue native (BN)-PAGE conditions we used, complexes III and IV were mainly detected in the wild-type (henceforth designated as WT) as two supercomplexes, III_2IV_2 and III_2IV (Figures 1E and 1F). High-molecular-weight complexes revealed with the Cyt1 and Cox2 antibodies were detected in the mutant *bcs1*-F342C. Two-dimensional BN-SDS analysis (2D) has shown the absence of Rip1 in these complexes, indicating that the low amount of Rip1 that accumulates in the *bcs1*-F342C mutant (70% versus WT, Figure 1G) was not incorporated into complex III. In addition, the typical pre-complex III (pre-III) previously observed in strains that fail to assemble Rip1 (for review, see Zara et al., 2009; Smith et al., 2012) showed a strong signal with anti-Cyt1 but a very weak signal with anti-Cox2, as shown on 2D gels. The weak anti-Cox2 signal in this region might correspond to partial dissociation of higher-molecular-weight Cox2-containing complexes, yielding some subcomplexes that comigrate with pre-III. Finally, the complexes around 440 kDa that are detected with anti-Cox2 in higher abundance in the mutants probably correspond to free complex IV dimers. Using a c-Myc-tagged version of the Qcr7 subunit of complex III, Cox2 was efficiently coimmunoprecipitated in the WT and the *bcs1*-F342C mutant, whereas Rip1 was only detected in the WT immunoprecipitate (Figure 1H). These findings indicate that pre-III can still interact with complex IV, but the integration of complex IV into supercomplexes is compromised.

ATP synthase in BN-PAGE was revealed using antibodies against the subunit Atp2 (Figure 1E). This enzyme was detected mainly as monomers (V) and dimers (V_2) both in WT and mutants. The faint band between V and V_2 (denoted with a #) also reacted with antibodies against several other ATP synthase subunits (Figure S2), suggesting that it could be a dimer that has lost some subunits during preparation and/or electrophoresis of the mitochondrial samples. In comparison to V and V_2, the amount of this band was too low to be detected in 2D (Figure 1F). An additional Atp2-containing complex of smaller size (sub-V) migrating ahead of free F_1 was repeatedly observed in the *bcs1*-F342C mutant as well as in Δ*bcs1* and Δ*rip1* strains, but not in the WT or in other mutants affecting complex III, indicating that sub-V resulted from a specific lack in Rip1 (Figures 1E and S2). Due to its low amount, this complex could no longer be detected in a 2D experiment (Figure 1F). Thus, the *bcs1*-F342C mutation only seems to have a slight effect on the integrity of ATP synthase.

Mutations in the Genes Encoding F_1 Subunits of ATP Synthase Rescue the *bcs1*-F342C Mutant

As extragenic compensatory mutations may uncover unpredictable networks of interacting cellular functions and proteins, we have applied this approach to the *bcs1*-F342C mutant in order to better understand how mitochondria modulate Bcs1 function. We analyzed four independent revertants of the *bcs1*-F342C mutant (called R2, R3, R12, and R18; Table S1) displaying various respiratory-sufficient growth phenotypes (Figure 2A). Each revertant resulted from a compensatory mutation located in another nuclear gene that we have identified through a combination of molecular cloning and gene candidate approaches (see Experimental Procedures). DNA sequencing showed that the *bcs1*-F342C mutation was still present in each revertant, and we found missense mutations within the genes *ATP1* (*atp1*-V68G) in R3 and *ATP2* (*atp2*-H400Y, *atp2*-V499F, and *atp2*-A48D) in R2, R12, and R18. The genes *ATP1* and *ATP2* encode the α and β subunits of the catalytic sector (F_1) of the ATP synthase (Figure 2B). Below, we describe a thorough biochemical analysis of the properties of the two mutations, *atp1*-V68G and *atp2*-A48D, that exhibited the strongest compensatory effects when associated with the *bcs1*-F342C mutation in the R3 and R18 revertants.

The level of mature Rip1 and the insertion of functional complex III within supercomplexes was substantially improved in the *bcs1*-F342C mutant by the *atp1*-V68G and *atp2*-A48D mutations, as revealed by SDS and BN-PAGE analyses (Figure 2C) and ubiquinol-cytochrome c oxidoreductase activity measurements (Figure 2D). As a result, the oxygen consumption rate in mitochondria was substantially improved, with values estimated at ~70% in R3 and ~30% in R18 with respect to WT, versus <10% in the *bcs1*-F342C mutant (Figure 2E and Table S2). The residual oxygen consumption activity in the *bcs1*-F342C mutant did not induce any significant mitochondrial ATP synthesis, whereas a substantial ATP production of ~35% and ~15% with respect to WT was observed in R3 and R18, respectively. Thus, the *atp1*-V68G and *atp2*-A48D mutations improve the assembly of functional complex III and its insertion into supercomplexes and restore mitochondrial ATP synthesis in the *bcs1*-F342C mutant.

The F_1 Mutations *atp1-V68G* and *atp2-A48D* Lead to a Strong Decrease in the ATP Synthase Assembly and Hydrolytic Activity

We next determined how the *atp1*-V68G and *atp2*-A48D mutations impact ATP synthase. Being recessive, these mutations were expected to partially impair the function of the two F_1 subunits. This possibility was examined first by measurements of the ATP hydrolytic activity of ATP synthase. Normally, when properly assembled into F_1 oligomers, the Atp1 and Atp2 proteins are responsible for ~80%–90% of the ATP hydrolytic activity of mitochondria. While this activity was mostly unaffected in the *bcs1*-F342C mutant, it was drastically reduced by 95% in R3 and 80% in R18 with respect to WT (Figure 3A and Table S3). Similar deficits in ATP hydrolytic activity were observed in the single mutants *atp1*-V68G and *atp2*-A48D. As revealed by BN-PAGE, both R3 and R18, as well as the single *atp1* and *atp2* mutants, had a reduced content of fully assembled F_1F_o complexes, and there was no indication of accumulation of free F_1 (Figure 3B). However, despite the reduced content in F_1, the steady levels of the Atp1 and Atp2 were essentially unaffected (Figure 3C). Previous work has shown that these proteins show a high tendency to form large inclusion bodies in the mitochondrial matrix when they cannot associate with each other (Ackerman and Tzagoloff, 1990; Lefebvre-Legendre et al., 2005). Aggregates strongly enriched in Atp1 and Atp2 proteins

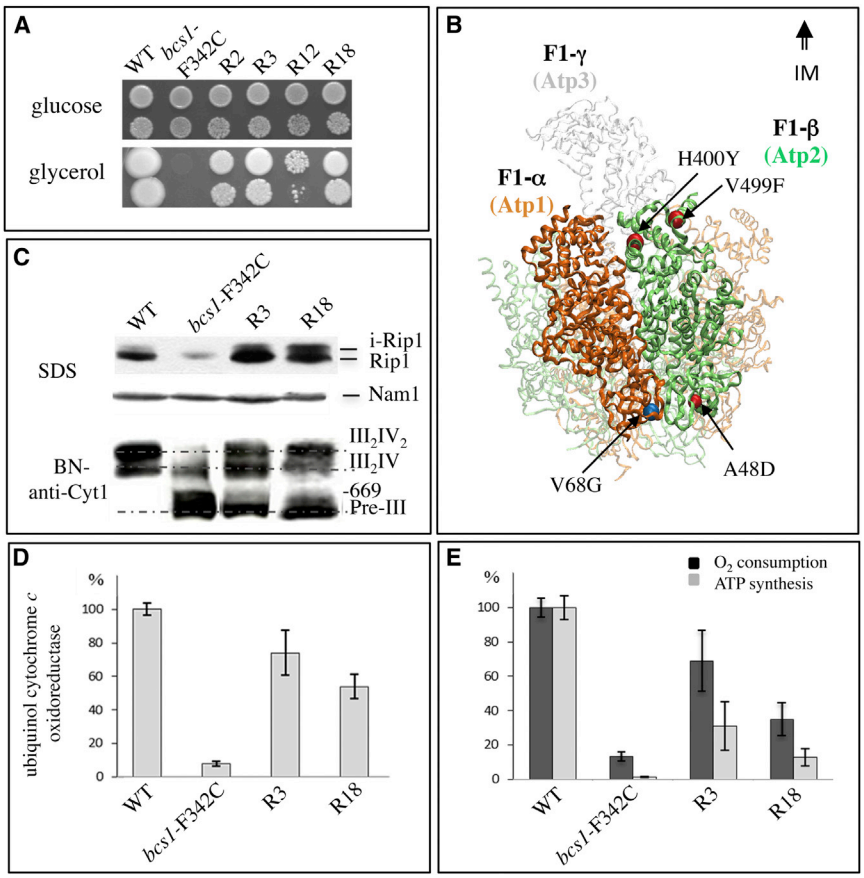

Figure 2. The *bcs1*-F342C Mutation Is Compensated for by Mutations in F_1 Subunits of the Mitochondrial ATP Synthase

R2, *bcs1*-F342C *atp2*-H400Y; R3, *bcs1*-F342C *atp1*-V68G; R12, *bcs1*-F342C *atp2*-V499F; R18, *bcs1*-F342C *atp2*-A48D.

(A) Dilution series of cells from WT, *bcs1*-F342C, and the four revertants, R2, R3, R12, and R18 (see Table S1) were grown for 3 days at 28°C.

(B) The α-F_1, β-F_1, and γ subunits (Atp1 in orange, Atp2 in green, Atp3 in gray) are represented according to the structure of the bovine ATP synthase (Abrahams et al., 1994). The figure was generated with the VMD 1.9.1. software. The mutations identified in the four revertants are represented as blue (*atp1*) and red (*atp2*) beads. The inner membrane (IM) is at the top of the figure.

(C) Mitochondrial proteins from WT, *bcs1*-F342C, R3, and R18 were analyzed by SDS-PAGE and BN-PAGE and immunoblotted with antibodies against Rip1 and Nam1 (SDS) or Cyt1 (BN) (see Figure 1F legend).

(D) The ubiquinol cytochrome *c* oxidoreductase activity (complex III activity) was measured in purified mitochondria. The activities of the three mutants are expressed as a percentage of the wild-type activity (1,800 nmol of reduced cytochrome *c*/min/mg of proteins). Data represent the mean of three independent experiments. Data are represented as mean ± SEM.

(E) The rates of oxygen consumption and ATP synthesis were measured on fresh, osmotically protected mitochondria with NADH as a respiratory substrate and after the addition of ADP (see Tables S2 and S3). Both the O_2 consumption and the ATP synthesis are represented as a percentage of the wild-type measurements. Data are represented as mean ± SEM.

were indeed observed on electronic micrographs of *atp1*-V68G and *atp2*-A48D cells (Figure S3). Thus, the lowering in mitochondrial ATP hydrolytic activity induced by the *atp1*-V68G and *atp2*-A48D mutations is mainly caused by a decreased ability of the Atp1 and Atp2 proteins to assemble with each other or by a diminished stability of the F_1 oligomers. As a result of this lower yield in F_1, the ATP synthase proton-translocating domain F_o, whose assembly is dependent on that of F_1 (Rak et al., 2009), also accumulated less efficiently in the *atp1*-V68G and *atp2*-A48D mutants, as shown by their low steady levels in the Atp6, a main component of the F_o (Figure 3C).

The F_1 Mutations *atp1-V68G* and *atp2-A48D* Lower the Energization of the Mitochondrial Inner Membrane by ATP

The influence of the *bcs1-F342C, atp1*-V68G and *atp2*-A48D mutations was further investigated by mitochondrial membrane potential (ΔΨ) in vitro analyses. ΔΨ mainly results from the proton translocation by the respiratory complexes and the ATP synthase. Consistent with its very low respiratory activity, the membrane was poorly energized by ethanol in the *bcs1*-F342C mutant in comparison to WT, whereas ΔΨ was restored in the revertants R3 and R18 due to their improved capacity to assemble complex III. In a first series of experiments (Figure 4A), we tested the effect of the addition of a small amount of ADP, which induces a ΔΨ consumption while imported into mitochondria. Return to the potential established before the addition of ADP, which reflects its phosphorylation by the ATP synthase, was null in the *bcs1*-F342C mutant and much slower in R3 and R18 than in the WT. In a second series of experiments (Figure 4B), we directly evaluated the proton pumping activity of the ATP synthase in the presence of a large excess of ATP. Before the addition of ATP, the mitochondria were treated with KCN in order to release, from the F_1, the IF1 peptide that inhibits the ATP hydrolytic activity of F_1 (Venard et al., 2003). Consistent with their high levels of assembled and functional F_1F_o complexes, both the WT and *bcs1*-F342 mutant can efficiently energize the membrane by hydrolyzing the exogeneous ATP, whereas R3 and R18 cannot, which is consistent with the ATP hydrolysis assays, showing the major impact of the *atp1* and *atp2* mutations on the ATP hydrolytic activity of F_1.

A Specific Mutation in the F_o Subunit Atp6 also Rescues the *bcs1-F342C* Mutant

In order to better understand the functional links between ATP synthase and Bcs1, we have tested other mutations in ATP synthase for their capacity to rescue the *bcs1*-F342C mutant. We selected three mutations (*atp6*-W136R, *atp6*-L183P, and

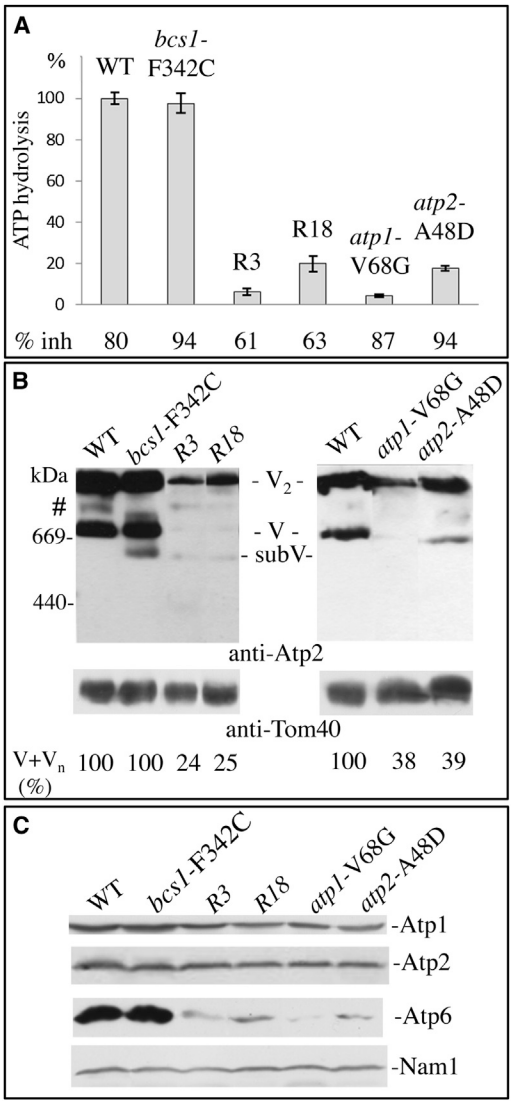

Figure 3. The *atp1*-V68G and *atp2*-A48D Mutations Affect the Assembly of ATP Synthase and Lead to a Strong Decrease in the Hydrolytic Activity of F_1

Mitochondria were purified from WT, *bcs1*-F342C, R3 (*bcs1*-F342C *atp1*-V68G), R18 (*bcs1*-F342C *atp2*-A48D), *atp1*-V68G, and *atp2*-A48D.

(A) Three independent ATP hydrolysis assays were performed on frozen and thawed mitochondria in the absence of osmotic protection and in the presence of saturating amounts of ATP. Specific enzyme activities are represented as a percentage of the wild-type activity (2,135 nM Pi/min/mg of protein). The percentage of inhibition of ATP hydrolysis by oligomycin is indicated. See Table S3 for complete data. Data are represented as mean ± SEM.

(B) BN-PAGE was immunoblotted with antibodies against Atp2 and Tom40, a protein of the mitochondrial outer membrane used as loading control.

(C) SDS-PAGE was immunoblotted with antibodies against Atp1, Atp2, Atp6, and the loading control Nam1. See also Figure S3.

atp6-L247P) affecting Atp6, an essential component of the F_o proton-translocating domain encoded by the mitochondrial genome. These mutations correspond to mutations found in human patients suffering from NARP (neuropathy, ataxia, and retinitis pigmentosa) or MILS (maternally inherited Leigh syndrome) (for review, see Houstek et al., 2006). Both the rate of mitochondrial ATP synthesis and hydrolysis are strongly reduced by 70% and 90%, respectively, in the *apt6*-W136R mutant, while the assembly/stability of the ATP synthase is normal (Kucharczyk et al., 2012, Figure 5A). The *atp6*-L183P and *atp6*-L247P partially compromise the assembly and/or stability of Atp6 within the ATP synthase, leading to mitochondrial ATP production deficits of 40%–60%, whereas the ATP hydrolytic activity of mitochondria is only modestly decreased (Kucharczyk et al., 2009; 2010). Each *atp6* mutation was combined with the *bcs1*-F342C mutation (see Table S1), and only the *atp6*-W136R mutation was able to restore the growth on glycerol (Figure 5B). In the *atp6*-W136R *bcs1*-F342C double mutant, complex III activity and the insertion of this complex into supercomplexes were partially recovered, whereas they were not in the *atp6*-L183P *bcs1*-F342C (Figures 5C and 5D). Thus, the F_1 mutations (*atp1*-V68G or *atp2*-A48D) and the F_o mutation (*atp6*-W136R) that all lead to a strong decrease in the rate of mitochondrial ATP hydrolysis can compensate for the defect in complex III assembly due to the *bcs1*-F342C mutation.

ATP Synthase Mutations Can also Rescue a *bcs1* Mutation Found to be Pathogenic in Humans

The results described above might hold promise for developing therapeutic pathways for human diseases caused by Bcs1 deficiencies. In this respect, since the *bcs1*-F342C mutation has no known equivalent in human patients, we wanted to know whether the ATP synthase-mediated compensation could also rescue, in yeast, a *bcs1* mutation that was known to be pathogenic in humans. We tested the mutation *bcs1*-F368I found in a patient with an early-onset encephalopathy (Fernandez-Vizarra et al., 2007). We constructed, in yeast, the *bcs1*-F401I mutation that is the equivalent of the human *bcs1*-F368I mutation (Figure S1). According to theoretical three-dimensional (3D) models of the human and yeast Bcs1 proteins, this mutation is located near the ATP binding site of Bcs1 (Figure 5E). The *bcs1*-F401I mutation led to a stringent respiratory growth deficiency (Figure 5F) and very severely compromised the activity of complex III (Figure 5G). As with the *bcs1*-F342C mutation, the steady-state levels and oligomerization of Bcs1 were not affected in the *bcs1*-F401I mutant (Figure 5E). Thus, the two mutations seem to have a very similar impact on Bcs1. Two of the ATP synthase mutations that rescue the *bcs1*-F342C mutant, one in F_1 (*atp1*-V68G) and one in F_o (*atp6*-W136R), were tested for their capacity to compensate the *bcs1*-F401I mutant. The two double mutants (*bcs1*-F401I, *atp1*-V68G and *bcs1*-F401I, *atp6*-W136R) were able to grow on respiratory substrates (Figure 5F) and showed an improved complex III activity (Figure 5G). These results suggest that Bcs1 mutations responsible for human diseases might be treatable by modulation of the ATP synthase activity.

Increasing the ATP Concentration Compensates for the In Vitro ATPase Deficiency of the *bcs1*-F342C Mutant

In order to test if and how the *bcs1*-F342C mutation affected the activity of Bcs1, we have set up an in vitro assay allowing the determination of its ATPase activity. We have purified WT and mutated Bcs1 proteins carrying a hexahistidine tag fused

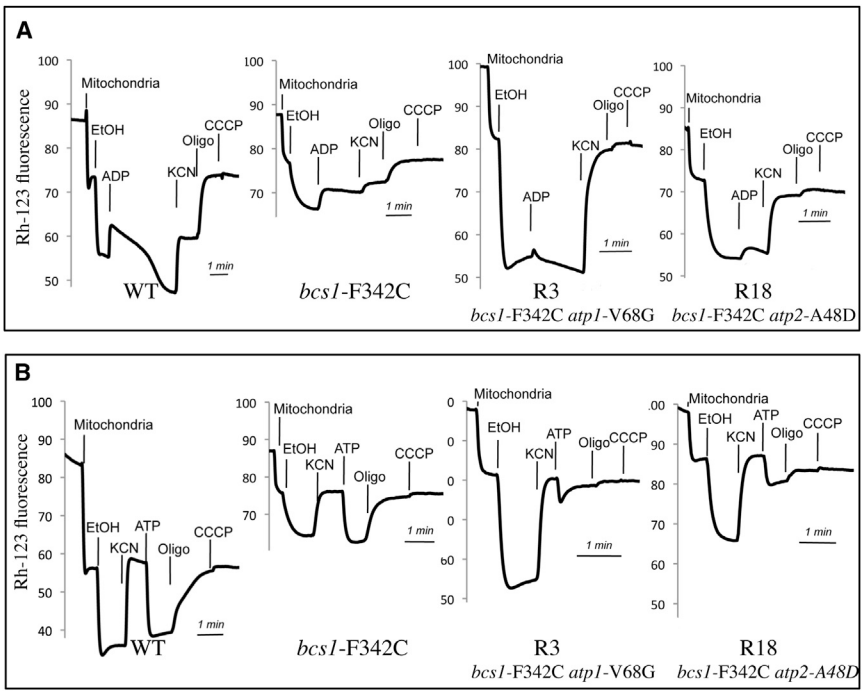

Figure 4. The *atp1*-V68G and *atp2*-A48D Mutations Lower the Potential of the Mitochondrial Inner Membrane by ATP

Membrane potential analyses were performed on fresh osmotically protected mitochondria from WT, *bcs1*-F342C, R3, and R18 using Rh-123, whose fluorescence decay is proportional to the mitochondrial membrane potential.

(A and B) A total of 50 μM ADP (A) or 1 mM ATP (B) was added to follow the energization due to ATP synthesis or hydrolysis, respectively. The other additions were 0.5 μg/ml Rh-123, 0.15 mg/ml of proteins, 10 μl ethanol, 0.2 mM KCN, 6 μg/ml oligomycin, and 3 μM CCCP. KCN, an inhibitor of complex IV, was used to collapse the membrane potential induced by the respiratory chain, oligomycin was used to block the ATP synthase, and CCCP was used to dissipate the proton gradient across the inner membrane. Experiments have been repeated three times.

to their C terminus (Figure S4). The tag had no influence on the chaperone activity of Bcs1, and both proteins kept their capacity to form oligomers both in vivo and after purification from mitochondrial digitonin extracts (Figure 6A). The ATP hydrolytic activity of the Bcs1 proteins was measured at different concentrations of ATP; at 2.5 and 5 mM, the *bcs1*-F342C protein had a rate of ATP hydrolysis 2- to 3-fold lower than that of the WT protein (Figure 6B). However, at higher ATP concentrations (10 or 20 mM), no significant difference was observed between the mutant and the WT (Figure 6C). Similar ATPase activities were obtained in the presence of oligomycin, which rules out the contamination by complex V during Bcs1 purification. Thus, it can be inferred that the reduced hydrolytic activity of *bcs1*-F342C is probably due to a lower affinity of the mutated protein for the nucleotide, and increasing its concentration in the assay compensates this deficiency.

DISCUSSION

Previous work has established that incorporation of the Rip1 protein into the yeast complex III involves a protein, Bcs1, belonging to the AAA protein family (Nobrega et al., 1992; Cruciat et al., 1999; 2000; Conte et al., 2011; Wagener et al., 2011). Here, we report that modulation of the ATP synthase activity can improve the activity of a mutated Bcs1 protein via its ATP dependency.

The mutant *bcs1*-F342C displayed large amounts of pre-III resulting from a block in Rip1 assembly. The immunoprecipitation (IP) data suggest that pre-III and complex IV can interact in the absence of Bcs1 and Rip1, as previously proposed with Rip1 variants (Cui et al., 2012). These interactions are consistent with current structural models that predict that Rip1 is not located at the interface between the two complexes (Heinemeyer et al., 2007). However, according to the BN-PAGE data, the integration of complex IV into supercomplexes is compromised, suggesting that Bcs1 and Rip1 are essential to maintaining the integrity of respiratory chain supercomplexes. Combined defects of OXPHOS complexes were also reported in *BCS1L*-deficient patients (Fernandez-Vizarra et al., 2007; Morán et al., 2010).

Unexpectedly, a main target for compensatory mutations rescuing the *bcs1*-F342C mutant was the ATP synthase. Four spontaneous compensatory mutations were identified as single amino acid changes in the two subunits, Atp1 and Atp2, that form the ATP synthase catalytic head. The functional consequences of two of these mutations, V68G in Atp1 and A48D in Atp2, were characterized. Both changes severely compromise the capacity of the Atp1 and Atp2 subunits to bind to each other, leading to their accumulation in the mitochondrial matrix as large aggregates. As a result, the content in fully assembled ATP synthase was substantially lowered, leading to a decrease in the enzyme's synthetic and hydrolytic activities. Nevertheless, in the revertant strains (*bcs1*-F342C + *atp1*-V68G or *atp2*-A48D), the assembly of Rip1 within the complex III was substantially improved, as compared to the single *bcs1*-F342C mutant.

To further understand how ATP synthase defects could improve complex III assembly in the *bcs1*-F342C mutant, we tested its compensation by other mutations of this enzyme. Substantial rescue was observed with the mutation W136R in the subunit Atp6 of the ATP synthase proton channel. This mutation had no effect on the assembly of ATP synthase but seriously impaired its functioning, as shown by strong deficits in both the ATP hydrolytic and synthetic activities of mitochondria (Kucharczyk et al., 2012). The *bcs1*-F342C mutant was not rescued by two other *atp6* mutations (L183P and L247P) that partially compromise incorporation and/or stability of Atp6 within the ATP synthase, and lead to similar decreases in the rate of ATP synthesis but with only minimal effect on the ATP hydrolytic activity.

It is difficult to understand how reducing the capacity of the ATP synthase to produce ATP could rescue Bcs1-mediated

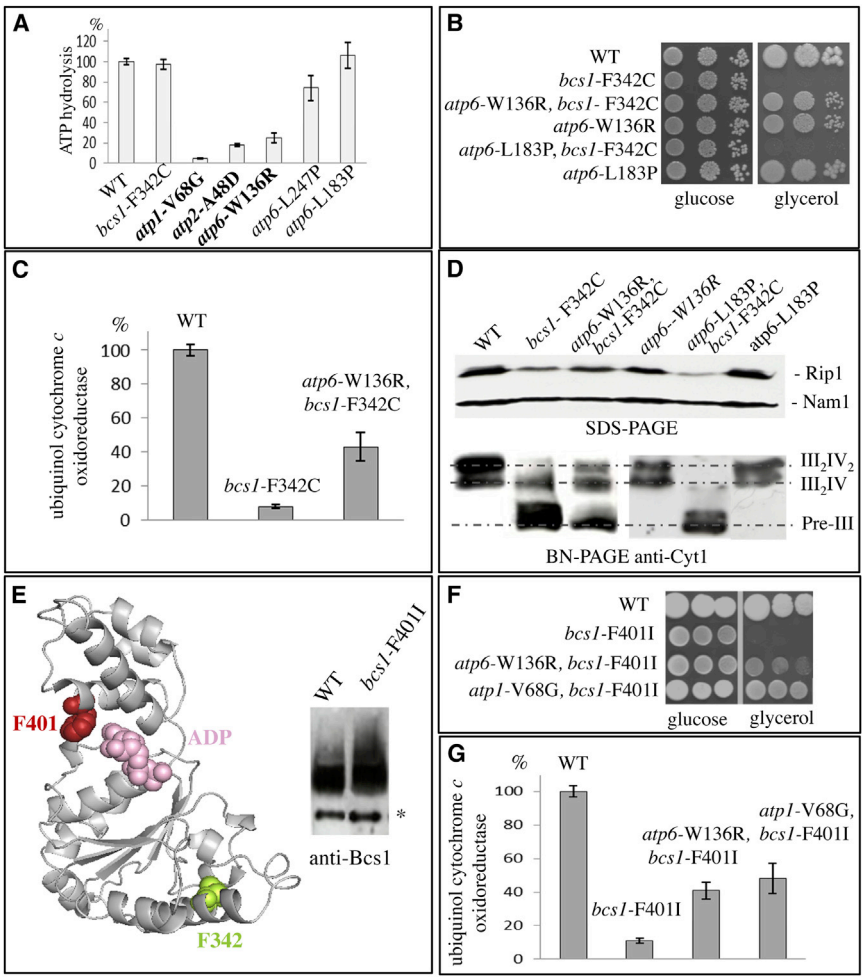

Figure 5. Mutations in Subunits of F_1 and F_O Rescue *bcs1* Mutations, One of which Is a Human Disease-Related Mutation Modeled in Yeast

(A) Comparison of ATP hydrolysis of *atp1*-V68G, *atp2*-V48D, and the three *atp6* mutants. Mutants that compensate for *bcs1-F342C* are in bold. See legend of Figure 3A and Table S3.

(B) Dilution series of cells of the WT, *bcs1*-F342C, *atp6*-W136R, *atp6*-L183P, and the double mutants *bcs1*-F342C and *atp6*-W136R or *atp6*-L183P were grown for 3 days at 28°C.

(C) Complex III activity as in legend of Figure 2D. Data are represented as mean ± SEM.

(D) SDS-PAGE and BN-PAGE analysis of mitochondrial proteins as in Figures 1E and 1G.

(E) Theoretical structural model of the AAA domain of the yeast Bcs1 with the positions of the amino acids F342, F401, and ADP. Mitochondrial complexes of WT and *bcs1-F401I* were analyzed by BN-PAGE and immunoblotted with anti-Bcs1 antibody as in Figure 1E.

(F) Dilution series of cells of WT, *bcs1-F401I*, and of the double mutants *atp6-W136R bcs1-F401I* and *atp1-V68G bcs1-F401I* were grown for 4 days at 28°C.

(G) Complex III activity as in legend of Figure 2D. Data are represented as mean ± SEM.

defects in complex III assembly. It is important to keep in mind that ATP synthase is a reversible enzyme that can hydrolyse ATP coupled to the pumping of protons out of the mitochondrial matrix through the F_o membrane domain (for review, see Ackerman and Tzagoloff, 2005). In the *bcs1*-F342C mutant, the electron flow and proton gradient generation are severely impaired due to a drastic effect on complex III assembly; the resulting level of ATP synthesized in this mutant is under the detection threshold. However, the ATP synthase is normally assembled and exhibits a wild-type hydrolytic activity that can be modulated by the compensatory mutations. Thus, rather than a reduced capacity of the ATP synthase to produce ATP, it is the low F_1-mediated ATP hydrolysis that is responsible for improving the assembly of complex III in the *bcs1*-F342C mutant.

As the steady-state levels and oligomerization of Bcs1 were not affected by the F342C mutation, it is probable that the less-efficient capacity to assemble Rip1 is due to an altered activity of the protein. The mutated residue is within the AAA domain of Bcs1, close to the SHR motif that is known to be required for ATP hydrolysis in other AAA proteins (Karata et al., 1999; Hanson and Whiteheart, 2005). Modeling of the phenylalanine-to-cysteine substitution in the theoretical structure of Bcs1 suggests that the interactions between the position 342 and conserved amino acids of the SRH motif are indeed modified (Figure S1). Thus, the activity of Bcs1 might be compromised by less-efficient ATP hydrolysis. This hypothesis is supported by the lower ATPase activity of the mutated, compared to the WT purified, Bcs1 protein at ATP concentrations of 2.5–5 mM, but nearly the same activity at higher concentrations. Thus, it can be inferred that the compensatory activity, conferred by a strong decrease in F_1-mediated ATP hydrolytic activity, results from a higher availability of ATP within mitochondria increasing the ATPase activity of the mutated Bcs1 protein and concomitant insertion of Rip1 to give fully assembled complex III. This would allow the reestablishment of a proton gradient and the synthesis of ATP by the remaining F_1-F_o complexes. The resulting ATP synthesis would further increase the matrix ATP content and stimulate Bcs1 activity. According to this suppressor mechanism, a *bcs1* mutation not affecting the AAA domain should not be suppressed by the ATP synthase mutations. This was indeed observed (data not shown).

The genetic interaction between Bcs1 and ATP synthase revealed by the present study leads us to propose a model in which the complex III biogenesis would be modulated by the energetic state of mitochondria (see Figure 7). When yeast cells rely on oxidative phosphorylation, the level of ATP inside mitochondria is high and exchanged against cytosolic ADP to provide the extramitochondrial compartment of the cell with energy. Large amounts of complex III are required. In cells producing ATP by fermentation, the intramitochondrial concentration of ATP is low, the glycolytic ATP is imported into mitochondria by the ADP/ATP translocator, and there is no

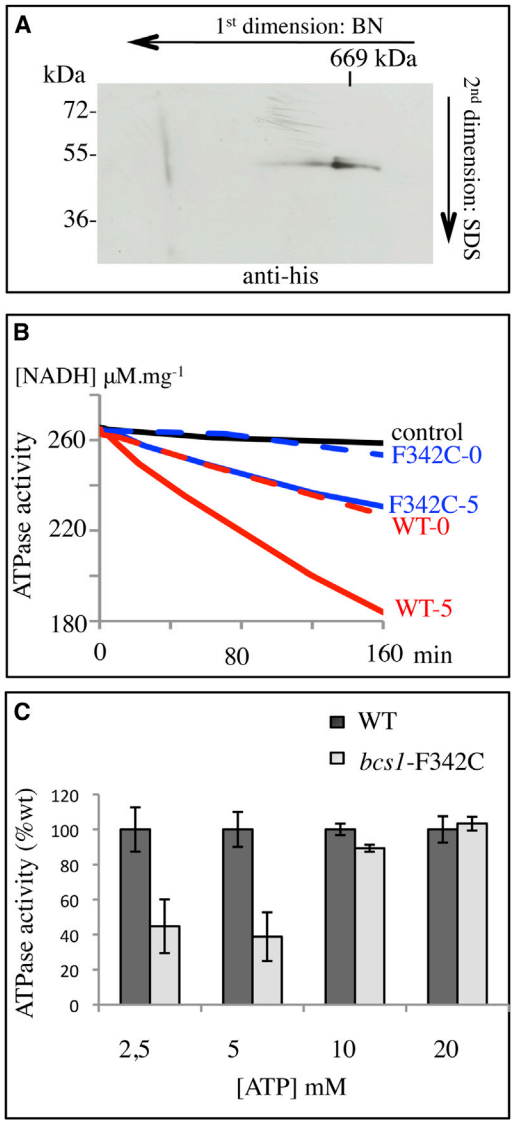

Figure 6. In Vitro ATPase Activity of Purified WT and Mutated Bcs1

(A) Ni-NTA-purified Bcs1 (see Figure S4) was analyzed by 2D BN-PAGE/SDS-PAGE and immunoblotted with anti-His antibody. Positions of size markers are indicated.

(B) The in vitro ATPase activity of Ni-NTA partially purified WT and mutated Bcs1 proteins was measured by monitoring NADH oxidation at 340 nm through a coupled reaction with pyruvate kinase (PK) and lactate dehydrogenase (LDH) that kept the ATP concentration constant during the assay. The absorbance decrease at 340 nm reflects ATPase activity. Control, no protein added; WT-0 or F342C-0, no ATP added; WT-5 or F342C-5, 5 mM ATP added.

(C) ATP consumption rates of the mutated versus WT Bcs1 at different ATP concentrations in the assay (2.5– 20 mM) are represented as mean values of three independent experiments. Data are represented as mean ± SEM.

need to produce large amounts of complex III. Thus, we propose that the ATP-dependent activity of Bcs1 is not just a requirement to exercise its chaperon activity, but also a way to couple the rate of complex III biogenesis to the energy-transducing activity of mitochondria.

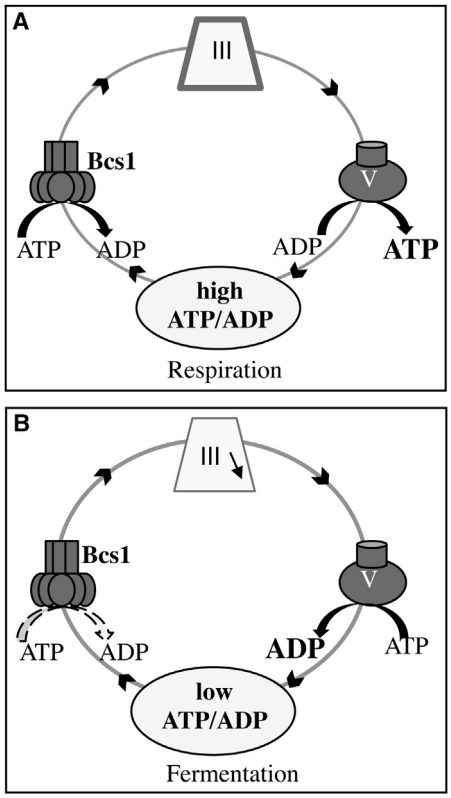

Figure 7. Schema for the Modulation of Complex III Biogenesis through the ATP-Dependent Activity of Bcs1

(A and B) The ATP/ADP ratio is known to be much higher in respiratory (ethanol) (A) than in fermentative (glucose) (B) conditions (Beauvoit et al., 1993; see Discussion).

The importance of ATP in the control of cellular activities is well established. There are numerous examples of such control in catabolic and anabolic pathways, like glycolysis, the Krebs cycle, and the electron transport chain of mitochondria. However, in all of these examples, ATP regulates the activity of an enzyme (e.g., cytochrome oxidase; Beauvoit and Rigoulet, 2001; Ramzan et al., 2010), whereas in the case of Bcs1, ATP could be used to modulate a late step in the assembly of an enzyme, complex III. This work shows that ATP might influence the biogenesis of an enzyme by controlling a protein specifically involved in its assembly. In the future, it would be interesting to determine whether other major ATP-dependent systems involved in mitochondrial quality control, like the m- and i-AAA proteases, are similarly modulated by the energetic activity of mitochondria.

The present study further defined the intramitochondrial adenine nucleotide pool as a potential target to treat Bcs1-based diseases, since this ATP-dependent compensatory mechanism is active on another yeast-modeled *bcs1* mutation found in a human patient. We recently showed that yeast models of ATP synthase disorders could be used for the screening of drugs active against human diseases caused by defects in this enzyme (Couplan et al., 2011). Our results indicate that such an approach might also be fruitful in the case of Bcs1-based disorders.

EXPERIMENTAL PROCEDURES

Strains and Media

S. cerevisiae strains are listed in Table S1. The nonfermentable media contain 2% glycerol, and the fermentable media contain either 2% glucose or 2% galactose with 0.1% glucose. Tetrad dissection was performed using a Singer MSM micromanipulator.

Genetic Identification of the Compensatory Mutations

Respiratory-competent revertants were isolated after plating independent subclones of the *bcs1*-F342C mutant on glycerol medium. Genetic crosses showed that the compensatory mutations were nuclear and extragenic and allowed the selection of strains carrying only the compensatory mutation associated to the WT *BCS1* gene. Further crosses suggested that the compensatory mutations of revertants R2, R12, and R18 are located in the same gene. We have constructed the double mutant carrying the compensatory mutations of R3 and R18 associated to the WT *BCS1* gene and shown that it exhibits a complete respiratory deficiency. After transformation of this double mutant with the wild-type genomic library, two classes of respiratory-competent transformants carrying *ATP1* or *ATP2* were isolated. Sequencing of these two genes revealed that R3 carries a mutation in *ATP1*, and R2, R12, and R18 carry mutations in *ATP2.*

Gene Deletion, Site-Directed Mutagenesis, and Epitope Tagging

The genes were deleted in the WT strain (CW252) by replacing the open reading frames (ORFs) with the *URA3*, *LEU2*, or *KanR* markers (see Table S1). The *bcs1*-F401I mutant was constructed by site-directed mutagenesis with the Stratagene QuikChange Kit and inserted at the chromosomal *BCS1* locus. Qcr7 as well as the WT and mutant Bcs1 proteins were tagged at their C termini with c-Myc or hexahistidine epitopes, respectively (Longtine et al., 1998). We verified that the introduction of the tag did not induce a respiratory deficiency. All the constructions were verified by PCR amplification and sequencing.

Mitochondria Preparation, SDS-PAGE, and BN-PAGE

Cells were grown overnight at 28°C in galactose medium, and mitochondria were isolated according to Lemaire and Dujardin (2008). Mitochondrial proteins were analyzed on 12% SDS-PAGE. For BN-PAGE, mitochondria were solubilized in digitonin (2%), and the complexes were separated on 5%–10% polyacrylamide gradient gels (Schägger and Pfeiffer, 2000; Lemaire and Dujardin, 2008). The BN-PAGE strips were placed on the 12% SDS-PAGE for the second dimension. Both SDS-PAGE and BN-PAGE were electrotransferred, and immunodetection was carried out using the chemiluminescent method from Pierce. Polyclonal antibodies against Cyt1, Bcs1, Cytb, and Nam1 were raised in the laboratory and used at a 1/30,000 ratio for Cyt1 and 1/5,000 for the others. The polyclonal Anti-Rip1 (1/3,000) is from N. Fisher (Liverpool, UK); the polyclonal antibodies against the subunits of ATP synthase (1/10,000) are from J. Velours (Bordeaux, France); the monoclonal anti-Cox2 (1/5,000) is from Molecular Probes; the monoclonal anti-c-Myc (1/20,000) is from J.M. Galan (Paris, France); the polyclonal anti-SDH (1/500) is from B. Guiard (Gif sur Yvette, France); and the anti-Tom40 is from C. Meisinger (Freiburg, Germany).

Coimmunoprecipitation Experiments

Mitochondria were solubilized in 50 mM Tris HCl (pH 7.4), 100 mM NaCl, 1% digitonine for 30 min at 4°C and centrifugated for 15 min at 100,000 × *g*. The supernatants were incubated with polyclonal anti-c-Myc antibodies coupled with agarose beads. Samples were incubated under gentle shaking for 90 min at 4°C. The beads were washed three times. The fractions were analyzed by western blotting experiments.

Determination of the Activities of the Respiratory Complexes III and IV

The activities were measured spectrophotometrically at 550 nm at 25°C on 2.5–10 μg of isolated mitochondria (Lemaire and Dujardin, 2008). The ubiquinol cytochrome *c* oxidoreductase (complex III) activity was assayed by the rate of reduction of cytochrome *c* in the presence of saturating amounts of decylubiquinol, and the cytochrome *c* oxidase (complex IV) activity was assayed by the rate of cytochrome *c* oxidation. The inhibitors, antimycin for complex III and KCN for complex IV, were used to test the specificity of the signal.

Membrane Potential, ATP Synthesis, and Hydrolysis Measurements

Variations of the membrane potential ($\Delta\psi$) were evaluated in respiration buffer by measurement of rhodamine 123 (Rh-123) fluorescence quenching with a SAFAS Monaco fluorescence spectrophotometer. Mitochondria were energized using ethanol as a respiratory substrate instead of NADH because fluorescence of the latter overlaps that of Rh-123. To determine ATP synthesis rates, mitochondria (0.3 mg) were placed in a 2 ml thermostatically controlled chamber at 28°C in respiration buffer. The reaction was started by the addition of 4 mM NADH and 1 mM ADP and stopped with 3.5% perchloric acid, 12.5 mM EDTA. Samples were then neutralized to pH 6.5 by addition of 2 M KOH/0.3 M MOPS. The luciferin/luciferase assay (Thermo Labsystems) was used to determine ATP concentrations. The specific ATPase activity at pH 8.4 of nonosmotically protected mitochondria (20 μg of proteins) was measured in the presence of saturating amounts of ATP with or without oligomycin (Lemaire and Dujardin, 2008).

Purification by Ni-NTA Chromatography of Bcs1 Proteins and Measurements of In Vitro ATPase Activity

A total of 10 mg of mitochondrial proteins was solubilized in 1 ml of buffer A (50 mM NaCl, 15% w/v glycerol, 15 mM imidazole, 50 mM sodium phosphate [pH 7.9]) with 2% digitonin. After 30 min of incubation at 4°C, the extract was clarified by centrifugation at 25,000 × *g* for 30 min at 4°C. The supernatant was mixed with 0.25 ml Ni-NTA agarose beads (QIAGEN) and washed with buffer A. After an overnight incubation at 4°C, the flow through was collected by centrifugation at 1,000 × *g* for 1 min at 4°C. Beads were washed with 40 volumes of buffer A by centrifugation at 1,000 × *g* for 1 min at 4°C, and Bcs1 proteins were eluted with buffer E (50 mM NaCl, 15% w/v glycerol, 250 mM imidazole, 50 mM sodium phosphate [pH 7.9]). The in vitro ATPase activity was measured at 28°C under magnetic stirring in 1 ml of activity buffer (20 units ml^{-1} of pyruvate kinase, 30 units ml^{-1} of lactate deshydrogenase, 2 mM phosphoenol pyruvate, 0.2 mM NADH, 5 mM $MgCl_2$, 50 mM HEPES [pH 7.5], and ATP at the concentrations 0, 2.5, 5, 10, or 20 mM). ATP concentrations of 0–5 mM were previously used by Augustin et al. (2009). The molar extinction coefficient of NADH at 340 nm was 6.22 M^{-1} cm^{-1}, and the path length was 1 cm.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.08.017.

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