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Prohibitins Interact Genetically with Atp23, a Novel Processing Peptidase and Chaperone for the F_1F_O-ATP Synthase

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The generation of cellular energy depends on the coordinated assembly of nuclear and mitochondrial-encoded proteins into multisubunit respiratory chain complexes in the inner membrane of mitochondria. Here, we describe the identification of a conserved metallopeptidase present in the intermembrane space, termed Atp23, which exerts dual activities during the biogenesis of the F_1F_O-ATP synthase. On one hand, Atp23 serves as a processing peptidase and mediates the maturation of the mitochondrial-encoded F_O-subunit Atp6 after its insertion into the inner membrane. On the other hand and independent of its proteolytic activity, Atp23 promotes the association of mature Atp6 with Atp9 oligomers. This assembly step is thus under the control of two substrate-specific chaperones, Atp10 and Atp23, which act on opposite sides of the inner membrane. Strikingly, both *ATP10* and *ATP23* were found to genetically interact with prohibitins, which build up large, ring-like assemblies with a proposed scaffolding function in the inner membrane. Our results therefore characterize not only a novel processing peptidase with chaperone activity in the mitochondrial intermembrane space but also link the function of prohibitins to the F_1F_O-ATP synthase complex.

INTRODUCTION

Mitochondria are dynamic organelles with essential catabolic and anabolic functions (Chan, 2006; McBride *et al.*, 2006). Their most prominent function, the production of cellular energy, is carried out by multisubunit respiratory chain complexes in the inner membrane, whose formation depends on the coordinated assembly of mitochondrial and nuclear encoded subunits (Costanzo and Fox, 1990). A multitude of nuclear-encoded proteins has been identified, which assist the formation of these complexes at all stages, from the synthesis of mitochondrial-encoded core subunits and their membrane insertion, to their assembly within the inner membrane.

This is exemplified by the F_1F_O-ATP synthase, which catalyzes the synthesis of ATP from ADP and organic phosphate using the electrochemical proton gradient across the inner membrane (Ackerman and Tzagoloff, 2005), but which has also a structural role for cristae morphology (Giraud *et al.*, 2002). Core subunits of the proton-conducting F_O-moiety of this enzyme are encoded within mitochondria and assemble with nuclear encoded subunits into functionally active F_1F_O-particles. Efficient assembly is ensured by mitochondrial Hsp60 (Gray *et al.*, 1990) and Hsp70 (Herrmann *et al.*, 1994) and several substrate-specific chaperone proteins present within mitochondria. The latter include Atp11, Atp12, and Fmc1 (Ackerman and Tzagoloff, 1990; Lefebvre-Legendre *et al.*, 2001), assembly factors of the matrix-exposed F_1-ATPase particle, as well as Atp10, which promotes the assembly of mitochondrial-encoded Atp6 into the membrane-embedded F_O-particle (Tzagoloff *et al.*, 2004).

Whereas these helper proteins assist the assembly of the F_1F_O-ATP synthase complexes in the inner membrane, a membrane-associated quality control system ensures the efficient removal of excess subunits (Nolden *et al.*, 2006). Two ubiquitous and conserved ATP-dependent proteolytic complexes, termed AAA proteases, degrade processively nonassembled proteins to peptides, which are either released from the organelle or further degraded to amino acids by oligopeptidases within mitochondria (Augustin *et al.*, 2005; Kambacheld *et al.*, 2005). In addition to their function for protein quality control, AAA proteases exert regulatory roles during mitochondrial biogenesis, as has been demonstrated for the *m*-AAA protease active on the matrix side of the inner membrane. The *m*-AAA protease mediates the maturation of the nuclear encoded ribosomal protein MrpL32 and thereby controls ribosomal assembly and the synthesis of mitochondrial-encoded proteins (Nolden *et al.*, 2005). Concomitantly, the absence of the *m*-AAA protease in yeast impairs the assembly of respiratory chain complexes and of the F_1F_O-ATP synthase and results in respiratory deficiency (Paul and Tzagoloff, 1995; Arlt *et al.*, 1998; Gallhun and Langer, 2004). The *m*-AAA protease thus can act both as a protein-degrading machine and a specific processing peptidase. The vast majority of nuclear encoded mitochondrial proteins, however, are targeted to mitochondria by N-terminal sorting signals that are cleaved off by specific, ATP-independent processing peptidases present in different compartments of mitochondria (Gakh *et al.*, 2002).

The *m*-AAA protease has been identified as part of a large suprastructure within the inner membrane containing the prohibitin complex (Steglich *et al.*, 1999). Two highly conserved prohibitin subunits, Phb1 and Phb2, assemble into a multimeric, ring complex that has a diameter of ~20–25 nm

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and was proposed to provide a scaffold within the membrane (Tatsuta *et al.*, 2005). Although highly conserved in eukaryotic cells, the function of prohibitins is only ill-defined (Nijtmans *et al.*, 2002; Mishra *et al.*, 2006). Deletion of either *PHB1* or *PHB2* in yeast results in the destabilization of the respective other subunit (Berger and Yaffe, 1998) and the accelerated degradation of nonassembled membrane proteins by the *m*-AAA protease (Steglich *et al.*, 1999). Therefore, prohibitins may regulate proteolysis by the *m*-AAA protease, either by directly interacting with substrate proteins or by modulating the enzymatic activity of the *m*-AAA protease. Yeast cells lacking prohibitins do not show apparent growth defects (Coates *et al.*, 1997); however, synthetic lethal interactions have been reported with mutations in several genes. These include *MDM10*, *MDM12*, and *MMM1*, involved in the maintenance of mitochondrial morphology and the import of β-barrel proteins in the outer membrane (Berger and Yaffe, 1998; Meisinger *et al.*, 2004), as well as *PSD1*, encoding a mitochondrial phosphatidyl decarboxylase (Birner *et al.*, 2003). A genetic interaction with prohibitins has also been described for *YTA10* and *YTA12*, coding for subunits of the *m*-AAA protease (Steglich *et al.*, 1999). The latter observation suggests additional functions of prohibitins within mitochondria that are not explained by their physical interaction with the *m*-AAA protease but are likely linked to its function.

Here, we identify the open reading frame *YNR020c* as being essential for the growth of prohibitin-deficient yeast cells. We demonstrate that *YNR020c* encodes a novel metallopeptidase of the mitochondrial intermembrane space, termed Atp23, with dual activities: it mediates maturation of mitochondrial-encoded Atp6 and chaperones its assembly into F_1F_O-ATP synthase complexes. Our findings therefore functionally link prohibitins to the F_1F_O-ATP synthase in the inner membrane.

MATERIALS AND METHODS

Cloning Procedures

ATP23 including 507 base pairs upstream of the start codon was amplified by PCR from genomic DNA and inserted into the BamHI site of centromer-based pFL38 (Bonneaud *et al.*, 1991). Point mutations were introduced into *ATP23* using the PCR-based Quik-Change mutagenesis kit (Stratagene, La Jolla, CA) and verified by DNA sequencing.

Yeast Strains and Growth Conditions

Yeast strains used in this study are derivatives of S288c (Brachmann *et al.*, 1998). The *ATP23* gene was disrupted using a *HIS3* cassette amplified by PCR from the plasmid pFA6a-*HIS3*MX6 (Longtine *et al.*, 1998). To avoid indirect effects on the expression of neighboring genes only base pairs 196-215 of *ATP23* were deleted. *PHB1* or *PHB2* were disrupted with a marker gene conferring resistance toward the antibiotic nourseothricin, which was amplified from the plasmid pAG25 (Goldstein and McCusker, 1999). *ATP10* was deleted using a *KAN* cassette (Longtine *et al.*, 1998). For C-terminal tagging of Atp23, a cassette containing a triple hemagglutinin (HA) tag was generated by PCR from the plasmid pFA6a-3HA-*KAN*MX6 (Longtine *et al.*, 1998).

Yeast strains were grown according to standard procedures on complete (YP) or synthetic media (SC) supplemented with 2% (wt/vol) glucose or, when indicated, 3% (vol/vol) glycerol. For tetrad dissection diploid cells were incubated on 1% (wt/vol) potassium acetate plates until tetrads had formed. Tetrads were dissected with a micromanipulator on YP containing 2% glucose (YPD). Tetrad dissection was evaluated by replicating spore clones on selective media containing G418 (250 μg/ml) and nourseothricin (100 μg/ml). To examine cell growth under various conditions, cells were grown in liquid YPD or selective media to an OD_{600} of ~1. Fivefold serial dilutions were made in sterile H_2O from a cell suspension containing 10^6 cells/ml, and aliquots (3.5 μl) were spotted onto the indicated plates that were incubated at 30°C for 2–5 d.

Assembly of Newly Synthesized Mitochondrial Encoded Subunits into F_1F_O-ATP Synthase Complexes

The assembly of the F_1F_O-ATP synthase was assessed by blue-native PAGE (BN-PAGE) essentially as described (Schägger, 2001; Wittig *et al.*, 2006). To generate a surplus of nuclear encoded subunits, wild-type and mutant strains were grown for 2 h in the presence of chloramphenicol (2 mg/ml) before mitochondria were isolated as described (Tatsuta and Langer, 2007). Mitochondria were resuspended at a concentration of 0.66 mg/ml in translation buffer [0.6 M sorbitol, 150 mM KCl, 15 mM potassium phosphate pH 7.4, 20 mM Tris/Cl, pH 7.4, 13 mM $MgSO_4$, 0.3% (wt/vol) BSA (fatty acid free), 4 mM ATP, 0.5 mM GTP, 6 mM α-ketoglutarate, 5 mM phosphoenolpyruvate, 0.1 mM each amino acid except methionine] that was supplemented with pyruvate kinase (0.5 mg/ml). After 2 min incubation at 30°C, ^{35}S methionine was added (0.5 μCi/μg mitochondria), and samples were further incubated at 30°C for 3 min. Mitochondrial translation was stopped by adding methionine (5 mM) and puromycin (0.03 mg/ml). After a further incubation for 2 min at 30°C, samples were cooled on ice for 5 min. Mitochondria were pelleted, washed with 0.6 M sorbitol, 1 mM EDTA, pH 8, 5 mM methionine, and then resuspended in translation buffer (0.66 mg/ml). Aliquots of the suspension (corresponding to 150 μg mitochondria) were incubated at 30°C for the indicated time periods. After washing, mitochondria were solubilized under constant mixing for 15 min at 4°C at a concentration of 7 mg/ml in 1.6% (vol/vol) Triton X-100, 50 mM NaCl, 5 mM 6-aminohexanoic acid, 50 mM imidazole/HCl, pH 7, 10% (vol/vol) glycerol, 50 mM potassium phosphate buffer, pH 7.4. After centrifugation for 20 min at 4°C at 20,000 × *g*, the supernatant was mixed with 1/10 1% (wt/vol) Coomassie in solubilization buffer and loaded onto a blue native gel (5–13%; Schägger, 2001). Gels were stained with Coomassie and analyzed by autoradiography. Assembled F_O-subunits of the ATP synthase were quantified by phosphoimaging using the program Quantity One (Bio-Rad, Hercules, CA).

Coimmunoprecipitation of Atp6 and Atp23

After labeling of mitochondrial translation products with [^{35}S]methionine as described previously (Tatsuta and Langer, 2007), wild-type mitochondria and mitochondria containing Atp23-HA were lysed at a concentration of 1 mg/ml in 2% (wt/vol) digitonin, 150 mM KAc, 4 mM MgAc, 30 mM Tris/Cl, pH 7.4, 1 mM PMSF, and 1 mM ATP. The suspension was vortexed for 30 s and cooled on ice for 30 s three times, mixed at 4°C for 15 min, and then centrifuged at 4°C at 125,000 × *g*. The supernatant was supplemented with polyclonal anti-HA antibodies (Roche, Mannheim, Germany) that had been coupled to protein A-Sepharose. Samples were incubated under gentle shaking for 60 min at 4°C and washed subsequently with lysis buffer and 10 mM Tris/Cl, pH 7.4. Immunocomplexes were dissociated in SDS sample buffer by vigorous shaking for 10 min at 30°C and 5 min at 95°C. The immunoprecipitates were analyzed by SDS-PAGE and Western blotting.

RESULTS

Deletion of ATP23 Is Synthetic Lethal with Δphb1 and Δphb2

To further characterize the cellular function of prohibitins, we performed a synthetic genetic array analysis for genes, which are essential for growth of Δ*phb1* or Δ*phb2* cells on glucose-containing media, using a deletion library of ~4850 nonessential yeast genes (Osman, Collart, and Langer, unpublished data). The uncharacterized ORF *YNR020c* on chromosome XIV was identified as a candidate gene and, based on experiments described below, was termed *ATP23*. To verify the genetic interaction of *ATP23* with *PHB1* and *PHB2*, we deleted *ATP23* by homologous recombination in a diploid yeast strain that was heterozygous for a deletion of *PHB1* or *PHB2*. After sporulation and tetrad dissection, cell growth of meiotic progenies was examined on YPD medium containing glucose as carbon source (Figure 1). The distribution of the gene deletions was monitored by plating the cells on media containing G418 or nourseothricin (data not shown). This analysis revealed that meiotic progenies lacking *ATP23* and either *PHB1* or *PHB2* were not viable or displayed a severe growth defect, demonstrating the genetic interaction of the genes.

Atp23 Resides in the Intermembrane Space of Mitochondria

We therefore examined the function of Atp23 in more detail and first determined its subcellular localization. The *ATP23* gene was modified by homologous recombination to allow expression of Atp23 carrying a hemagglutinin-epitope at its C-terminus. Expression of Atp23-HA restored growth defects associated with a deletion of *ATP23*, indicating func-

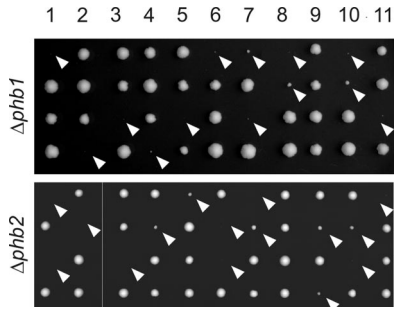

Figure 1. Synthetic lethal interaction of *ATP23* with *PHB1* and *PHB2*. Tetrad dissection of 11 asci derived from a Δ*atp23/ATP23* Δ*phb1/PHB1* diploid strain (top) and a Δ*atp23/ATP23* Δ*phb2/PHB2* diploid strain (bottom). Ascospores were dissected on YPD and incubated at 30°C for 3 d. Genotypes were inferred from the distribution of the markers linked to the deletions. Double mutants are indicated with arrowheads.

tionality of the modified protein (data not shown). On subcellular fractionation, Atp23-HA was exclusively detected in the mitochondrial fraction (Figure 2A). It showed a behavior similar to Tom40, a component of the protein translocase in the outer membrane, whereas cytosolic Bmh1 was exclusively detected in the postmitochondrial fraction (Figure 2A). These results were confirmed by in vitro experiments that demonstrated the posttranslational import of ^{35}S-labeled Atp23 into isolated mitochondria (data not shown).

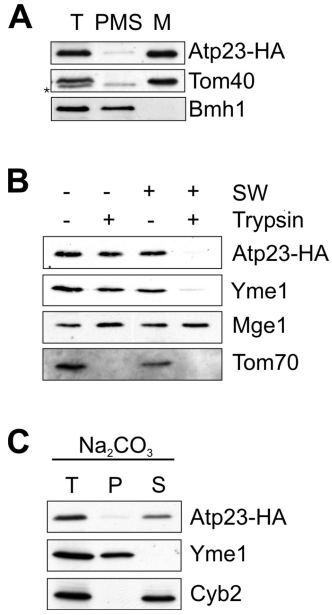

Figure 2. Atp23 is an intermembrane space protein of mitochondria. (A) Mitochondria were isolated from a strain expressing Atp23-HA. Whole cell extracts (T), postmitochondrial supernatant (PMS), and isolated mitochondria (M) were analyzed by SDS-PAGE and immunoblotting using Tom40 as a mitochondrial and Bmh1 as a cytosolic marker protein. A band unspecifically cross-reacting with the Tom40-specific antiserum is marked with an asterisk. (B) Mitochondria harboring Atp23-HA were treated with trypsin (50 μg/ml) with and without hypotonic disruption (SW) of the outer membrane as described (Leonhard *et al.*, 2000). Samples were subjected to SDS-PAGE followed by immunoblotting. Fractionation was monitored using the outer membrane protein Tom70, the intermembrane space protein Yme1 and the matrix-localized protein Mge1 as controls. (C) Isolated mitochondria were treated with sodium carbonate (pH 11.5, T) and subjected to ultracentrifugation to obtain soluble (S) and insoluble (P) fractions, which were analyzed by SDS-PAGE and immunoblotting. The integral inner membrane protein Yme1 and the soluble intermembrane space protein cytochrome b_2 (Cyb2) were used as controls.

Inspection of the amino acid sequence of Atp23 with a series of protein prediction programs did not provide evidence for the presence of a membrane-spanning segment in Atp23. Consistently, Atp23-HA was almost exclusively recovered from the soluble fraction upon alkaline extraction of mitochondrial membranes at pH 11.5, i.e., behaved like the L-lactate dehydrogenase cytochrome b_2 soluble in the intermembrane space (Figure 2C). In contrast, the integral inner membrane protein Yme1 remained in the pellet fraction under these conditions (Figure 2C). We conclude from these experiments that Atp23 is localized to the intermembrane space of mitochondria.

Atp23 Is Essential for the Maintenance of Respiratory Competence

To characterize the function of Atp23 within mitochondria, *ATP23* was deleted by homologous recombination and the growth of Δ*atp23* cells on various carbon sources was examined (Figure 3A). Deletion of *ATP23* resulted in the formation of small colonies under fermenting growth conditions in the presence of glucose, whereas cell growth was completely inhibited under respiring conditions on glycerol-containing medium (Figure 3A). This *petite* phenotype indicates an essential function of Atp23 for the formation of the respiratory chain in the inner membrane.

We therefore examined the steady state concentration of various subunits of respiratory chain complexes in Δ*atp23* mitochondria by immunoblotting (Figure 3B). In agreement with the observed respiratory deficiency, a number of respiratory chain subunits accumulated at drastically reduced levels in Δ*atp23* cells. These included Atp6 and Atp18, subunits of the F_1F_O-ATP synthase (complex V), cytochrome *b* (complex III), and subunits 1 and 3 of cytochrome *c* oxidase (complex IV; Figure 3B). The reduced steady state concentrations of subunits of various respiratory chain complexes indicate a rather profound effect of Atp23 on the respiratory chain.

A pleiotropic effect on the respiratory chain is characteristic of mutations in nuclear genes that affect the maintenance or expression of the mitochondrial genome encoding essential respiratory chain subunits. Mating of Δ*atp23* cells with *mit*$^-$ strains harboring point mutations in the mitochondrial *COX3* gene as well as staining of Δ*atp23* cells with 4′,6-diamidino-2-phenylindole revealed the presence of mtDNA in Δ*atp23* cells, excluding the possibility that a general loss of mtDNA causes pleiotropic defects in these cells (data not shown). Of note, we observed an increased tendency of mtDNA loss in Δ*atp23* cells grown under fer-

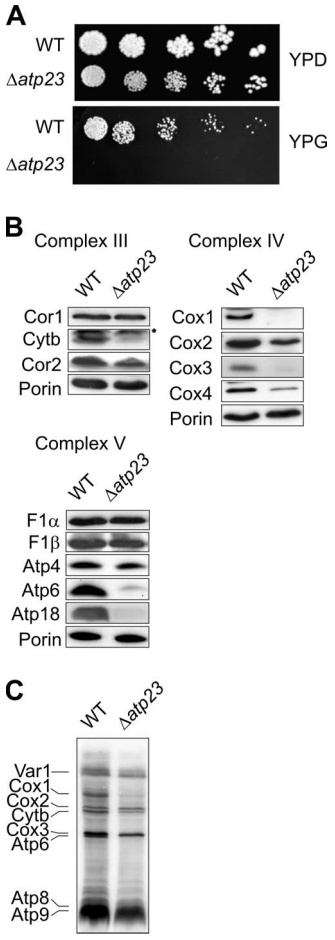

Figure 3. Atp23 is essential for respiratory growth. (A) Serial dilutions of wild-type (WT) and Δ*atp23* cells were plated on YPD and YPG plates. Strains were grown at 30°C. (B) Steady state levels of various subunits of the respiratory chain were examined by immunoblotting of mitochondria (50 μg) derived from Δ*atp23* and wild-type (WT) cells. A band unspecifically cross-reacting with cytochrome *b* (Cytb)-specific antiserum is marked with an asterisk. (C) Synthesis of mitochondrial-encoded proteins in wild-type (WT) and Δ*atp23* mitochondria. Mitochondrial translation products were synthesized in the presence of [^{35}S]methionine. Mitochondrial proteins were separated by SDS-PAGE and analyzed by autoradiography. The efficiency of Cox1 labeling varied in different experiments (data not shown).

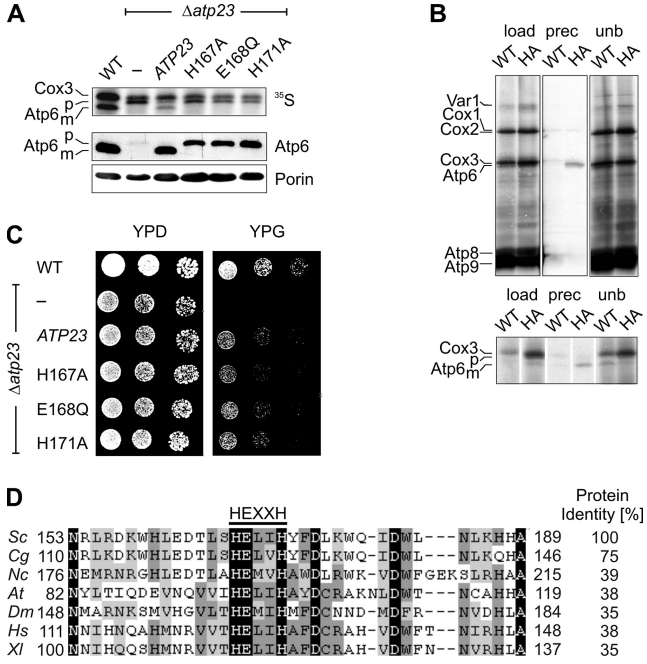

Figure 4. Dual activity of Atp23 within mitochondria. (A) Accumulation of uncleaved Atp6 in mitochondria harboring mutant Atp23. Mitochondrial protein synthesis was carried out in the presence of [^{35}S]methionine in mitochondria isolated from wild-type (WT) cells and from Δ*atp23* cells that expressed Atp23 variants carrying mutations in the consensus metal binding site (Atp23^{H167A}, Atp23^{E168Q}, Atp23^{H171A}) when indicated. Mitochondrial proteins were analyzed by SDS-PAGE and Western blotting. An autoradiograph of the membrane is shown in the top panel (^{35}S), an immunoblot analysis with Atp6- and porin-specific antisera in the middle and bottom panels. p, precursor form; m, mature form. (B) Binding of newly synthesized Atp6 to Atp23-HA. After labeling of mitochondrial translation products with [^{35}S]methionine in wild-type (WT) mitochondria and mitochondria harboring Atp23-HA (HA), mitochondria were lysed with digitonin. Mitochondrial extracts (load, 5% of total) were subjected to coimmunoprecipitation with HA-specific antiserum. The precipitates (prec) and unbound material (unb, 10% of total) were analyzed by SDS-PAGE and autoradiography. The top panel shows the autoradiograph including all mitochondrial translation products. In the bottom panel, a part of an autoradiograph of a high-resolution gel is shown including Cox3, Atp6 precursor (p), and mature Atp6 (m). (C) Respiratory growth of Δ*atp23* cells expressing mutant Atp23. Atp23 variants containing mutations in the consensus metal-binding site (Atp23^{H167A}, Atp23^{E168Q}, Atp23^{H171A}) were expressed in Δ*atp23* cells. Fivefold serial dilutions of wild-type (WT) and mutant cells were plated on YPD and YPG and incubated for 2 d at 30°C. (D) Multiple sequence alignment of Atp23 and homologues using AlignX. Sequences surrounding the consensus metal-binding sites are shown. Sequence identities between whole proteins according to Blastp are indicated. *Sc, S. cerevisiae* Atp23 (Ynr020c), *Cg, Candida glabrata* CAG62785; *Nc, Neurospora crassa* XP322193; *At, Arabidopsis thaliana* Ku70-binding family protein NP_566205; *Dm, Drosophila melanogaster* CG5131-PA; *Hs, Homo sapiens* KUB3; and *Xl, Xenopus laevis* KUB3-homologue. Identical amino acids are shown in black, conserved residues in dark gray, and similar residues in light gray.

menting conditions. This suggests that Atp23, though not essential, affects the stability of mtDNA.

We analyzed mitochondrial translation in wild-type and Δ*atp23* mitochondria and labeled mitochondrial-encoded proteins with [^{35}S]methionine (Figure 3C). Radiolabeled mitochondrial-encoded proteins accumulated irrespective of the presence of Atp23 (Figure 3C). This finding substantiates the presence of mtDNA in these mitochondria and demonstrates that Atp23 is not required for mitochondrial translation. It therefore appears that either Atp23 has pleiotropic effects on various respiratory chain complexes or that some of the deficiencies are indirectly caused by an impaired assembly of one of these complexes (Marzuki *et al.*, 1989; Paul *et al.*, 1989).

Atp23 Mediates Maturation of Newly Synthesized Atp6

In the course of these experiments, we noted that newly synthesized Atp6 accumulated at a larger molecular weight in Δ*atp23* cells when compared with wild-type cells. This became more apparent when a high-resolution SDS-PAGE was performed after labeling of mitochondrial-encoded proteins in mitochondria isolated from wild-type and Δ*atp23* cells (Figure 4A). Consistently, Western blot analysis of mitochondria lacking Atp23 with an Atp6-specific antiserum indicated that a larger form of Atp6 was present at drasti-

cally reduced steady state concentrations (Figure 4A). Previous studies have demonstrated that Atp6 is synthesized as a precursor form carrying an N-terminal extension of 10 amino acids that is subsequently cleaved off by an unknown peptidase (Michon *et al.,* 1988). Our findings therefore suggested that Atp23 is required for the maturation of newly synthesized Atp6, which exposes its N-terminal amino acid residues to the intermembrane space.

To exclude indirect effects of a deletion of *ATP23* on Atp6 maturation, we examined whether both proteins directly interact with each other by coimmunoprecipitation. Mitochondrial-encoded proteins were synthesized in the presence of [^{35}S]methionine in wild-type mitochondria and mitochondria harboring Atp23-HA (Figure 4B). After completion of translation, mitochondrial membranes were solubilized with digitonin and subjected to coimmunoprecipitation using a HA-specific antiserum. While newly synthesized polypeptides were not detectable in the precipitate derived from wild-type mitochondria, Atp6 was precipitated with Atp23 when Atp23-HA–containing mitochondria were analyzed (Figure 4B). High-resolution SDS-PAGE analysis revealed binding of mature Atp6 to Atp23 (Figure 4B). These results demonstrate that newly synthesized Atp6 interacts directly with Atp23. It should be noted that no other mitochondrial-encoded polypeptides including Atp8 and Atp9 were found in association with Atp23, suggesting a specific effect of Atp23 on the biogenesis of Atp6.

The analysis of the protein sequence of Atp23 identified a consensus metal-binding motif HEliH, formed by amino acid residues 167-171 of Atp23, which is characteristic of metallopeptidases and which is highly conserved in homologous proteins present in various eukaryotic organisms, including mammals and plants (Figure 4D). This observation indicates that Atp23 by itself may exert peptidase activity and mediate maturation of Atp6 within mitochondria. To substantiate this hypothesis, we mutated histidine residues 167 and 171, which should serve as metal-binding ligands, and the putative catalytically active glutamate 168. The corresponding residues have been demonstrated to be essential for the proteolytic activity of various metallopeptidases. The Atp23 variants were expressed from a centromer-based plasmid under the control of the endogenous *ATP23* promoter in Δ*atp23* cells and respiratory growth on glycerol-containing medium was examined (Figure 4C). Strikingly, respiratory growth of Δ*atp23* cells was restored upon expression of Atp23 and all variants thereof.

These findings suggest that either Atp23 does not exert proteolytic activity or that Atp23 mediates Atp6 maturation, which however is not essential for the maintenance of respiratory growth. To distinguish between these possibilities, we analyzed the processing of Atp6 in Δ*atp23* mitochondria harboring Atp23 or the variants Atp23^{H167A}, Atp23^{E168Q}, or Atp23^{H171A} (Figure 4A). Mitochondrial-encoded proteins were synthesized in the presence of [^{35}S]methionine, and the accumulation of newly synthesized Atp6 was monitored by SDS-PAGE. Although mature Atp6 was formed in Δ*atp23* mitochondria containing Atp23, only the precursor form of Atp6 was detected in the presence of Atp23 variants carrying point mutations in the predicted proteolytic center (Figure 4A). Immunoblotting with Atp6-specific antiserum revealed that Atp6 was stabilized upon expression of the mutant variants of Atp23 and accumulated in its precursor form in these mitochondria, but in its mature form in the presence of plasmid-borne Atp23 (Figure 4A).

We conclude that the integrity of the metal binding motif of Atp23 is essential for the maturation of Atp6. This observation and the physical interaction of both proteins strongly suggest that Atp23 acts as the processing peptidase of Atp6. Moreover, our findings demonstrate that Atp6 processing is not required for maintenance of respiratory growth. In the presence of proteolytically inactive Atp23, the steady state concentration of Atp6 within mitochondria and respiratory growth of Δ*atp23* cells was restored. It therefore appears that Atp23 has a dual activity within mitochondria, only one of them depending on its proteolytic activity.

The Assembly of the F_O-Particle of the ATP Synthase Depends on Atp23 But Not on Its Proteolytic Activity

To further characterize the nonproteolytic function of Atp23, we analyzed the assembly of respiratory chain complexes in mitochondria lacking Atp23 or harboring Atp23 mutant variants by blue-native PAGE (BN-PAGE). Gentle solubilization of mitochondrial membranes with digitonin preserves supercomplexes composed of different respiratory chain complexes as well as a dimeric form of F_1F_O-ATP synthase complexes (Arnold *et al.,* 1998). These complexes can be identified by immunoblotting using antisera directed against subunits of these complexes. Digitonin-extracts of wild-type and Δ*atp23* mitochondria were fractionated by BN-PAGE, followed by immunoblotting with antisera directed against subunit 2 of cytochrome *c* oxidase (Cox2). Two high-molecular-mass assemblies containing Cox2 were detected in wild-type mitochondria, which correspond to supercomplexes built up of two copies of complex III and one or two copies of complex IV. In agreement with the reduced steady state concentration of some subunits of both complexes (see Figure 3B), both assemblies accumulated in significantly reduced amounts in the absence Atp23 (Figure 5A). A more drastic effect, however, was observed upon immunoblotting with antibodies directed against Atp6 and the α-subunit of the F_1-particle (Figure 5A). Monomeric or dimeric F_1F_O-ATP synthase complexes were hardly detected in Δ*atp23* mitochondria indicating defective assembly (Figure 5A). Notably, a major band corresponding to a native molecular mass of ~440 kDa accumulated in the absence of Atp23, which cross-reacted with $F_1\alpha$-specific but not Atp4- or Atp6-specific antibodies (Figure 5A; data not shown) and therefore most likely represents the assembled F_1-particle. Thus, Atp23 is dispensable for the formation of the F_1-ATPase, but specifically affects the assembly of the membrane-embedded F_O-particle. This function does not depend on the proteolytic activity of Atp23. Expression of Atp23 variants harboring point mutations in the consensus metal binding motif in Δ*atp23* cells restored the assembly of F_1F_O-ATP synthase complexes (Figure 5A).

In further experiments, we developed an assay that allowed us to monitor directly the insertion of newly synthesized, mitochondrial-encoded F_O-subunits into the F_1F_O-ATP synthase complex. Mitochondria were isolated from wild-type and Δ*atp23* cells grown in the presence of chloramphenicol. This treatment results in the reversible inhibition of mitochondrial protein synthesis and leads to the accumulation of assembly-competent intermediates by nuclear-encoded subunits of the F_1F_O-ATP synthase. Mitochondrial translation was initiated in the presence of [^{35}S]methionine in wild-type and Δ*atp23* mitochondria and incorporation of newly synthesized F_O-subunits Atp6, Atp8, and Atp9 into the F_1F_O-ATP synthase was assessed by BN-PAGE analysis (Figure 5B). Deletion of *ATP23* completely inhibited the assembly of the mitochondrial-encoded F_O-subunits into the F_1F_O-ATP synthase, which was observed in wild-type mitochondria (Figure 5B). Expression of Atp23^{E168Q} lacking the catalytic active glutamate residue in the proteolytic center of Atp23

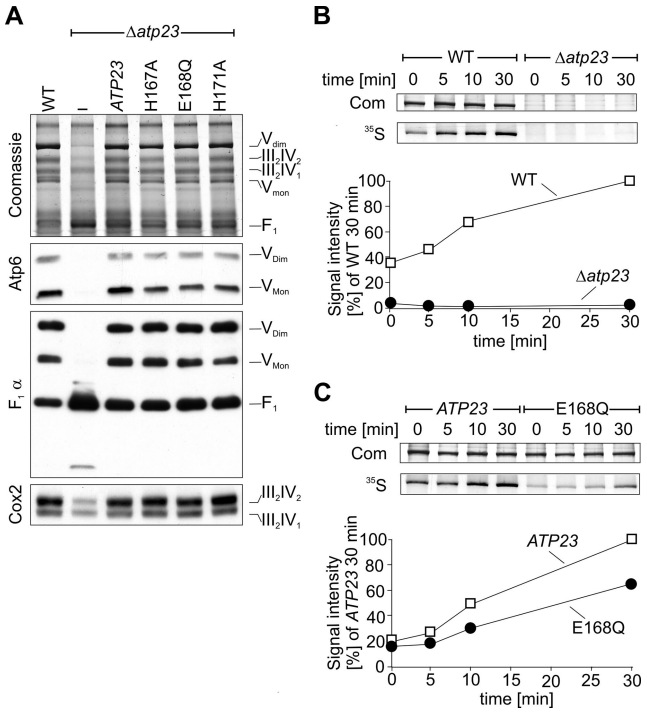

Figure 5. Atp23 is required for the assembly of the F_O-particle of the F_1F_O-ATP synthase independent of its proteolytic activity. (A) Wild-type and Δ*atp23* mitochondria (100 μg), which contain Atp23 variants carrying mutations in the consensus metal binding site ($Atp23^{H167A}$, $Atp23^{E168Q}$, $Atp23^{H171A}$) when indicated, were solubilized in digitonin (1.8%) and analyzed by BN-PAGE followed by Coomassie staining (top panel) or immunoblotting (lower three panels) using polyclonal antisera directed against Atp6, the α-subunit of the F_1-particle ($F_1\alpha$), or subunit 2 of cytochrome *c* oxidase (Cox2). The position of supercomplexes containing complex III and IV (III_2IV_2; III_2IV_1) and of monomeric (V_{mon}) and dimeric (V_{dim}) F_1F_O-ATP synthase complexes are indicated. (B and C) Assembly of newly synthesized, mitochondrial-encoded F_O-subunits Atp6, Atp8, and Atp9 into F_1F_O-ATP synthase complexes. Cells were grown in the presence of chloramphenicol before mitochondria were isolated. Mitochondrial-encoded proteins were labeled with [^{35}S]methionine and, after inhibition of translation, mitochondria were further incubated at 30°C for the time indicated to allow the assembly of newly synthesized subunits. Mitochondria (150 μg) were solubilized in 1.6% (vol/vol) Triton X-100 and subjected to BN-PAGE. Only monomeric F_1F_O-ATP synthase is detectable under these conditions (Arnold *et al.*, 1998). Coomassie stained gels (Com) and autoradiographs (^{35}S) are shown in the top panel. Incorporated Radioactivity incorporated into monomeric ATP synthase was quantified by phosphoimaging and corrected for different labeling efficiencies in various mitochondria. (B) Analysis of F_1F_O-ATP synthase assembly in wild-type (WT) and Δ*atp23* mitochondria. (C) Analysis of F_1F_O-ATP synthase assembly in Δ*atp23* mitochondria harboring Atp23 (*ATP23*) or the mutant variant $Atp23^{E168Q}$ (E168Q).

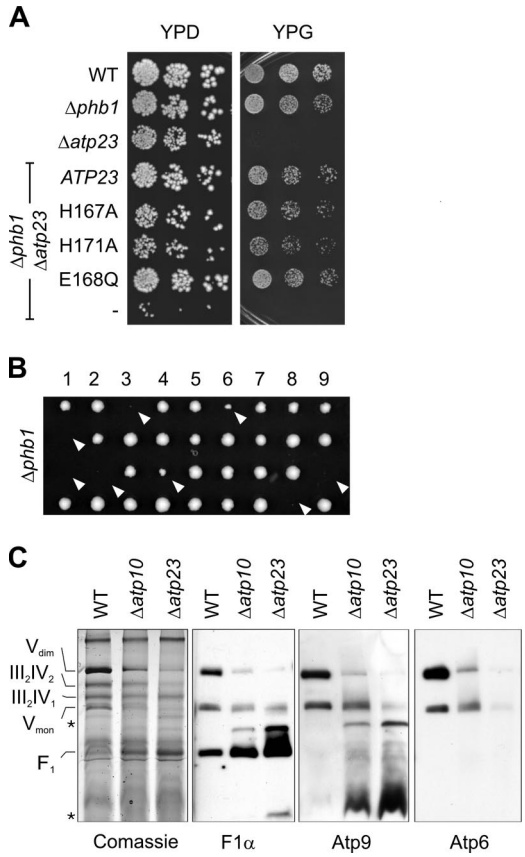

Figure 6. Genetic interaction of *PHB1* with *ATP23* and *ATP10* controlling Atp6 assembly into the F_O-particle. (A) Proteolytically inactive Atp23 allows growth of Δ*phb1*Δ*atp23* cells. Tetrads derived from diploid Δ*phb1*/*PHB1* Δ*atp23* cells expressing Atp23 or its proteolytically inactive variants $Atp23^{H167A}$, $Atp23^{E168Q}$, $Atp23^{H171A}$ were dissected. Ascospores carrying deletion of both *PHB1* and *ATP23* and expressing Atp23 or variants thereof were isolated and examined for growth on YPD and YPG at 30°C. (B) Synthetic lethal interaction of *PHB1* with *ATP10*. The genetic interaction of *ATP10* with *PHB1* was confirmed by tetrad analysis as described in Figure 1 for *ATP23*. Similarly, a synthetic lethal interaction was observed between *ATP10* and *PHB2* (data not shown). (C) Impaired assembly of F_1F_O-ATP synthase complexes in Δ*atp10* and Δ*atp23* mitochondria. The assembly of the F_1F_O-ATP synthase was analyzed by BN-PAGE after solubilization of mitochondrial membranes with digitonin as described in Figure 5A. Assembly intermediates detected in Δ*atp23* and, to a lower extent, in Δ*atp10* mitochondria are marked with asterisks.

restored assembly in Δ*atp23* mitochondria, which occurred with only slightly reduced kinetics in the presence of $Atp23^{E168Q}$ (Figure 5C). These observations further substantiate the role of Atp23 for the assembly of the F_1F_O-ATP synthase complex and suggest that maturation of Atp6 alleviates negative effects of the presequence on Atp6 assembly.

Atp23 and Atp10 Affect F_O-Assembly at a Similar Step

Our findings indicate that Atp23 affects newly synthesized Atp6 in two ways: it mediates Atp6 maturation and promotes the assembly of Atp6 into the F_O-particle in the mitochondrial inner membrane. To examine which of these activities of Atp23 is essential for the growth of Δ*phb1* and Δ*phb2* cells, we generated a diploid yeast strain that was heterozygous for deletions of both *PHB1* and *ATP23*. Atp23 and variants harboring point mutations in the proteolytic center were expressed in these cells. After sporulation and tetrad dissection, haploid progenies lacking genomic copies of both *PHB1* and *ATP23* but expressing plasmid-borne Atp23 or mutants thereof were isolated. Cell growth of Δ*phb1*Δ*atp23* cells was restored by all Atp23 variants irrespective of the presence of mutations in the consensus metal binding motif of Atp23 (Figure 6A). Thus, the assembly of Atp6 into the F_O-particle rather than Atp6 maturation is essential for cell growth in the absence of prohibitins.

The subunit-specific chaperone Atp10 can be cross-linked to newly synthesized Atp6 and assists its assembly into the

F_O-particle (Tzagoloff *et al.,* 2004). Strikingly, we identified *ATP10* as another candidate gene in our synthetic genetic array analysis to be essential for cell growth in the absence of *PHB1* or *PHB2*. We therefore confirmed this genetic interaction by deleting *ATP10* by homologous recombination in a diploid yeast strain that was heterozygous for a deletion of *PHB1* or *PHB2*. After sporulation and tetrad dissection, cell growth of meiotic progenies was examined on YPD medium containing glucose as carbon source (Figure 6B). Haploid cells receiving deletions of *ATP10* and either *PHB1* or *PHB2* were not viable demonstrating the synthetic lethal interaction of these genes (Figure 6B). Thus, the growth of prohibitin-deficient cells depends on two genes, *ATP10* and *ATP23*, which both affect the assembly of the F_O-particle in mitochondria.

Previous studies have demonstrated that Atp10 assists the binding of Atp6 to an oligomer of Atp9 at late stages of F_O-assembly (Tzagoloff *et al.,* 2004). To examine whether Atp23 affects the assembly of Atp6 in a similar manner, we analyzed the formation of the F_1F_O-ATP synthase in parallel in Δ*atp23* and Δ*atp10* mitochondria using BN-PAGE (Figure 6C). Immunoblotting with $F_1\alpha$-specific antiserum detected two additional assemblies containing $F_1\alpha$-subunits in both Δ*atp10* and Δ*atp23* mitochondria, which were absent in wild-type mitochondria: one larger and one significantly smaller than the assembled F_1-particle (Figure 6C). For further characterization, we probed for the presence of Atp9, Atp6, and Atp4 in these complexes by immunoblotting. While Atp4 and Atp6 were not present in either complex (Figure 6B; data not shown), Atp9 was detected in an intermediate complex as was the $F_1\alpha$-subunit both in Δ*atp10* and Δ*atp23* mitochondria (Figure 6C). Thus, our BN-PAGE analysis reveals the accumulation of similar-sized intermediate complexes in the absence of Atp10 and Atp23, which contain the F_1-subunit α and Atp9. These findings strongly suggest that Atp23 and Atp10 control similar steps in the assembly of the F_1F_O-ATP synthase, most likely the assembly of Atp6 with Atp9 oligomers.

DISCUSSION

We characterize in the present article Atp23 as a novel processing peptidase in the mitochondrial intermembrane space that exerts chaperone function during the assembly of the F_1F_O-ATP synthase. Atp23 mediates the maturation of newly synthesized Atp6 and, independent of its proteolytic activity, promotes the subsequent assembly of Atp6 into the membrane-embedded F_O-particle (Figure 7). The assembly of mitochondrial-encoded Atp6 is thus under the control of two substrate-specific chaperones, Atp10 and Atp23, acting on the matrix and the intermembrane space side of the inner membrane, respectively.[1]

Atp6 is synthesized within mitochondria as a precursor protein, from which 10 N-terminal amino acids are cleaved off (Michon *et al.,* 1988). Maturation of Atp6 was found to depend on Atp23 and on the integrity of the consensus metal binding site, HEliH, characteristic of metallopeptidases. Together with the observed physical interaction of Atp23 with newly synthesized Atp6, this strongly suggests that Atp23 serves as a metal-dependent processing peptidase for Atp6, which removes N-terminal amino acids when they become accessible in the intermembrane space. Atp23 therefore represents the second processing peptidase in this compartment

[1] Similar findings are reported in a related study by Zeng *et al.* (2007) published in this issue of *MBC*.

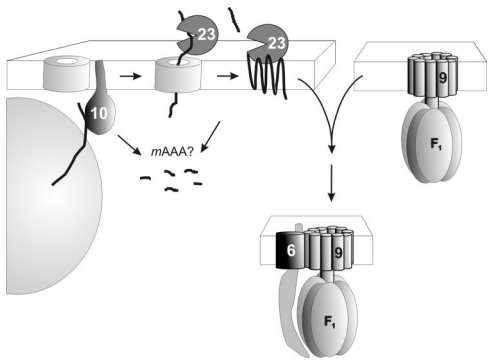

Figure 7. Model of Atp6 assembly into F_1F_O-ATP synthase complexes. See text for details. The translocase mediating membrane insertion of Atp6 has not been identified. 10, Atp10; 23, Atp23; 9, Atp9; *m*AAA, *m*-AAA protease.

besides the IMP protease, which mediates maturation of several nuclear-encoded proteins and of mitochondrial-encoded Cox2 (Gakh *et al.,* 2002). It remains to be determined whether other substrates of Atp23 exist. Notably, the human homologue of Atp6 lacks the N-terminal extension, which is cleaved off from the newly synthesized yeast protein. Nevertheless, Atp23 including the consensus metal binding site is highly conserved from yeast to man, pointing to further substrate proteins present in mammalian mitochondria.

A dual activity of Atp23 was unraveled by the mutational analysis of its proteolytic center. Cells harboring proteolytically inactive Atp23 were deficient in the maturation of Atp6 but were able to grow on nonfermentable carbon sources and contained fully assembled F_1F_O-ATP synthase. These findings demonstrate that the formation of a functionally active ATP synthase complex does not require processing of Atp6, but the presence of Atp23 in the intermembrane space. Atp23 directly interacts with mature Atp6 and mediates its assembly with Atp9 constituting the proton-translocating channel in the inner membrane (Figure 7). Assembly of Atp6 also depends on another substrate-specific chaperone, Atp10 (Tzagoloff *et al.,* 2004). Consistently, similar-sized intermediate complexes were detected in Atp10- and Atp23-deficient mitochondria. Given the membrane topology of Atp10 exposing a domain to the matrix space (Ackermann and Tzagoloff, 1990), it is conceivable that newly synthesized Atp6 initially interacts with Atp10 before it is bound by Atp23 in the intermembrane space (Figure 7).

How these substrate-specific chaperones ensure the assembly of the F_O-moiety remains to be established. A detailed analysis is made difficult by the increased turnover of Atp6 in the absence of Atp23 or Atp10, which thus are at the interface between assembly and proteolytic processes. Atp23 may be directly involved in the insertion of newly synthesized Atp6 into the inner membrane by trapping the N-tail of Atp6 in the intermembrane space. Alternatively, Atp23 may stabilize membrane-inserted Atp6 in an assembly-competent conformation. According to this scenario, the protective effect against proteolysis would simply result from the efficient assembly of Atp6 into F_O-complexes. This is reminiscent of Cox20, which is required for the maturation by IMP protease of Cox2, the second mitochondrial-encoded protein synthesized as a preprotein (Hell *et al.,* 2000). Evidence for Cox20-induced conformational changes in newly synthesized Cox2 was recently obtained analyzing the turnover of nonassembled Cox2 (Graef, Seewald, and Langer,

unpublished results). However, although a role of Cox20 for the assembly of mature Cox2 remained speculative, our findings demonstrate a chaperone function of Atp23 during the assembly of F_1F_O-ATP synthase, independent of Atp6 maturation. Finally, it cannot be excluded that the main function of Atp23 is to protect newly synthesized Atp6 against proteolysis and thereby increase the time frame available for Atp6 assembly. Accordingly, inhibition of Atp6 proteolysis should alleviate the requirement of Atp23 for the assembly of the F_1F_O-ATP synthase. A direct examination of this possibility, however, awaits the identification of proteases involved in the turnover of Atp6. Impairment of *m*-AAA protease activity in Δ*atp23* mitochondria did not result in the stabilization of the precursor form of Atp6 suggesting the involvement of additional proteases in its proteolytic breakdown (unpublished observations).

We have identified *ATP23* as a gene that is essential for the growth of prohibitin-deficient cells, providing genetic evidence for a functional link of prohibitins to the F_1F_O-ATP synthase. Cell growth is not impaired by the loss of the F_1F_O-ATP synthase activity per se because we did not observe a genetic interaction of prohibitins with subunits of the F_1-ATPase or other subunits of the ATP synthase complex. This includes also the accessory subunit e, which is only present in dimeric F_1F_O-ATP synthase complexes and whose loss leads to aberrant cristae formation (Arselin *et al.*, 2004). It therefore appears that an impaired mitochondrial ultrastructure by itself is not on the basis of the genetic interaction of prohibitins with Atp23. Strikingly, deletion of either *ATP23* or *ATP10* inhibits growth of prohibitin-deficient cells, suggesting the accumulation of a specific assembly intermediate that is hazardous for cell growth in the absence of prohibitins. A partially assembled F_O-moiety lacking Atp6 may for instance allow proton-leakage through the membrane dissipating the inner membrane potential. Prohibitins have been proposed to exert chaperone function (Nijtmans *et al.*, 2000) and thereby might mask the deleterious effect of these assembly intermediates. However, as we did not obtain any evidence for a direct interaction of the prohibitin complex with nonassembled inner membrane proteins, we favor another scenario. Increasing evidence suggest a higher order organization of respiratory chain complexes that ensures efficient metabolite channelling and maintains a high membrane potential (Everard-Gigot *et al.*, 2005; Bornhovd *et al.*, 2006). Both oligomeric ATP synthase as well as ring-like prohibitin complexes have been proposed to exert scaffolding functions and may define functional microdomains in the inner membrane (Steglich *et al.*, 1999; Bornhovd *et al.*, 2006). Their loss in the absence of both complexes together with the accumulation of deleterious F_O-assembly intermediates may therefore promote the breakdown of the membrane potential across the inner membrane and inhibit cell growth.

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