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Control of the biogenesis of the OXPHOS complexes and their interactions in *Saccharomyces cerevisiae*

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“The day we stop playing will be the day we stop learning.”

William Glasser

Résumé

Contrôle de la biogenèse des complexes OXPHOS et des leurs interactions chez *Saccharomyces cerevisiae*

Le complexe III de la chaine respiratoire mitochondriale (OXPHOS III) chez *S. cerevisiae* est assemblé à partir de dix sous-unités structurales codées par le génome soit nucléaire, soit mitochondrial et fait intervenir une douzaine de protéines extrinsèques au complexe. Nous avons étudié l'une d'entre elle, Bcs1, une ATPase oligomérique conservée de la famille des protéines AAA (ATPases Associated with diverse cellular Activities), qui contrôle la dernière étape de l'assemblage du complexe III. Chez l'Homme, des mutations dans l'orthologue de *BCS1*, *BCS1L*, sont associées à différentes maladies. Nous avons montré que des mutations dans les résidus conservés du domaine AAA de Bcs1 peuvent être compensées par des mutations dans les sous-unités de l'ATP synthase mitochondriale (OXPHOS V). Ces mutations compensatrices diminuent toutes l'activité d'hydrolyse de l'ATP de l'enzyme et nous avons proposé que la biogenèse du complexe III puisse être modulée selon l'état énergétique mitochondrial par Bcs1 *via* sa dépendance à l'ATP. Nous avons aussi identifié des mutations compensatrices dans d'autres gènes et le cas particulier de la délétion du *RRF1*, facteur général du recyclage des ribosomes mitochondriaux, a été étudié. Nous avons montré que l'absence de Rrf1 a un effet différent sur la stabilité et la traduction des divers ARNm mitochondriaux. Nos résultats suggèrent une coopération entre les facteurs généraux et les facteurs spécifiques de la traduction mitochondriale dans le contrôle de l'expression des sous-unités des complexes OXPHOS traduites dans la mitochondrie

Summary

Control of the biogenesis of the OXPHOS complexes and their interactions in *Saccharomyces cerevisiae*

OXPHOS complexes are multi-subunit complexes embedded in the inner mitochondrial membrane. We have studied the assembly factor Bcs1 that is a membrane-bound AAA-ATPase, required for the assembly of complex III. Mutations in the human gene *BCS1L* are responsible for various mild to lethal pathologies. Extragenic compensatory mutations able to restore the assembly of complex III in yeast *bcs1* mutants were found in different genes not directly connected to the complex, revealing new networks of protein interactions. Mutations in catalytic subunits of ATP synthase were identified and thoroughly characterized. This work has allowed us to propose a novel regulatory loop *via* the ATP-dependent activity of Bcs1 protein, connecting the production of mitochondrial complex III and the activity of the ATP synthase. Moreover, these results hold promise for the development of therapies, targeting the mitochondrial adenine nucleotide pool, in treatment of BCS1-based disorders. We also show that the absence of *RRF1*, a mitochondrial ribosome recycling factor, is able to compensate defects of *bcs1* mutants. Deletion of RRF1 has a differential impact on the stability and translation of mitochondrial mRNAs. Our results suggest cooperation between general and specific translation factors in controlling the expression of mtDNA-encoded subunits of the OXPHOS complexes.

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List of Abbreviations

ADP – adenosine diphosphate

ADP – adenosine tripohosphate

BNGE – blue native gel electrophoresis

Fe/S – iron sulfur

IM/MIM – mitochondrial inner membrane

IMS – mitochondrial intermembrane space

ISC – iron sulfur cluster

kDa – kilo dalton

min – minutes

mRNA – messenger ribonucleic acid

mtDNA – mitochondrial DNA

NADH - nicotinamide adenine dinucleotide

nDNA – nuclear DNA

NTP – nucleotide triphosphate

OM/MOM – mitochondrial outer membrane

ORF – open reading frame

OXPHOS – oxidative phosphorylation

PCR – polymerase chain reaction

ROS – reactive oxygen species

SDS-PAGE – sodium dodecyl sulphate - polycrylamide gel electrophoresis

UTR – untranslated region

Introduction

Ia - Introducing mitochondria, their origin, structure and function

1. Theories on mitochondrial origin

Modern mitochondria are known to be semi-autonomous and multifunctional organelles of eukaryotic cells, but tracing their evolution from their ancestral bacterial origin to a present-day organelle is still proving to be a complex task. It is widely accepted that mitochondria-containing eukaryotic cells originated from a single ancestral endosymbiotic event, but pieces of the puzzle are still missing and eukaryotic/mitochondrial origin still stands as a challenging question for evolutionary biology. Many models of the endosymbiont theory have been proposed over the years (reviewed in Martin et al., 2001), but can be mainly represented by two themes, referred to as the "archaezoan scenario" and the "symbiogenesis scenario" (Koonin, 2010; Fig. I-1). Main difference between the two theories lies in the timing

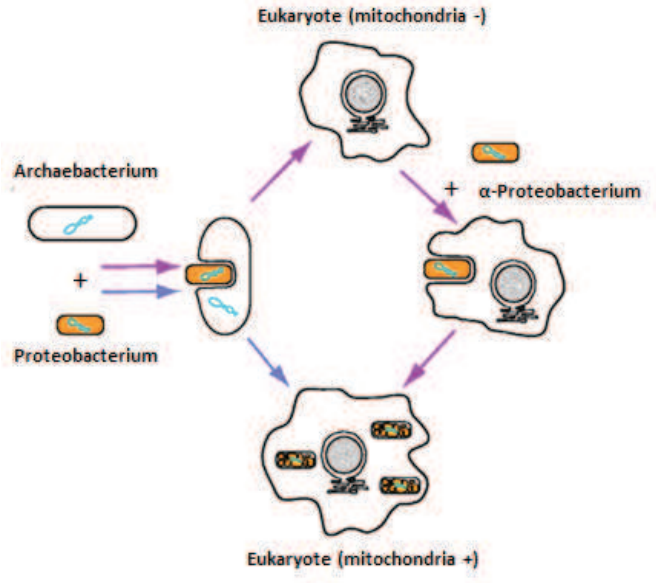


Figure I-1 Symbiogenesis vs. archaezoan hypotheses
Blue arrows - Simultaneous creation of the eukaryotic nucleus (gray) and mitochondrion (orange) by fusion of a hydrogen-requiring, methanogenic Archaebacterium (host) with a hydrogen-producing α-Proteobacterium (symbiont). Magenta arrows - An amitochondriate eukaryote is formed by fusion of an Archaebacterium and a Proteobacterium (archezoan, mito$^-$) , followed by acquisition of the mitochondrion through endosymbiosis with an α- Proteobacterium. Bacterial and mitochondrial genomes are blue. (Adapted from Gray, 1999; Martin and Müller, 1998).

of proto-mitochondrion formation with respect to the formation of the nucleus: either the endosimbiosis occurred at the same time as the formation of the eukaryotic cell, or it arrived only after the ancestral cell was already essentially eukaryotic.

1.1. The archaezoan scenario

The archaezoan scenario is more closely related to the classical endosymbiont hypothesis of mitochondrial origin, popularized in the early seventies by Lynn Margulis's book "the origin of eukaryotic cells", stating that mitochondria evolved from a bacterial progenitor through symbiosis with an amitochondrial eukaryotic cell. These primitive eukaryotes were formally put in the Archaezoa kingdom. Organisms such as *Entamoeba* and *Trichomonas*, considered as modern descendants of the Archaezoa, were thought to be important for understanding the early evolution of pre-mitochondrial eukaryotes. However, mitochondria or related organelles such as hydrogenosomes and mitosomes were eventually discovered in all known lineages of Archaezoa (Mai et al., 1999; Tovar et al., 2003), largely leading to the abandon of this hypothesis.

1.2. The symbiogenesis scenario

As the archaezoan hypothesis was discarded the symbiogenesis scenario started gaining more ground. According to this hypothesis the host cell was a strictly anaerobic, strictly autotrophic and hydrogen dependent archaebacterium, while the symbiont was an eubacterium, a facultative anaerobe that produced hydrogen as a waste product of anaerobic heterotrophic metabolism. The host's dependence on hydrogen would constitute a selective force that irreversibly bound one symbiotic partner to the other (Martin and Müller, 1998). This theory proposes that distinctive features of eukaryotic cells arose once the endosymbiosis took place, the evolution of the nucleus being a defensive system resulting from the exposure of the archaeal host genome to the eubacterial DNA. Although it has not been ultimately proven, the symbiogenesis scenario seems to explain the accumulated data on mitochondrial origin better than the archezoan scenario (Koonin, 2010).

2. General overview on mitochondrial DNA

The mitochondrion maintains its own DNA (mtDNA), organized in nucleoids, and also performs protein synthesis. In the yeast *Saccharomyces cerevisiae*, cytoplasmatic and mitochondrial protein syntheses were differentiated *in vivo* through the use of antibiotics (Clark-Walker and Linnane, 1966), which provided first insights into the mtDNA-encoded proteins. mtDNA generally encodes catalytic subunits of the complexes of the respiratory chain with tRNA and rRNA components of the mitochondrial translation system, but it can also contain additional proteins; in S*. cerevisiae*, besides seven subunits of complexes involved in oxidative phosphorylation (OXPHOS), twenty two tRNAs and three rRNAs, one small ribosomal subunit protein (Var1) is also of mitochondrial origin (Fig. I-2B). The complete sequencing of several mammalian mtDNAs, including human, also revealed a small number of proteins (thirteen in humans) that are still encoded by the organelle's DNA. Although at a first glance mitochondrial genomes appear to be rather small (~17kb in *H.sapiens*), this is not at all a general rule in eukaryotes; in fact, there are extraordinary variations in size, coding capacity, intron content, expression patterns and topology of mtDNAs when investigating non-animal species (Fig. I-2A). Sharp contrast can be observed when comparing the 6 kb mtDNA of

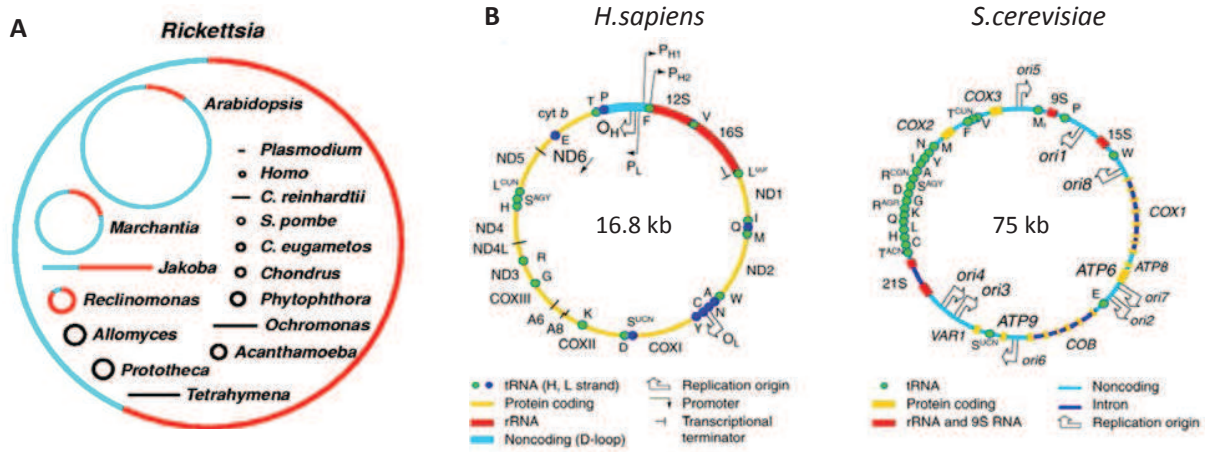


Figure I-2 Size and gene content of mitochondrial genomes.
A) Circles and lines represent circular and linear genome shapes, respectively. For genomes >60 kbp, the DNA coding for genes with known functions (red) is distinguished from that coding for unidentified ORFs and intergenic sequences (blue). B) Comparison between mtDNAs of *H.sapiens* and intron-containing S.cerevisiae. ND1-6, ND4L

are subunits of complex I; COXI(1), COXII(2), COXIII(3) are subunits of complex IV; cytb (COB) is a subunit of complex III; A6(ATP6), A8(ATP8) and ATP9 are subunits of ATP synthase. (Adapted from Gray, 1999; Jacobs, 2001).

Plasmodium falciparum, the human malaria parasite, with mtDNAs greater than 200kb in some flowering plants, including the largest known, 11.3 Mb mtDNA, belonging to *Silene conica* (Sloan et al., 2012). The phylogenetic origin of the mitochondrial genome's eubacterial ancestry has been intensively investigated and allowed to pinpoint the α-class of Proteobacteria (*Alphaproteobacteria*) as the lineage mitochondria originated from and to narrow it further to the order of Rikettsiales (Gray 2012 and references therein). However, it is still unclear if mitochondria actually branch within Rikettsiales. Moreover, members of both recently uncovered α-Proteobacterial oceanic clades, SAR11 and OMAC (Oceanic Mitochondrial Affiliated Clade) were proposed to be the closest free-living relatives of mitochondria (Brindefalk et al., 2011; Georgiades et al., 2011). Expansion of databases containing mitochondrial and bacterial sequences together with more sophisticated methods in constructing the phylogenetic trees may clarify this point in the future.

3. Mitochondrial inheritance and dynamics

3.1. Mitochondrial inheritance

Mitochondria cannot be created *de novo* and so individuals can only inherit their mitochondria through division of the parental organelle. In most of the sexually-reproducing organisms paternal mitochondria are excluded or removed from the zygote, so that mitochondria are maternally inherited. According to the theory of ageing (Allen, 1996) a separation of labour between germ line mitochondria of the two sexes explains their uniparental inheritance. This theory postulates that mitochondrial function is detrimental to the fidelity of mitochondrial replication. Mitochondrial electron transport and ATP production are accompanied by generation of free radical oxygen species (ROS), which can damage the mitochondrial genome. As the correct synthesis and functioning of the respiratory chain require undamaged mtDNA these events form a positive feed-back loop where incorrect functioning of

the OXPHOS system causes mutations, and mutations in turn cause even more errors in the system. This conflict may be resolved by having separate sexes: paternal gametes have more ROS-damaged mtDNA, given their high energy demand for both motility and massive production, while the maternal immobile gametes are produced in a small number with repressed bioenergetical function, making them more suitable for faithful mtDNA transmission to the offspring. However, biparental mitochondria inheritance exists for a number of organisms; it is the usual inheritance mechanism in *S. cerevisiae* and it has been described for some species of mussels (Hoeh et al., 1991). A few cases of paternal mtDNA leakage have been reported for animal species, including mice and a single case in humans (Gyllensten et al., 1991; Hoeh et al., 1991; Schwartz and Vissing, 2002).

3.2. Mitochondria are extremely dynamic

Mitochondria are often described as being organized in tubular networks, but they are highly dynamic, frequently changing shape and size and moving throughout the cell. The first proteins with a role in mitochondrial dynamics and inheritance were identified in *S. cerevisiae* (Burgess et al., 1994; Sogo and Yaffe, 1994), and most of these are maintained through evolution, making the budding yeast the model of choice for studying mitochondrial dynamics. Mitochondrion is a multi-layered organelle with at least four distinct structural parts: the outer membrane, the intermembrane space (IMS), the inner membrane and the matrix. The structural and functional identity of these compartments is maintained during the dynamic behavior of the organelle.

Changes in mitochondrial morphology are governed by two dynamically opposing processes: fusion and fission (Sesaki and Jensen, 1999), which are balanced in response to the cellular environment. This is important for maintaining the bioenergetic function of mitochondria and the integrity of the mtDNA. Pathways of mitochondrial fusion and fission will be briefly described here; for a more detailed explanation see (Hoppins et al., 2007; Zhao et al., 2012) and references therein.

3.3. Mitochondrial fusion

The fusion complex in *S. cerevisiae* is formed by two conserved GTPases, Fzo1, an integral protein of the outer membrane (mitofusins 1 and 2 in mammals), and Mgm1, an IMS exposed inner membrane-associated protein (Opa1 in mammals). Yeast Mgm1 exists in two isoforms, l-Mgm1 (long form) and s-Mgm1 (short form). l-Mgm1 is a transmembrane protein which exposes its GTPase domain in the IMS, while the s-Mgm1 lacks the transmembrane segment, which is generated through protein cleavage by the protease Pcp1 of the inner membrane. Nevertheless, the s-Mgm1 remains tightly associated with the IMS side of

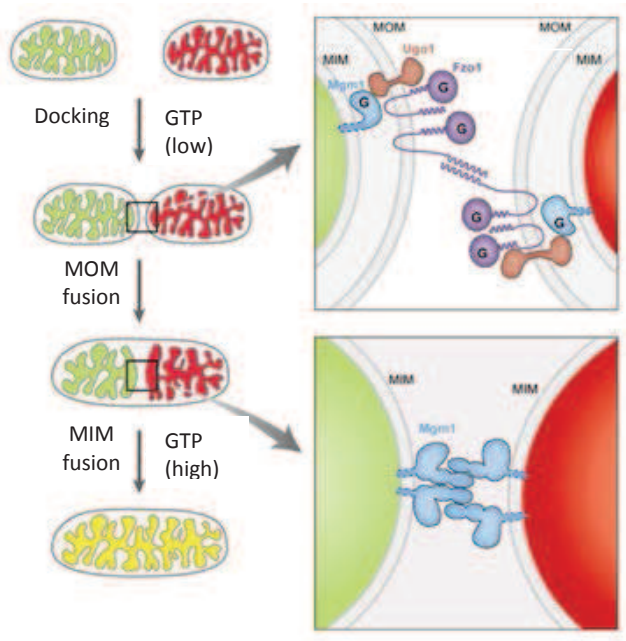


Figure I-3 Model for mitochondrial fusion in *S. cerevisiae*
Mitochondrial fusion requires the sequential interaction of the outer mitochondrial membranes and the inner mitochondrial membranes. Fusion of the outer membrane requires GTP and a proton gradient. Fusion of the inner membrane requires GTP and the electrical component of the inner membrane potential. MOM, MIM are the outer membrane and the inner membrane, respectively. (Adapted from Hoppins et al. 2007)

the inner membrane, and both isoforms function together in fusion events. In *S. cerevisiae*, a protein named Ugo1, embedded in the outer membrane and exposing domains to both cytosol and the IMS binds to both Fzo1 and Mgm1, thus connecting the outer and the inner mitochondrial membranes. No ortholog of Ugo1 has been found in mammals so far. The GTPase activities of Fzo1 and Mgm1, as well as the membrane potential, are required for

membrane fusion but not for the formation of the fusion complex with Ugo1. The exact series of events occurring during the mitochondrial fusion are not completely elucidated; still the process can be divided into at least three steps: docking, outer membrane fusion, inner membrane fusion (Fig. I-3).

3.4. Mitochondrial fission

Mitochondrial fission relies on dynamin-related GTPases, called Dnm1 in yeast and Drp1 in humans. The GTP-driven self-assembly and GTPase activity of these proteins are required for fission (Fig. I-4). Dnm1 exists in highly dynamic cytosolic and membrane-bound assemblies.

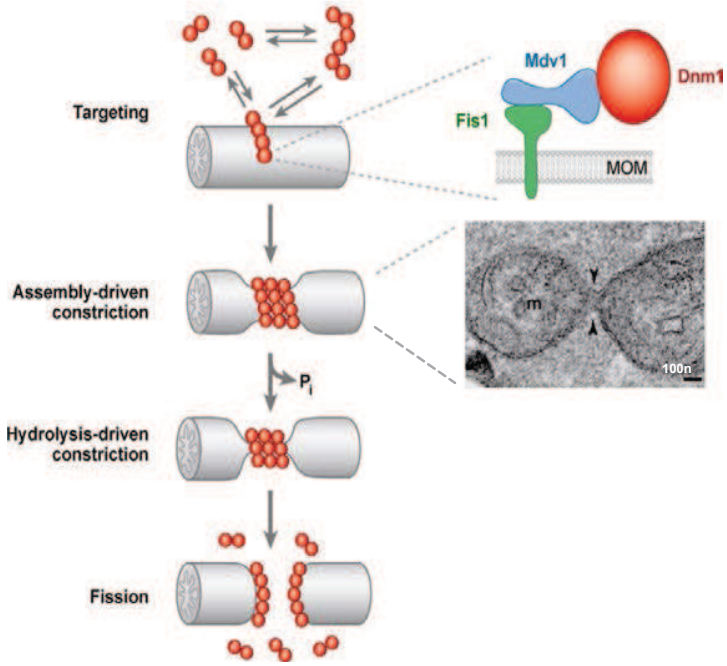


Figure I-4 Model for Dnm1-mediated mitochondrial fission in *S. cerevisiae.*
The thin section electron microscopy analysis of yeast cells shows mitochondrial constriction intermediates. (Adapted from (Hoppins et al., 2007).

Another protein is essential for fission, Fis1 (hFis1 in humans), an integral outer membrane protein exposing its N-terminal domain to the cytoplasm. Fis1 interacts with two structurally similar proteins, Mdv1 and Caf4, and these are thought to promote the recruitment and stable assembly of Dnm1 oligomers on the outer membrane. Dnm1 forms ring-like structures around

the mitochondrial tubule and mediates mechanical membrane scission due to the conformational changes upon GTP hydrolysis.

3.5. Mitochondria-ER interactions

Mitochondria physically interact with the endoplasmic reticulum forming highly dynamic lipid raft-like structures in mammals, termed MAMs (Mitochondrially Associated endoplasmic reticulum Membranes). Regulation of calcium homeostasis, cholesterol and phospholipid metabolism and trafficking involves MAM interface and its dysfunction could play a role in the onset of neurodegenerative diseases in humans (Schon and Area-Gomez 2013). In *S. cerevisiae,* the outer membrane is bound to the ER through the ER-mitochondria encounter structure (ERMES), which contain the ER protein Mmm1, the outer membrane proteins Mdm10, Mdm12 and Mdm34 (Fig. I-5A, 5B) and a GTPase Gem1 and participate in efficient inter-organellar phospholipid exchange, calcium signaling and protein import (Kornmann et al., 2009; Michel and Kornmann, 2012). Contacts between the two organelles have been shown to occur near the mitochondrial fission sites and replicating nucleoids of mtDNA and are important for the maintenance of a correct mitochondrial morphology and inheritance of mtDNA upon fission (Nezich and Youle, 2013; Fig. I-5C, 5D).

A similar protein complex has been described for the inner membrane, called MINOS (Mitochondrial INner membrane Organizing System), constituted of at least six inner membrane proteins: the core Mitofilin/Fcj1 and Mio10 with Aim5, Aim13, Aim37 and Mio27 (von der Malsburg et al., 2011). MINOS dynamically interacts with the mitochondrial protein import machinery (see later). ERMES and MINOS have multiple genetic and biochemical interactions and are part of a larger ER-mitochondria organizing network (ERMIONE). ERMIONE is proposed to function as a regulatory system that structurally and functionally links all mitochondrial compartments to the ER and regulates mitochondrial function and biogenesis (for review van der Laan et al., 2012; Nezich and Youle, 2013).

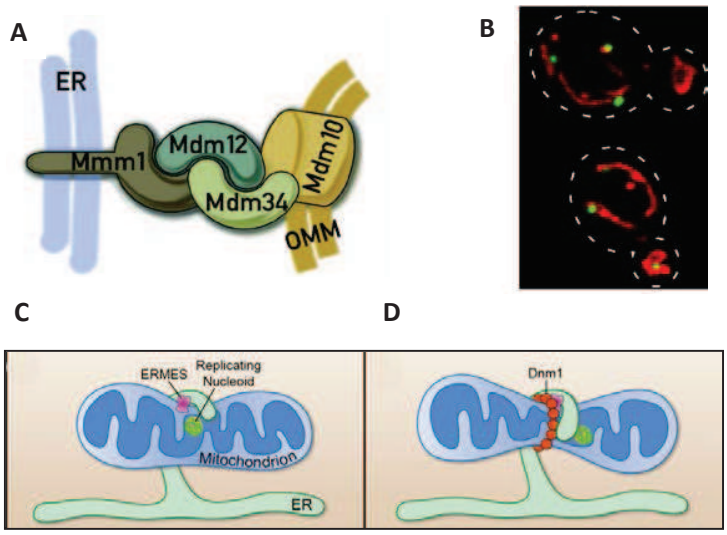


Figure I-5 ERMES complex in *S. cerevisiae.*
A) Mmm1 is an ER protein, while Mdm10 is an OMM β-barrel. Mdm12 is a cytosolic protein. Mdm34 is associated with the OMM through an unclear mechanism. B**)** The ERMES complex is formed in a few discrete foci per cell. Here a green fluorescent protein (GFP)-tagged version of Mdm34 (green) is imaged together with a marker for the mitochondria (red). The outlines of the cells are shown as broken lines. (from Michel and Kornmann 2012) C) ERMES complex (pink) localizes to the region where the endoplasmic reticulum (ER) makes contact with a mitochondrion in yeast, and where the nucleoid that contains the mitochondrial DNA is replicating. D) The mitochondrion undergoes constriction at this contact site, which allows a ring of Dnm1 (red) to form around it. This leads to further constriction and, ultimately, to the division of the mitochondrion (adapted from Nezich and Youle, 2013).

4. Compartmentalization of mitochondria is vital to their biology

4.1. The outer membrane

4.1.1. The outer membrane as a gateway for entering the mitochondrion

It has been known for some time that the outer membrane is fairly permeable (Zinser et al., 1991) allowing the free diffusion of small molecules (<5 kDa) through its conserved voltage dependent anionic channels, porins, but it is also a site for active mitochondrial protein uptake. There are generally 1000-1500 proteins operating inside the mitochondrion, yet its genome encodes just a handful of the core proteins of the respiratory chain; therefore, most mitochondrial proteins are encoded by the nuclear DNA (nDNA), synthesized on cytosolic ribosomes and need to be imported into the mitochondrion (different import machineries in *S.*

cerevisiae mitochondria are presented in Fig I-6). The proteins may find their way through the cytosol to the mitochondrial outer membrane by virtue of different targeting elements, one of which is the classical positively charged N-terminal presequence, as well as their interaction with cytosolic factors (Young et al. 2003). Additionally, numerous mRNAs encoding mitochondrial proteins were found to be localized at the outer membrane, where their translation is likely coupled to the import of the resulting proteins (Devaux et al., 2010; Gadir et al., 2011; Saint-Georges et al., 2008).

4.1.2. Outer membrane active import system

Upon arrival on the outer membrane the proteins engage with components of the Translocase of the Outer Membrane (TOM complex). The TOM complex acts as a receptor for precursor proteins and it also forms a channel for their translocation into mitochondria. Seven different subunits compose this complex: (i) Tom40, the β-barrel subunit that forms the traslocation pore, (ii) Tom22, the central receptor of the complex, (iii) three small Tom proteins, Tom5, Tom6 and Tom7, important for TOM assembly and stability and (iv) the peripheral receptors Tom20 and Tom70, involved in precursor recognition. Our knowledge of TOM structure and function derives mostly from research on *S. cerevisiae*, but the components and main features are conserved from yeast to humans (Gebert et al. 2011 and references therein). The outer membrane contains a number of β-barrel proteins (Tom40, porins) and their assembly is mediated by SAM complex (Sorting and Assembly Machinery). SAM complex is evolutionarily conserved form bacterial BAM complex (β-barrel Assembly Machinery) (Voulhoux, 2003), its central β-barrel component Sam50 being homologus to BAM's central subunit BamA. After translocation across the outer membrane by the TOM complex, the hydrophobic β-barrel precursors are chaperoned by small Tim proteins and delivered to SAM for membrane integration. The membrane insertion of the TOM complex itself is also mediated by SAM (Meisinger et al., 2004).

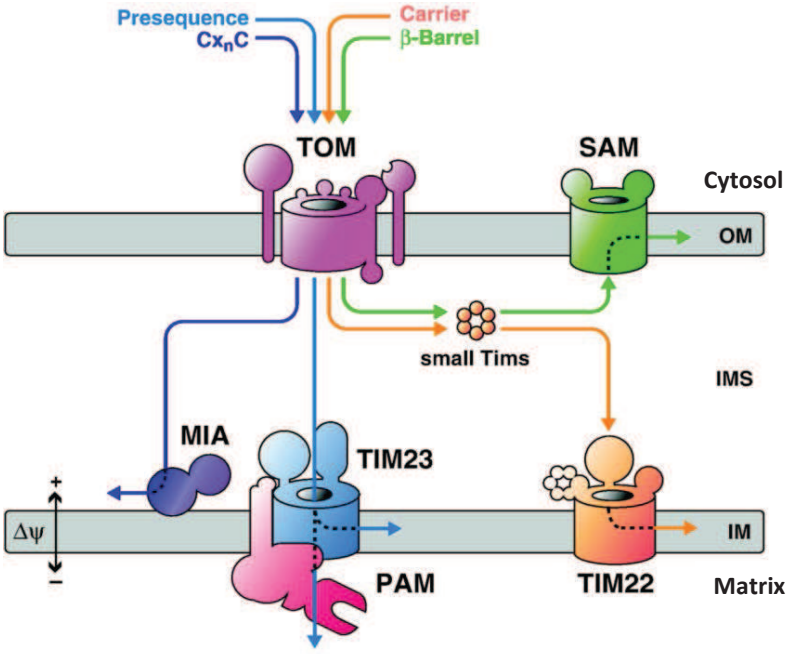


Figure I-6 Translocation machineries in *S. cerevisiae.*
Different targeting signals direct nuclear encoded precursor proteins on specific transport routes to their final localization within mitochondria. After translocation of precursors through the general translocase of the outer membrane (TOM complex), distinct downstream import pathways diverge in the intermembrane space (IMS): Biogenesis of β-barrel proteins of the outer membrane (OM) requires the small Tim chaperones of the IMS and the sorting and assembly machinery (SAM). Proteins of the IMS that contain cysteine-rich signals (CxnC) are imported via the mitochondrial intermembrane space import and assembly (MIA) pathway. Carrier proteins of the inner membrane (IM) are transported with the help of the small Tims and the TIM22 complex. Presequence-containing proteins are inserted into the inner membrane or imported into the matrix by the TIM23 complex. Matrix translocation requires the activity of the presequence translocase-associated import motor (PAM). (Dudek et al., 2013)

4.2. The intermembrane space

4.2.1. The solute and protein environment of the IMS differs from that of the cytosol

Given the presence of numerous porines in the outer membrane, the solute environments of the IMS and cytosol were considered to be similar. However, at least in terms of pH and redox environment, the IMS has been shown to be distinct from the cytosol (Porcelli et al., 2005) and important for metabolite transport in and out of the matrix (Herrmann and Riemer 2010; Fig. I-7).

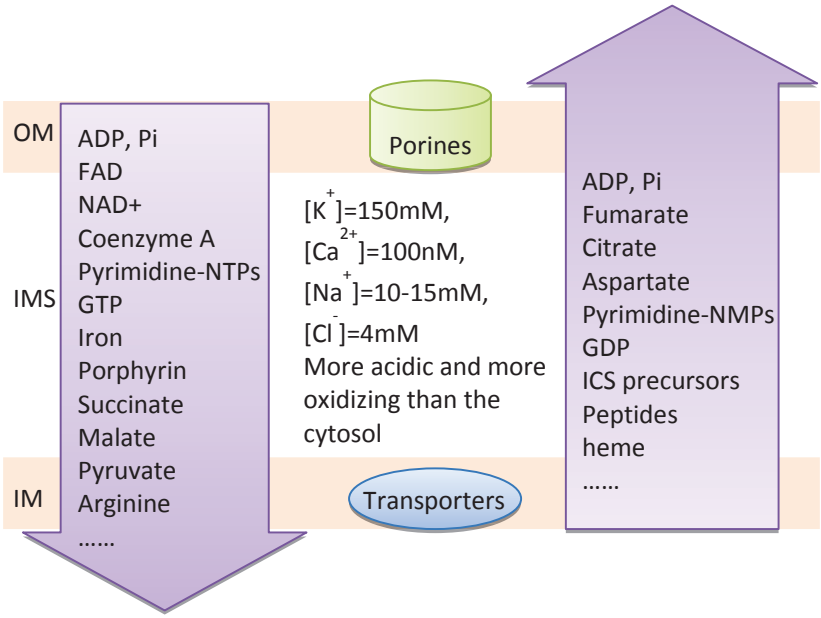


Figure I-7 The IMS serves as a transport hub between the cytosol and the matrix.
Small molecules diffuse across the outer membrane (OM) via the relatively large openings of porins. In contrast, translocation across the inner membrane (IM) is driven by active transport processes (primary or secondary) through numerous dedicated transport systems, including members of the mitochondrial carrier family and ABC transporters. Taken together, these transport processes influence the small-molecule composition of the IMS (Adapted from Herrmann and Riemer 2010)

The annotation of the protein complement of the IMS has recently been revised, leaving 49 proteins, all encoded in the nucleus (Vogtle et al., 2012). Most of them are soluble, although a number of peripheral inner or outer membrane proteins whose enzymatic activity takes place in the IMS are also considered to be proteins of the intermembrane space. Most IMS proteins lack obvious presequences, and their proper folding after translocation across the outer membrane is considered to be crucial for their retention in the IMS. This can be achieved in different ways, such as by binding to partner proteins in the outer/inner membrane or upon addition of prostetic groups, as in the case of cytochome *c* (Dumont et al., 1991). Additionally, the oxydation-dependent MIA pathway (Mitochondria Intermembrane space Assembly), relying on two essential components, the chaperon Mia40 and the sulfhydryl oxidase Erv1, is engaged in folding of numerous IMS proteins that contain cystein residues (for review Stojanovski et al., 2012).

4.2.2. Proteases in the IMS

Since correct folding of the IMS proteins is so important for their sorting, another crucial issue in the IMS is protein quality control. The IMS contains several proteases that process or degrade misfolded proteins, and frequently display additional chaperon functions. As an example, Yme1 is a conserved homohexameric ATPase of the mitochondrial inner membrane (i-AAA), which exposes its catalytic site in the IMS (Leonhard et al. 1996; Weber et al. 1996). Yme1 unfolds and degrades mostly inner membrane proteins that expose domains in the IMS, and it is proposed to do so in virtue of its chaperon-like, conformation-sensing AAA domain (Leonhard et al., 1999). Additional roles of Yme1 in import and folding of a number of mitochondrial proteins have been reported as well (Fiumera et al., 2009; Rainey et al., 2006; Schreiner et al., 2012). Proteases of the IMS can also have more specific targets and an example of this is the conserved peptidase Atp23. One of the substrates of Atp23 is the subunit 6 (Atp6) of ATP synthase. Atp23 cleaves the presequence of Atp6 and provides chaperoning for its assembly within the late intermediate of ATP synthase (Osman et al. 2007; Zeng et al. 2007).

4.3. The inner membrane

4.3.1. Structure of the inner membrane

Several models of the IM structure have been proposed, mainly based on the transmission electron microscopy studies. For a long time, the “baffle model” has been the most widely used (Palade, 1952), depicting the structure of the inner membrane as random wide invaginations, the cristae (Fig. I-8A). An alternative to this model was proposed soon afterwards and suggested the cristae to be independent membrane lamellae (Sjostrand, 1953). More recently, results obtained using advanced imaging techniques show that the IM can be divided into the inner boundary membrane, which is parallel to the outer membrane and encloses the matrix, and the cristae that appear to be tubular and discrete structures in the matrix, linked to the inner boundary membrane through cristae junctions (Logan, 2006; Mannella, 2006; Fig I-8B).

It was also shown that the morphology of the cristae changes with the changing of the metabolic state of mitochondria; the biogenesis of cristae appears to be directly linked to the biogenesis of the oxydative phosphorylation (OXPHOS) complexes, and in particular to the dimerization of ATP synthase (Paumard et al., 2002). The compartmentalization of the inner membrane into cristae, cristae junctions and inner boundary membrane has physiological consequences on both the small molecule environment (such as ATP, ADP, H+, ROS) and the protein composition of the different sub-compartments (Herrmann and Riemer 2010; Fig. I-9A, B). This reflects the organization and optimization of different cellular processes confined to each compartment.

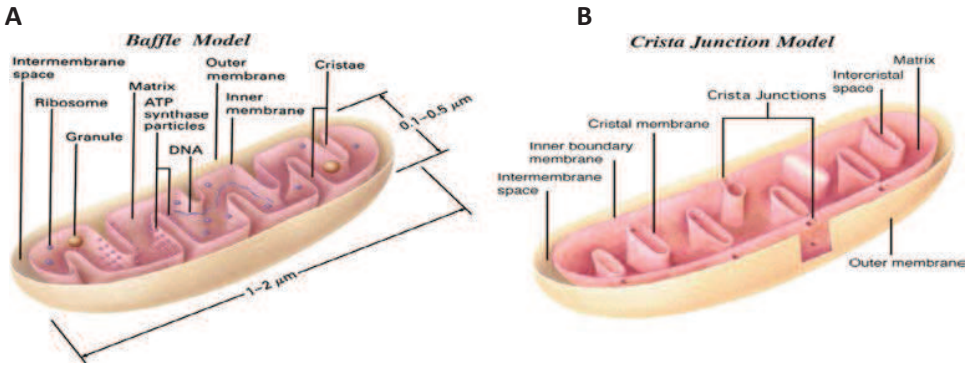


Figure I-8 Models of the inner membrane structure.
A) Classical text book or "baffle" model. B) Cristae junction model; instead of wide invaginations of the inner membrane, tight narrow membranous junctions connect cristae to the IMS. (Logan, 2006).

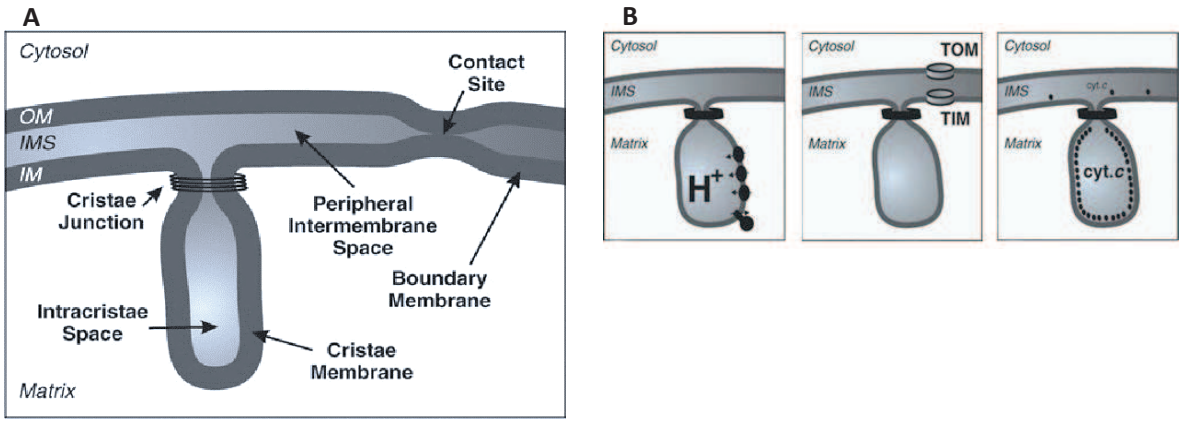


Figure I-9 Subcompartments of the inner membrane.
A) The inner membrane can be divided in the inner boundary membrane and cristae membrane. B) Different compartments of the inner membrane differ in their protein composition. Complexes of the respiratory chain and cytochrome *c* are enriched in the cristae membrane while TOM and TIM complexes are predominantly present in the inner boundary membrane (Adapted from Herrmann and Riemer 2010).

4.3.2. Import machineries of the inner membrane

In order for proteins to cross or assemble in the IM sophisticated import machineries are required; these are the complexes TIM23 and TIM22 (for review Dudek et al., 2013; Gebert et al., 2011a; see Fig I-6). TIM23 consists of three core components: the channel forming Tim23, Tim17 and the receptor Tim50. When the regulatory subunit Tim21 associates with the core complex it promotes the interactions between the complexes TIM23 and TOM (Mokranjac, 2005), resulting in interactions between the outer and the inner membrane, also called membrane contact sites. In the absence of other partners this four-subunit machine works in the stop-transfer mode ($TIM23_{sort}$), releasing the precursors laterally into the lipid phase. If precursors need to be translocated into the matrix the TIM core complex associates with the ATP driven motor PAM (Presequence translacase-Associated import Motor), consisting of the heat shock protein mtHsp70, membrane anchor Tim44, the nucleotide exchange factor Mge1, and chaperons Pam16, 17 and 18. When it functions in the translocation mode the TIM machinery is referred to as the $TIM23_{motor}$. The initiation of translocation is dependent on the membrane potential, while the hydrolysis of ATP by Hsp70 provides the energy necessary to finish the import of proteins into the matrix (Krayl et al., 2006). In *S. cerevisiae*, Both Tim21 and Pam16/18 were found to interact with respiratory supercomplexes formed by complexes III and IV, which would support the translocation of precursors under conditions when membrane potential is limiting. The association of TIM23 with complexes of the respiratory chain may act as the regulatory switch between the $TIM23_{sort}$ and the $TIM23_{motor}$ modes of function (Wiedemann et al. 2007; Dienhart and Stuart 2008).

There is another import pathway reserved for metabolite carriers, such as the ADP/ATP transporter. Once translocated through the TOM complex, the carrier precursor binds to Tim9-10 chaperons that guide the precursor through the IMS and deliver it to the TIM22 complex, a twin-pore-forming translocase. TIM22 is formed out of three integral membrane subunits, Tim22, Tim54 and Tim18 and the small Tim complex (Tim9-Tim10-Tim12) (Gebert et al., 2008). An additional protein, Sdh3, is both a subunit of respiratory complex II and TIM22, where it is

necessary for the assembly of Tim18 (Gebert et al., 2011b). The insertion of carrier proteins into the inner membrane is potential dependent.

4.3.3. Mitochondrial carrier family controls the permeability of the inner membrane

A number of proteins transport amino acids, nucleotides, metabolites and co-factors in a bidirectional fashion across the inner membrane. These transporters are essential for the homeostasis of different metabolic functions of the mitochondria as they allow shuttling of metabolic products between mitochondrial compartments. Mitochondrial transporters belong to a "family of mitochondrial carriers", characterized by tandemly repeated hydrophobic sequences with a particular motif P–X–D/E–X–X–K/R–X–K/R–(20–30 residues)–D/E–G–(5 residues)–K/R–G (for more detailed information see (Palmieri et al., 2006 and references therein). The ATP/ADP transporter and the phosphate carrier, which supply the matrix with substrates for the synthesis of ATP, the uncoupling protein of brown adipose tissue, the GTP/GDP carrier, NAD^+ uniporter, succinate-fumarate, aspartate/glutammate and the dicarboxylate carriers are only some of the transporters identified in the carrier family. Operating mechanisms of the transporters are variable, including uniport, symport and antiport, and can be dependent on the proton motive force. Furthermore, the understanding of the physiological role of carriers is also medically important as their defects are linked to some human diseases.

4.4. The matrix

4.4.1. Compartmentalization inside the matrix

Numerous multisubunit enzymatic complexes are situated in the matrix, such as pyruvate dehydrogenase complex (PDC), which catalyzes the irreversible oxidation of pyruvate to acetyl-CoA, or the enzymes of TCA (tricarboxylic acid) cycle that oxidize acetyl-CoA to produce energy and redox carriers. Metabolites deriving from these reactions and from the

functioning of the mitochondrial respiratory chain are also contained in this compartment. In higher eukaryotes the matrix has an important role in the modulation of Ca^{2+} storage in the interplay with the ER (Pizzo and Pozzan, 2007), which can regulate a variety of processes, including apoptosis and necrosis. It has been proposed that enzymes are spatially organized in the matrix, as metabolons, particles containing a part or the whole protein complement of a particular pathway, allowing rapid solute diffusion through the remaining aqueous space (Partikian et al., 1998). An example of metabolon could be the PDC complex, which is found to be heterogeneously distributed in hot spots along mitochondrial tubules in human fibroblasts (Margineantu et al., 2002).

4.4.2. Proteases in the matrix

As the matrix is a protein-rich environment, the protein processing, quality control and turnover are of prime importance (for review see Anand et al. 2013; Teixeira and Glaser 2013). Proteins that are targeted to the matrix usually present a positively charged N-terminal presequence, which is cleaved once they reach the matrix by the mitochondrial processing peptidase (MPP). MPP is a heterodimeric metalloprotease that presents the substrate-binding α subunit and the proteolytically active β subunit. Additional proteases have been described in this compartment, such as the ATP-dependent m-AAA, which degrades unassembled integral membrane proteins and the soluble Lon/PIM1 which regulates the turnover of soluble proteins in the matrix.

4.4.3. Biogenesis of iron-sulfur clusters (ISC) in the matrix

The eukaryotic proteins bearing the iron-sulfur clusters (Fe/S proteins) are involved in metabolism, the regulation of gene expression and electron transport and are present in mitochondria, the cytoplasm and the nucleus (Fig. I-10). The maturation of all cellular Fe/S proteins requires the mitochondrial ISC-assembly and export machineries (Kispal et al., 1999). The ISC assembly complex generates most of the cellular ISC clusters and resembles to its

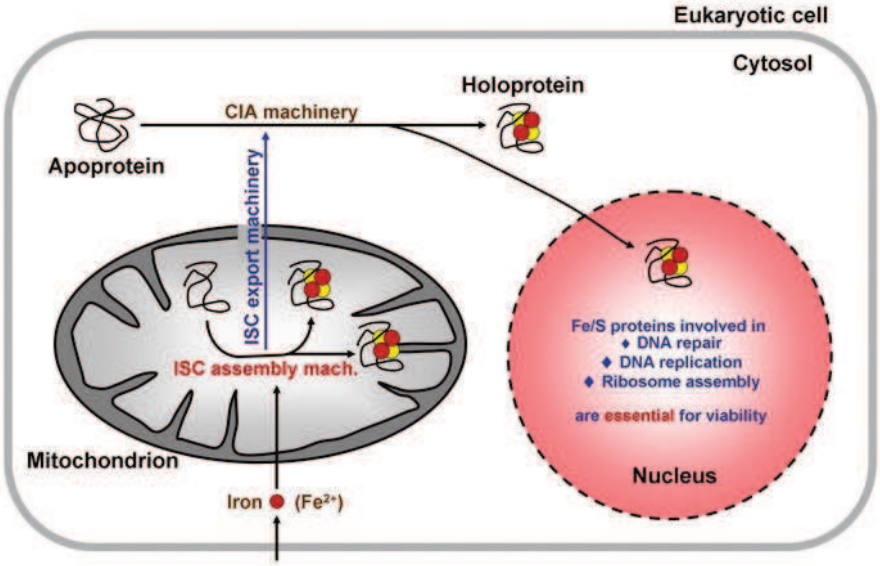


Figure I-10 Biogenesis of Fe/S proteins in eukaryotes.
Mitochondrial Fe/S proteins require the iron-sulfur cluster (ISC) assembly machinery which was inherited from bacteria during evolution. Cytosolic and nuclear Fe/S protein assembly also depends on the function of this machinery, yet additionally requires the mitochondrial ISC export apparatus and the cytosolic iron-sulfur protein assembly (CIA) machinery. (R. Lill laboratory)

bacterial counterpart (Zheng et al., 1998), as it was probably inherited from the eubacterial ancestor of mitochondria. The basic scheme for ISC biogenesis, mostly studied in *S.cerevisiae*, involves *de novo* synthesis on scaffold proteins that can bind both iron and sulfur donor proteins (Lill and Mühlenhoff, 2005; Wiedemann et al., 2006). The sulfur is transferred from conserved cysteine residues of a pyridoxal phosphate-dependent cysteine desulfurase, while the iron binding protein frataxin is believed to function as the iron donor. Most mitochondrial Fe/S apoproteins are complexed with neosynthesized ISCs after their import into the matrix. Components of these machineries are well conserved from yeast to humans. The mitochondrial inner membrane transporter Atm1 (ABCB7 in humans) and the sulphydryl oxidase Erv1 in the IMS are required for the exportation of a still poorly defined ISC-containing intermediate, synthesized in the matrix by the ISC-assembly complex. This component is necessary for the maturation of cytosolic and nuclear Fe/S proteins by the cytosolic iron-sulfur protein assembly complex (CIA) (Lill, 2009).

Ib - OXPHOS complexes in eukaryotes

1. Basics on oxidative phosphorylation

Oxidative phosphorylation is the final biochemical process in the series of events that transform energy from food-derived carbon compounds to ATP, the energy-storing molecule of the cell. At the beginning of the oxidative phosphorylation the energy is "stocked" in electrons with high redox potential of the TCA cycle intermediates NADH and $FADH_2$. The electrons enter the respiratory chain where they are transferred between the complexes I-IV to the ultimate electron acceptor, the molecular oxygen. These multisubunit membrane embedded enzymes contain multiple oxidation-reduction centers, including quinones, flavins, hemes and Fe/S clusters. Three complexes of the respiratory chain, I, III and IV, act as electron driven proton pumps and transfer protons from the matrix to the IMS during the electron flow, creating an uneven distribution of protons across the IM and with it the pH gradient and the transmembrane electrochemical potential. In this way the electron-motive force has been transformed into proton-motive force, which is used by another multimeric enzyme of the IM, the ATP synthase, to synthesize ATP from ADP and Pi. The proton-motive force is converted into phosphoryl transfer potential in the ATP molecule, which can be exported throughout the cell, *via* the ATP/ADP transporter, to fuel different energy-requiring reactions (Fig. I-11).

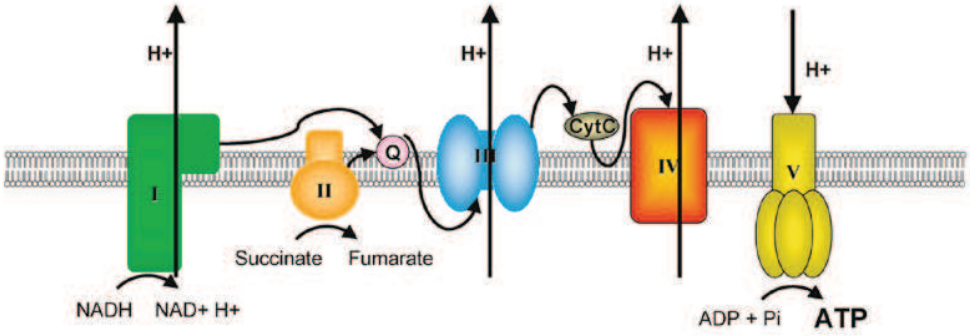


Figure I-11 Schematic representation of the OXPHOS complexes.
Complex I NADH dehydrogenase; complex II succinate dehydrogenase; complex III ubiquinol cytochrome c oxidoreductase; complex IV cytochrome c oxidase; complex V ATP synthase (adapted from Cuperus et al., 2009)

2. *S. cerevisiae* as a model organism to study the respiratory function

The budding yeast *Saccharmoyces cerevisiae* is a unicellular eukaryote with a short generation time (~2 hours), easy to maintain and culture. Its genome has been sequenced and public since 1996, and it is continuously updated at Saccharomyces Genome Database (SGD). A distinct feature of yeast makes it particularly suitable for studies on the respiratory function: it is a facultative anaerobe, meaning that, it can survive and proliferate in absence of the functional respiratory chain by synthesizing ATP through the glycolytic pathway (fermentation). The metabolic switch from respiration to fermentation depends on the oxygen as well as the carbon source available; glucose will be used for fermentation while "respiratory" substrates, such as glycerol or lactate will stimulate respiration in absence of glucose. This means that mutants blocking the respiratory function can be maintained on a fermentable media (Fig. I-12), and therefore analyzed thoroughly. *S. cerevisiae* can support the complete loss of mtDNA, becoming a *rho*^{0} strain.

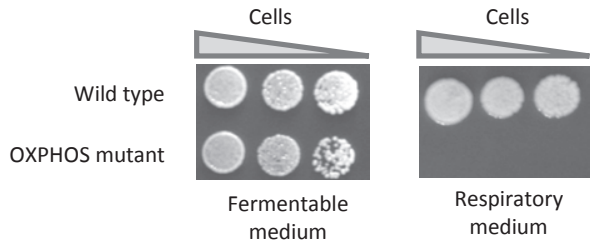


Figure I-12 Growth of yeast cells on fermentable and respiratory media
Serial dilutions of wild type and mutant yeast cells were spotted on solid media. Mutants impairing OXPHOS function cannot grow on respiratory substrates, but can be maintained and analyzed on fermentable media

S. cerevisiae shares the complex cellular structure and basic cellular mechanisms with higher eukaryotes, allowing the comparison between these systems, but it is far easier to manipulate. Efficient deletion and manipulation of *S. cerevisiae* genes can be accomplished by transformation of cells and subsequent homologous recombination; it is also one of the two organisms (the other being the single cell green alga *Chlamydomonas reinhardtii*) that allow site directed mutagenesis of mitochondrial DNA. The budding yeast can be maintained as a diploid strain, or as a haploid one after sporulation and tetrad dissection which permits important genetic analyses to be performed. Moreover, numerous molecular and biochemical tools have

also been developed for yeast research, and it has frequently been the organism of choice for the study of mitochondrial function, as mentioned above on several occasions. The abundance of the available information helps the development of hypotheses and putting the pieces together when researching different areas of mitochondrial function.

2.1. *Complex I*

The composition of most respiratory complexes and their subunits in *S. cerevisiae* is remarkably conserved, with the important exception of the complex I (type-I NADH dehydrogenase or NADH:ubiquinone oxidoreductase). Complex I is the largest of the respiratory complexes, with 45 subunits in humans and 14 conserved core components in bacterial equivalents (Brandt, 2006). This complex catalyzes the rate limiting transfer of two electrons from NADH to ubiquinone and it is the entry point for most of the electrons in the respiratory chain. Complex I translocates protons from the matrix to the IMS and this activity is associated with high ROS production. Deficiency of complex I is linked to the development of neurodegenerative diseases in humans, in particular Parkinson's disesase (Dawson, 2003).

Although present in most bacteria, in *S. cerevisiae*, as well as in a number of other species of yeasts, the complex I has been replaced with three single component type-II NADH dehydrogenases (Feng et al., 2012; Fig. I-13). Two external NADH dehydrogenases (Nde1, Nde2) and one internal (Ndi1) oxidize NADH to NAD^+ in the IMS and matrix, respectively, contributing to the NADH/NAD^+ homeostasis (Luttik et al. 1998; Melo et al. 2004). These enzymes catalyze

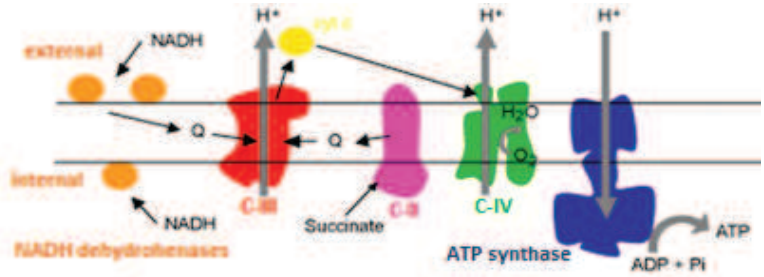


Figure I-13 Schematic representation of the respiratory chain in *S. cerevisiae* (G. Dujardin laboratory)

the two electron transfer from NADH to quinones, which are fed to the rest of the respiratory chain, but do not translocate protons across the inner membrane and are resistant to inhibitors of complex I.

Direct studies of the classical complex I function using *S. cerevisiae* have to be excluded, but interesting questions arise from studies of the alternative system. From an evolutionary point of view it will be interesting to understand how and why the largest respiratory enzyme has been lost insome organisms, but further research on this subject may be important for molecular medicine as well. Ndi1 is a potential therapeutic agent for complex I-related human diseases, since its expression in several complex I deficient mammalian models was shown to restore mitochondrial respiratory activity (Marella et al., 2009, 2010). Furthermore, in malaria-causing parasites the complex I has also been replaced by the alternative dehydrogenases, which could serve as specific targets for the development of new inhibitor drugs, as suggested recently for *Plasmodium falciparum* (Biagini et al., 2012).

3. Proton-pumping respiratory complexes in *S. cerevisiae*

3.1. The complex III (ubiquinol:cytochrome c oxydoreductase)

The complex III functions as a homodimer that contains 10 subunits per monomer in yeast and eleven in mammals (Brandt et al., 1993; Iwata, 1998; Hunte et al., 2000; Table 1; Fig. I-14). Structures derived from different organisms show that the overall organization of the complex III is well conserved.

3.1.1 Catalytic core of complex III

The three-subunit catalytic core of complex III is made of heme containing cytochrome *b* (Cytb) and cytochrome *c1* (Cyt1) and the 2Fe/2S-containing protein Rip1.

Subunits of complex III	
B. taurus	*S. cerevisiae*
SU1 (*UQCRC1*)	Cor1 (*COR1*)
SU2 (*UQCRC2*)	Cor2 (*COR2*)
SU3 (*MT-CYB*)	Cytb (*CYTB*)
SU4 (*CYC1*)	Cyt1 (*CYT1*)
SU5 (*UQCRFS1*)	Rip1 (*RIP1*)
SU6 (*UQCRQ*)	Qcr7 (*QCR7*)
SU7 (*UQCRB*)	Qcr8 (*QCR8*)
SU8 (*UQCRH*)	Qcr6 (*QCR6*)
SU9 (*UQCRFS1*)	/
SU10 (*UQCR10)*	Qcr9 (*QCR9*)
SU11 (*UQCR11*)	Qcr10 (*QCR10*)

Table I-1 Subunits of eukaryotic complexes III from *B. taurus* and *S. cerevisiae*.
Gene names are in italic. SU9 is the presequence of the catalytic Fe/S protein that stays in the mammalian complex as an additional subunit

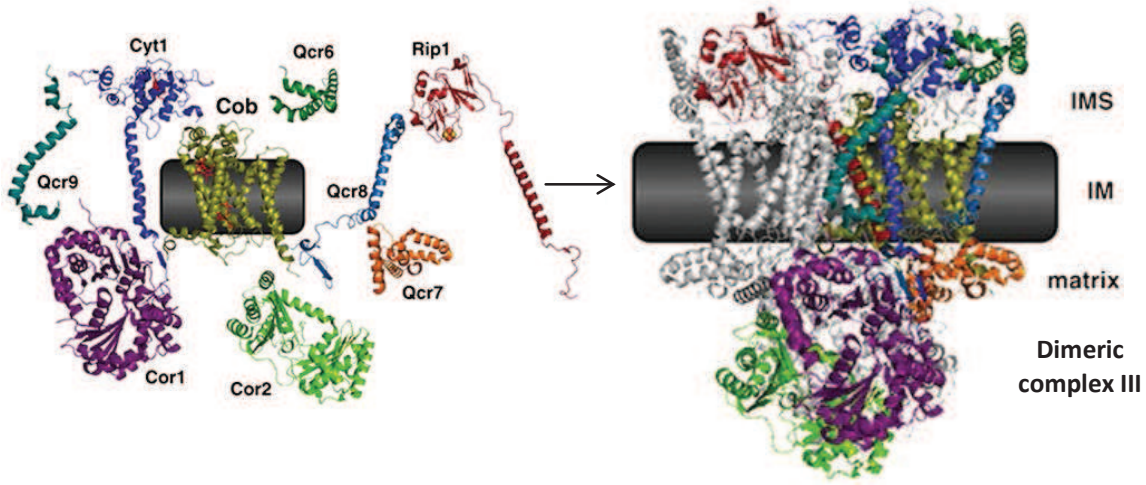


Figure I-14 Subunits of the complex III in *S. cerevisiae*.
Individual subunits of one monomer are shown surrounding Cytb (Cob), the integral membrane protein that initiates the complex biogenesis (left). The location of the subunits in the mature dimeric complex is shown on the right. The structure from PDB accession number 3CX5 was used to generate the figure. Qcr10 is not crystalized (from Smith et al. 2012).

Cytb is the only mtDNA encoded subunit of this complex, a highly hydrophobic, eight transmembrane segment-containing protein which exposes its N and C-terminal domains to the matrix. The synthesis of Cytb is tightly coupled to the assembly process. Stability of *CYTB* mRNA depends on Cbp1, which binds to its 5' untranslated region (5'UTR) (Chen and Dieckmann, 1997), and its translation is activated by two ribosome-interacting proteins, Cbs1 and Cbs2, which bind to the same region of the mRNA. As soon as the protein arrives at the ribosomal exit tunnel it interacts with the Cbp3/Cbp6 complex that, together with an additional protein Cbp4, stabilizes Cytb while it is complexed with the heme cofactors and assembled with Qcr7 and Qcr8. The Cbp3/Cbp6 complex has an additional role in the regulation of Cytb synthesis; the interaction of the complex with the ribosome promotes efficient translation of *CYTB* mRNA, thus linking the translation to the assembly of Cytb (Gruschke et al., 2011; Fig. I-15). This type of coordination between the synthesis and the membrane assembly of a given protein has been reported for subunits of complex IV and the ATP synthase (see later), and it resembles the process termed "control by epistasis of synthesis", found to adjust the translation of certain chloroplast mRNAs to the assembly of resulting proteins in complexes of the photosynthetic system (Choquet et al. 2000; Wostrikoff and Stern 2007).

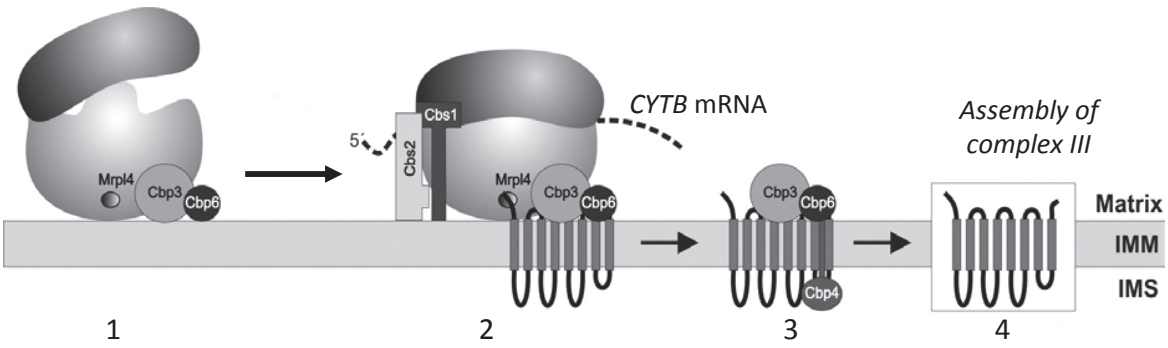


Figure I-15 Hypothetical model for the role of the Cbp3–Cbp6 complex during biogenesis of cytochrome b.
In the absence of an mRNA, the Cbp3–Cbp6 complex is bound to mitochondrial ribosomes (1). *CYTB* mRNA binds to the ribosome, and its efficient translation is promoted when the Cbp3–Cbp6 complex is ribosome bound (2). Cbp3 and Cbp6 then interact with the newly synthesized cytochrome *b*. Cbp4 is recruited (3), and this complex accompanies the subsequent assembly of cytochrome *b* with nuclear subunits of complex III (4). IMM, inner mitochondrial membrane; IMS, intermembrane space (Adapted from Gruschke et al. 2009).

Cyt1 is a single segment transmembrane protein. Heme *c* is covalently attached to the N-terminal of Cyt1 by the action of the heme lyases of the IMS (Bernard, 2003). This domain is

also important for the interaction with the mobile electron carrier cytochrome *c* and the dimerization of the complex III.

Rip1 exposes its catalytic C-terminal domain to the IMS, but it is imported to the matrix prior to its assembly with the rest of the complex in the inner membrane (Hartl et al., 1986). This may be due to the matrix localization of the ISC assembly machinery, which is the presumed source of the protein's 2Fe/2S cluster. In yeast, imported Rip1 undergoes a double proteolytic cleavage, firstly by the matrix processing peptidase (MPP), which removes its presequence and forms the intermediate Rip1 (i-Rip1), and then by mitochondrial intermediate peptidase (MIP), which removes an additional eight amino acids from the amino terminal to make mature Rip1 (m-Rip1), although this final modification is not required for the assembly and activity of Rip1 (Fu et al. 1990; Nett et al. 1997).

In the mammalian enzyme the presequence is cleaved in one-step by MPP and stays in the complex as an additional subunit. The C-terminal of Rip1 is a globular domain connected to its single transmembrane helix by a short linker region (7-9 amino acids). The linker region permits movements of the globular domain, which are essential for the electron transferring activity of the complex (see below). Rip1 is positioned so that the globular domain interacts with the catalytic subunits of one monomer while its transmembrane segment crosses the homodimer interface of complex III and resides in the adjacent monomer, making Rip1 essential for the dimerization of the complex.

3.1.2. Dynamic assembly process of complex III

The assembly of complex III is a modular pathway that proceeds through the sequential IM-insertion of several assembly intermediates of the complex (for review Zara et al. 2009; Smith et al. 2012, Fig. I-16). The first sub-complex contains Cytb and two small nDNA encoded subunits, Qcr7 and Qcr8.

3.1.3. Evidence for the Cyt1-containing complexes

Several sub-complexes forming between Cyt1 and Cor1 or Cor2 have been proposed (Zara et al. 2007), although the final position of these subunits in the fully assembled complex III is quite distant. Cor1 and Cor2 proteins reside in the matrix (Tzagoloff et al. 1986; Oudshoorn et al. 1987) where Cor1 is bound to the IM and shares an interface with Cytb. Cor2 is linked to the rest of the complex through a direct interaction with Cor1. The Cyt1-Cor1-Cor2 complex has never been purified, but the proposed interactions could reflect associations of preproteins, before their processing and assembly within the complex. This is based on the high homology

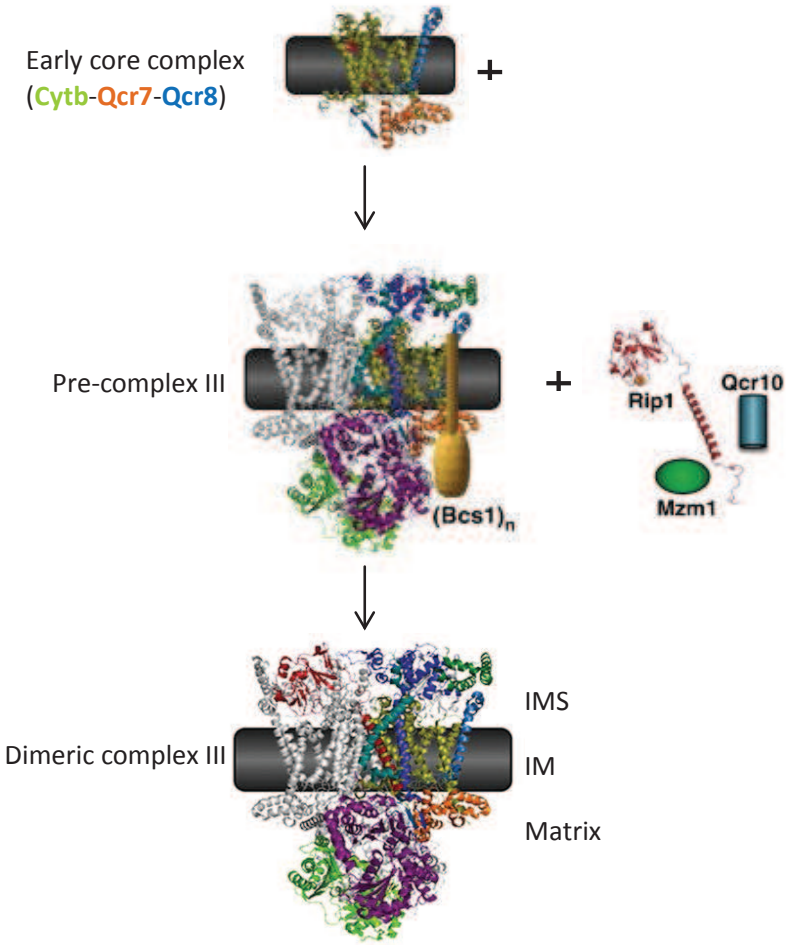


Figure I-16 Model for the assembly of functional complex III.
The assembly of complex III proceeds from the early core subcomplex to the pre-complex III via addition of the catalytic Cyt1 subunit and several supernumerary subunits. The subunits interact in a variety of smaller subcomplexes (see text for details) and with the assembly factors Bca1, Bcs1 and Mzm1. The Qcr10 subunit is not present in the yeast crystal structure, but it is the final subunit to be inserted into the complex. The structure from PDB accession number 3CX5 was used to generate the figure.(Adapted from Smith et al. 2012)

that the Cor1 and Cor2 proteins have with the matrix processing peptidases (MPP). Although in yeast the two proteins have no proteolytic activity, due to the incomplete active site, they still serve as matrix processing peptidases in plants (Braun et al., 1992).

3.1.4. The pre-complex III and final steps of assembly

Even if the association between the Core proteins and Cyt1 is not completely clear the three proteins are unambiguously found associated with the early core complex in the late assembly intermediate, also called the pre-complex III (Zara et al., 2009). Pre-complex III also contains Qcr6 and Qcr9, gathering in this way most of the complex III subunits into a late protease-resistant intermediate of assembly. But as the pre-complex III lacks Qcr10, and more importantly Rip1, it lacks catalytic activity as well. In the final steps of the assembly Rip1 and Qcr10 are inserted into the pre-complex III.

3.1.5. Assembly of complex III requires the assistance of non-subunit proteins

The coordination of the assembly of mtDNA and nDNA encoded subunits into a fully functional multimeric protein complex is an intricate process which requires assistance from additional proteins. The assembly of complex III relies on twelve proteins in yeast, listed in Table 2.

Bca1 is an assembly factor found in fungi, with no obvious ortholog in plants or metazoans (Mathieu et al., 2011). Bca1 contains a single transmembrane segment, exposing its large globular C-terminal domain to the IMS. Bca1 is found in high molecular weight complex of approximately 600 KDa, and its absence decreases levels of Rip1 and levels of pre-complex III when the assembly of Rip1 is impaired. It is thought to accompany the assembly of complex III prior to Rip1 insertion.

Mzm1 acts late in the assembly of complex III, at the insertion of Rip1. Mzm1 has a mutated LYR motif, characteristic of a protein family thought to associate with Fe/S-containing proteins. During the assembly of complex III Mzm1 was shown to stabilize Rip1 in the matrix

and protect it against proteolytic degradation, prior to its insertion into the pre-complex III (Atkinson et al., 2011).

Protein	Function
Cbs1	Translational activator of *CYTB* mRNA
Cbs2	Translational activator of *CYTB* mRNA
Cbp1	Stability and translation of *CYTB* mRNA
Cbp2	Splicing factor of *CYTB* pre-mRNA
Cbp3	Translational activator of *CYTB* mRNA; also interacts in a complex with newly synthesized Cytb
Cbp4	Interacts in a complex with newly synthesized Cytb
Cbp6	Translational activator of *CYTB* mRNA; also interacts in a complex with newly synthesized Cytb
Cyt2	Heme lyase involved in maturation of cytochrome c_1
Cyc2	Protein involved in maturation of cytochromes c and c_1
Bca1	Protein involved in early biogenesis of complex III in fungi
Bcs1	ATPase required for Rip1 insertion into the complex III
Mzm1	Protein involved in stabilization of Rip1

Table I-2 Known assembly factors of complexes III of *S. cerevisiae* and their function

The actual insertion of Rip1 into the IM and its assembly with the rest of the complex III is ATP-dependent and requires the action of another conserved assembly factor, Bcs1, an inner membrane ATPase that has had a central role in my PhD project and will be discussed more in detail in the Results section.

Tetratricopeptide 19, a protein of the inner membrane, was shown to physically interact with complex III in mice (Ghezzi et al., 2011). Orthologs of this protein in fungi are not known.

3.1.6. Activity of complex III – the Q cycle

Complex III catalyses the transfer of electrons from ubiquinol to cytochrome *c* and translocates four protons to the IMS during this process. The complex has two quinone binding

sites, Q_o (or Q_P, oxidation site) and Q_i (or Q_N, reduction site). Both sites are on Cytb, but on the opposite sides of the inner membrane.

It is widely accepted that complex III operates according to the Q-cycle hypothesis, originally proposed by Peter Mitchel (1976). The Q cycle is a two step process, resulting in the translocation of four protons to the IMS. In the first step, the ubiquinol molecule binds the Q_o site (UQH_2) while the ubiquinone binds at the Q_i site (UQ). UQH_2 transfers two electrons to the enzyme, which take separate pathways and this bifurcation at the Q_o site results in higher energetic yield compared to the linear pathway (Crofts et al. 1999).

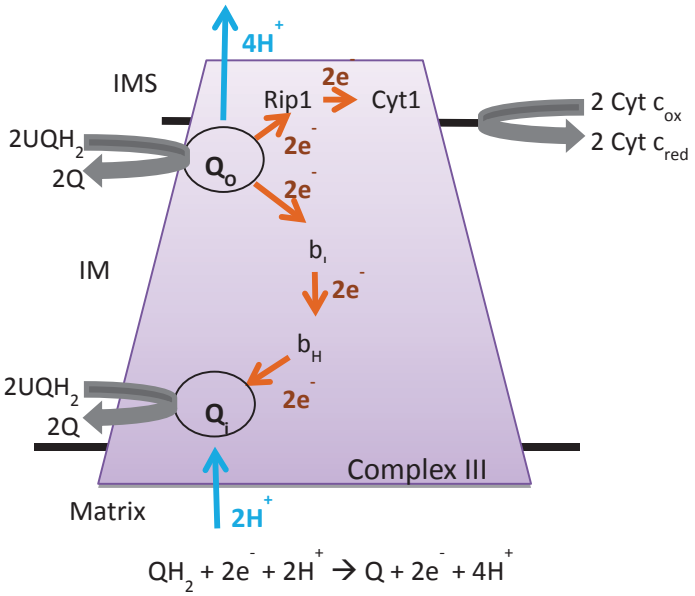


Figure I-17 Schematic representation of the Q cycle of the respiratory complex III
UQH_2 and UQ are ubiquinol and ubiquinone, respectively. IMS and IM are intermembrane space and the inner membrane, respectively. See text for details.

The first electron takes the high potential pathway, going to the oxidized cytochrome *c* *via* Rip1 and Cyt1. The electron is transferred from Cyt1 to cytochrome *c* in a rapid heme-to-heme manner (Lange and Hunte, 2002) and the reduced molecule of Cyt *c* can dissociate from the complex. The second electron from UQH_2 is transferred to the low potential hemes b_L and b_H, which reduces a ubiquinone molecule bound to the Q_i site, producing a semiquinone radical.
The second round of the cycle is necessary to reduce the semiquinone at the Q_i site to ubiquinol, which finally results in four protons transferred to the IMS (two from the matrix, two from the IM) and the reduction of two molecules of cytochrome *c* (Fig. I-17).

3.1.7. Mutations affecting the human complex III lead to disease

Disease-causing mutations of complex III are not the most frequently encountered mutations compared with those found in the complex I or tRNAs (www.mitomap.org), still they are not so rare and have been linked to a plethora of clinical syndromes. For most, if not all, mitochondrial mutations the clinical symptoms vary greatly, and mutations of complex III are no exception. Human cells contain a high copy number of mtDNA, mutated and wild type mitochondrial genomes can coexist in the same cell or the same mitochondrion (heteroplasmy). The proportion of the two co-habitant genomes can influence the expression, onset and severity of a particular disease, and a ''threshold'' effect (up to a certain point the wild type genome compensates for the mutated one) is often observed. Still, some general observations can be made: skeletal muscle involvement, exercise intolerance, lactic acidosis and neurological impairment generally appear as common features in mitochondrial diseases.

3.1.8. Most of the reported human mutations affect MTCYTB

In complex III related pathologies most of the reported mutations are found in mitochondrially-encoded Cytb (*MTCYTB*) (reviewed in (Meunier et al., 2012). Although activity assays for OXPHOS complexes using very small amounts of biological samples have been developed (Bénit et al., 2006), scarce availability of biological material available from patients may slow clinical studies. *S. cerevisiae* can be a used as a model organism in studies of *CYTB* mutations. Point mutations in *CYTB* can be introduced in the mtDNA of *S. cerevisiae* and functionally assayed (Ding et al., 2008). Moreover, yeast mitochondria are homoplasmic for a given mutation and share most of the structural and functional features of the human enzyme (50% sequence identity between cytochromes b of the two species).

Besides cytochrome b, two other nuclear subunits of the complex, SU6 and SU7, have been found mutated in patients, leading to hepatic dysfunction, lactic acidosis and psychomotor retardation (Haut et al., 2003; Barel et al., 2008).

3.1.9. TTC19 (Tetratricopeptide 19)

The cases of TTC19 mutations were recently reported in individuals with progressive encephalopathy that can have a late onset (one of the patients showed symptoms at age 42) (Ghezzi et al., 2011). *Drosophila melanogaster* was used as a knock-out model showing that the disruption of the gene is associated with severe neurological abnormalities in this organism. Mutations found in patients affect the correct assembly of complex III, but allow the formation of intermediates containing Core proteins, suggesting that TTC19 could act early in the assembly of complex III.

3.1.10. BCS1L

More than twenty pathological mutations have been reported for the *BCS1L* gene. The mutations spread throughout the protein sequence and cause variable pathologies with differently severe onset and outcome, which can be divided in three main groups, according to their clinical symptomes (Table 3).

The Bjornstad syndrome, an autosomal recessive disorder characterized by neurosensory hearing loss and brittle hair (*pili torti*), constitutes the first group and it is the least severe of BCS1L-linked disorders. Mutations that cause the Bjornstad syndrome affect the activity and the assembly of complex III, and have been associated with increased ROS production (Hinson et al., 2007).

The second group encompasses a variety of disorders that display developmental delays, liver dysfunction and variable degree of muscular problems and exercise intolerance, termed the complex III deficiencies (Blázquez et al., 2009; Fernandez-Vizarra et al., 2007; Gil-Borlado et al., 2009; de Lonlay et al., 2001). Mutations were found to severely decrease the activity of complex III, while the assembly process is not always affected.

In the third group there is the most common and the most severe syndrome associated with *BCS1L* mutation: the GRACILE syndrome (Growth Retardation, Aminoaciduria, Cholestasis, Iron overload, Lactacidosis, and Early death) (Visapää et al., 2002). This is an autosomal

Mutation	Syndrome	Reference
S277N	Complex III deficiency	De Lonlay et al., 2001 Moràn et al., 2011
P99L		
R155P		
V353M		
S78G	GRACILE syndrome	Visapaa et al., 2002 Kotarsky et al., 2010 Moràn et al., 2010
R56stop	Complex III deficiency	
V327A		
R144Q		
R45C	Complex III deficiency	De Merleir et al., 2003
R183H	Bjornstad syndrome	Hinson et al., 2007 Moràn et al., 2010
R306H		
R184C		
G35R		
R114W		
Q302E		
R291stop		
I106stop		
R184C	Complex III deficiency	Fernandez-Vizarra et al., 2007
R73C		
F368I		
R183C		
T50A	Complex III deficiency	Blazquez et al., 2009
G129R	Complex III deficiency	Tuppen et al., 2010

Table I-3 Reported mutations in the BCS1L protein and the associated pathologies

recessive disease, characterized by multiple system failure and early death. This disorder is more common in patients of Finnish descent, where it was first identified and all patients examined so far harbored the pathogenic Ser79Gly exchange in the *BCS1L* gene (Kotarsky et al., 2010). The mutation could render the protein less stable since lowered levels of BCS1L were found in several different tissues from patients. The accumulation of RISP (Rip1), the assembly

and the activity of complex III were also found to be decreased in GRACILE patients. The groups investigating the syndrome hypothesized on additional functions of BCS1L in mitochondria, in particular in the iron metabolism, given that the iron overload is a prominent feature of the syndrome. Still, no conclusive data exist on this point.

BCS1L was reported to interact with LETM1, a mitochondrial inner membrane protein involved in maintaining the mitochondrial tubulation and cell viability, mutations of which are associated with Wolff-Hirchhorn syndrome, a multisystemic disorder (Tamai et al., 2008). Interactions between the yeast homolog of LETM1, Mdm38, and Bcs1 have not been reported.

The S78G substitution in BCS1L, responsible for the GRACILE syndrome, has recently been introduced in mouse, which is the first viable mammalian model for a complex III deficiency (Levéen et al., 2011). The homozygous mutant mice from 3 weeks of age had lowered amounts of BCS1L and lowered assembly and activity of complex III, while the complex was correctly assembled in younger animals. Symptomatic animals presented glycogen depletion in liver, growth defects, cirrhosis, tubulopathy, lactic acidosis and a short lifespan, but not the iron overload.

3.2. The complex IV (cytochrome c oxidase)

The crystal structure of the complex IV from bovine heart mitochondria was the first to be published for a respiratory chain complex (Tsukihara et al., 1995). Complex IV is a functional dimer that consists of eleven subunits in *S. cerevisiae* (thirteen in mammals), three of which are encoded by the mtDNA (Fig. I-18, Table 4). Complex IV transfers electrons from cytochrome *c* to molecular oxygen, reducing it to water, and couples this transfer to the extrusion of four protons to the IMS. The assembly of this large enzyme is relies on a multitude of assembly factors; in fact, more than thirty complex IV assembly factors have been identified (for detailed list of the assembly factors of complex IV see Soto et al., 2012).

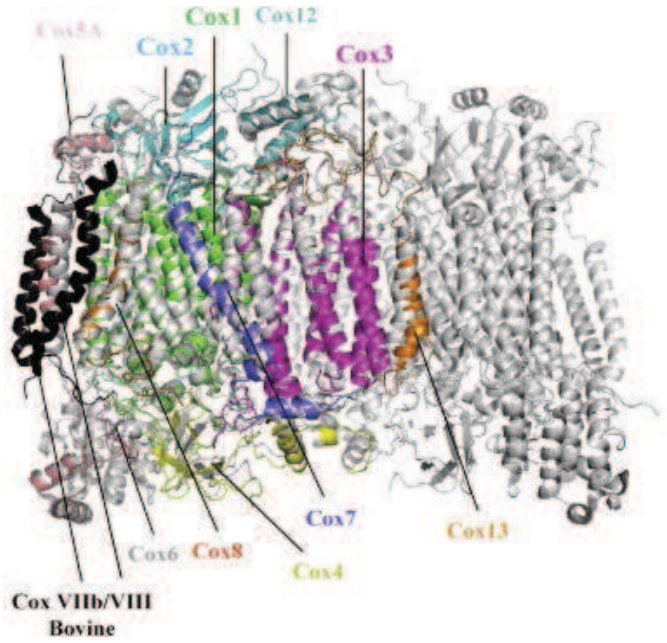


Figure I-18 Superposition of homology models of the complexes IV from yeast and bovine.
The whole, dimeric structure of bovine complex IV (drawn from PDB ID: 1V54) is shown in light grey. Each of the eleven subunits of yeast complex is modelled on its counterpart in one half of the bovine dimer only and is colour-coded to aid recognition. The two bovine subunits without homologues in yeast complex IV are shown in black. (Maréchal et al., 2012)

3.2.1. Auxiliary proteins coordinate the synthesis of the catalytic core of complex IV

The mitochondrial-encoded Cox1, Cox2 and Cox3 make up the active core of the complex IV in most respiring organisms. However, exceptions where *COX2* or *COX3* gene have been transferred to the nucleus can be cited, as in the case of *COX3* in the photosynthetic alga *C. reinharditii* (Perez-Martinez et al., 2002). Cox1, Cox2 and Cox3 are all hydrophobic and membrane embedded, containing twelve, two and seven transmembrane segments, respectively.

Synthesis of the catalytic core of the complex IV in mitochondria of *S. cerevisiae* relies on the assistance of several translation activators for each of the three subunits (reviewed in Herrmann et al., 2013; Towpik, 2005), that usually recognize the 5'-untranslated regions (5'UTR) of their target mRNAs. Synthesis of Cox1 requires at least two proteins, Mss51 and Pet309 (Decoster et al., 1990; Manthey and McEwen 1995). Cox2 relies on Pet111 (Mulero and

Fox, 1993) while the synergic action of three proteins, Pet54, Pet122 and Pet494 is necessary for the synthesis of Cox3 (Brown et al. 1994). These activators of translation have been identified mostly through genetic studies, and their detailed mechanism of action and interactions is still not completely elucidated. Most of the translational activators have been found to interact with each other, both genetically and physically (Naithani et al. 2003; Fiori et al. 2005). This spatial and temporal clustering of factors that activate translation of core subunits was proposed to co-localize the translation of the three mitochondrial mRNAs to facilitate the subsequent assembly process (for review Ott and Herrmann 2010). For their insertion into the inner membrane the three core proteins of complex IV rely on the hydrophobic, five-transmembrane segment-containing inner membrane protein Oxa1. Oxa1 acts as a general membrane insertion machinery for mtDNA encoded proteins and has a conserved function in

humans (Bonnefoy et al. 1994; Hell et al. 2001). The matrix-exposed C-terminal of Oxa1 interacts with the mitoribosomes to facilitate the co-translational membrane insertion of hydrophobic mitochondrial proteins (Szyrach et al., 2003). The rate of *COX1* mRNA translation is tightly coupled to the amount of correctly assembled Cox1 into the membrane and with nuclear subunits of the enzyme (Perez-Martinez et al. 2003; Barrientos et al. 2004).

The translational activator of Cox1, Mss51, can bind both Cox1 mRNA and newly synthesized Cox1, and has a central role in this assembly-controlled translation regulation (Barrientos et al., 2004; Fontanesi et al., 2009; Khalimonchuk and Rödel, 2005; Mick et al., 2007, 2010; Perez-Martinez et al., 2003, 2009) (described in detail in the Results).

Cox2 is synthesized as a precursor protein and as such is inserted in the inner membrane, where it is stabilized through interaction with the chaperon Cox20. The pre-sequence of Cox2 is cleaved by the inner membrane peptidase complex, IMP (Hell et al. 2000; Nunnari et al. 1993).

Cox3 is the last member of the catalytic core, important for the stabilization of Cox1 and Cox2 (Brunori et al., 1987), and possibly for modulation of the proton transfer across the membrane, which has been proposed for the bacterial equivalent of complex IV (Hosler et al. 2006).

S. cerevisiae		*H. sapiens*	
Gene	Protein	Gene	Protein
Catalytic core (mtDNA encoded structural subunits)			
COX1	Cox1	MTCOXI	COX1
COX2	Cox2	MTCOXII	COX2
COX3	Cox3	MTCOXIII	COX3
Core protective shield (nDNA encoded structural subunits)			
COX4	Cox4	COXVb	COX5b
COX5a	Cox5a	COXIV-1	COX4-1
COX5b	Cox5b	COXIV-2	COX4-2
COX6	Cox6	COXVa	COX5a
COX7	Cox7	COXVIIa	COX7a
COX8	Cox8	COXVIIc	COX7c
COX9	Cox7a	COXVIc	COX6c
–	–	COXVIIb	COX7b
–	–	COXVIII	COX8
COX12	Cox12 (Cox9)	COXVIb	COX6b
COX13	Cox13 (Cox10)	COXVIa	COX6a

Table I-4 Subunits of the respiratory complexes IV in *S. cerevisiae* and *H. sapiens*

3.2.2. Heme cofactors of Cox1

Cox1 carries two heme molecules (*a* and a_3), necessary for its folding and stability and is essential for the correct assembly and catalysis of complex IV (Carr and Winge, 2003; Kim et al., 2012). At least three assembly factors, Cox10, Cox15 and Shy1 (COX10, COX15, SURF1 in humans) have a role in this process (Glerum and Tzagoloff 1994; Barros et al. 2001; Smith 2004). Additional proteins have been proposed to participate in the heme insertion into Cox1, such as Coa2 (Cytochrome Oxydase Assembly 2) (Bestwick et al., 2010), but as the precise knowledge of the factors and mechanisms for this process is still lacking, the involvement of other assembly factors may be reported in the future.

3.2.3. Cox1 and Cox2 bind copper ions

Both Cox1 and Cox2 contain copper ions in their respective Cu_B and Cu_A copper centers, whose formation occurs after the insertion of the two proteins in the inner membrane and requires the assistance of several assembly factors. Cox17 is a hydrophilic protein of the IMS that binds and transfers copper ions to Cox11 and Sco1, which deliver the ions to the copper binding sites in Cox1 and Cox2, respectively (Glerum et al. 1996; Horng et al. 2004). The exact mechanism of the ion transfer between Cox11 and Cox1 remains to be elucidated, while Sco1 was shown to directly interact with Cox2 during the transfer of copper ions to its IMS-protruding copper binding site (Lode et al., 2000). How the copper ions reach Cox17 in the IMS is another open question, in particular since the matrix copper pool is thought to be the source of complex IV copper ions (Cobine et al., 2004). It has been proposed that copper from the matrix is transferred to Cox17 *via* an uncharacterized membrane transporter coupled to the ''copper-transfer pathway'', which involves several copper-binding proteins of the IMS, such as Cox19, Cox 23, Pet191, Cmc1 and Cmc2 (reviewed in Horn and Barrientos 2008).

3.2.4. Assembly of complex IV

The complex IV assembles through a complicated and not fully understood sequential pathway for which a number of assembly intermediates and different assembly models have been described. It is clear that the regulation of the synthesis and maturation of the three catalytic subunits is the heart of the assembly process (reviewed in Mick et al. 2011, Fig I-19).

Cox1 is stabilized in different intermediaries involving the assembly factors Mss51, Cox14, Coa1, Coa3 and Shy1, and is the first mitochondrial subunit to assemble with imported subunits of nuclear origin, Cox5 and Cox6. Processed Cox2 containing copper ions and Cox3 can both associate with Cox1-Cox5-Cox6 complex, but it is unclear which one does so first. Subunits that assemble at the next step are Cox4, Cox7, Cox8 and Cox9, but little is known about the sequence of their incorporation. However, it is known that Cox7, Cox8 and Cox9 form a complex, assisted by the assembly factor Pet100, prior to their incorporation within the earlier intermediate; yet this appears puzzling given their final position in the mature enzyme. The mature complex is formed after the addition of Cox12 and Cox13.

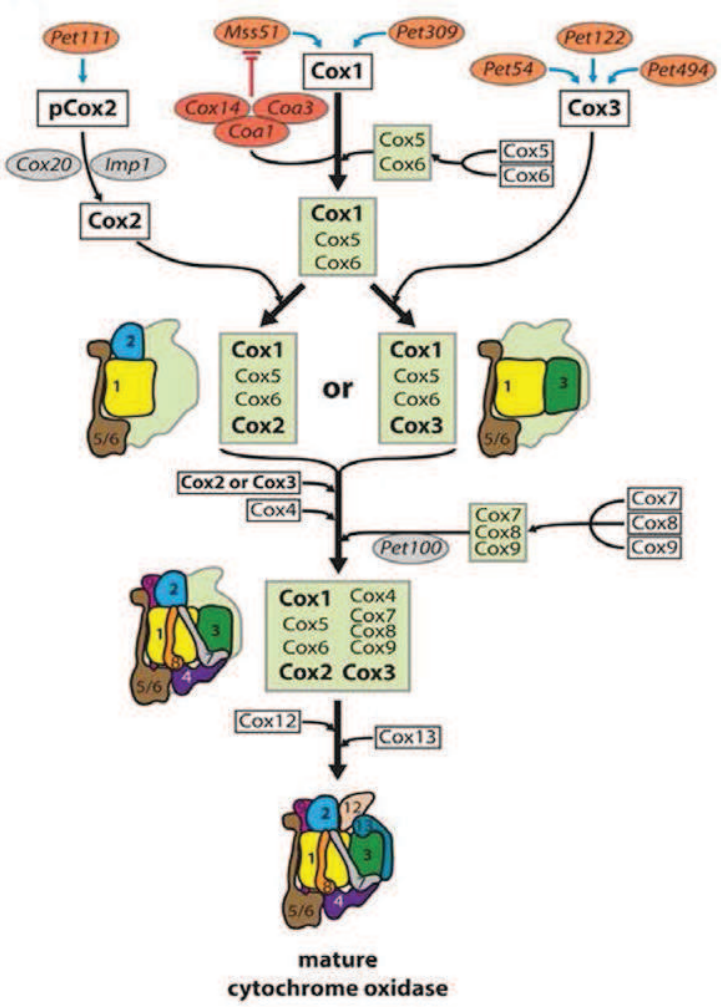


Figure I-19 Model of complex IV assembly (see text).
White boxes denote structural subunits and green boxes denote reported assembly intermediate complexes. Schematic drawings represent assembly intermediates in a structural context. The subunits are numbered after their respective protein (for example, 1 stands for Cox1, pCox2 is a precursor of Cox2) and are colour-coded; for simplification, the Cox5–Cox6 subcomplex (5/6) is shown in brown. Orange ovals represent translational activators, red and grey ovals represent assembly factors (Adapted from Mick et al. 2011)

3.2.5. The catalysis of complex IV

Despite intensive research, the exact mechanism of electron transfer and proton pumping of the complex IV is still not fully understood. The catalysis of complex IV will only be briefly described here; for more detailed reading and references see Wikström 2004; Lucas et al. 2011. Molecules of cytochrome *c*, which have been reduced by the complex III, bind to Cox2

and transfer electrons to the two copper atoms of the Cu_A center. From this point, electrons go from Cu_A to the heme *a* and subsequently to the heme a_3-Cu_B binucleated site in Cox1, which is the site of oxygen reduction. Although this is the generally acepted point of view for the electron flow in complex IV, a direct transfer of electrons from Cu_A to heme a_3-Cu_B site has not been excluded either. For the transfer of four electrons from cytochrome *c* the complex IV takes up eight protons from the matrix, four of which are substrates in the reduction of oxygen to water and four are transferred to the IMS. A number of models were proposed to explain the catalysis of the complex IV. In some of those models the electron transfer between the Cu_A and heme *a* is believed to be associated to the uptake of one proton, which is released to the IMS as the electron is passed to heme a_3-Cu_B site. An additional proton is thought to be translocated to the IMS upon the reduction of the oxygen to water at the a_3-Cu_B site. Distinct paths for proton translocation across the enzyme have been described (pathways D and K), formed out of charged amino acids inside the enzyme structure, embedded in the inner membrane, that appear to have different roles in proton transfer from the heme a_3-Cu_B site. An additional proton uptake pathway could exist, associated to the Cu_A-heme *a* electron transfer (pathway H) (Fig. I-20).

3.2.6. Disease-linked mutations in the catalytic subunits of complex IV

Complex IV deficiency is commonly encountered in human respiratory chain pathologies, and it is mostly due to mutations in the assembly factors of this complex. Only a low number of disease-causing mutations have been found in the three catalytic core subunits (Barrientos et al. 2002), and among the eleven remaining structural subunits only mutations in *COXVIB* gene have been reported (Massa et al., 2008). Clinical symptoms of complex IV deficiency are very variable and include encephalopathy, myopathy and motoneuron disease-like presentations.

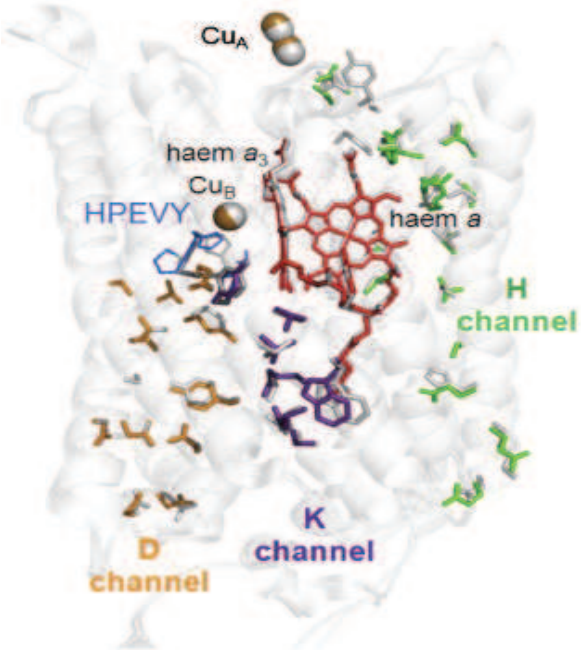


Figure I-20 Heme/copper centers and possible proton channels in complex IV.
Positions of heme/copper centers and hydrophilic residues possibly forming the K (purple), D (orange) and H (green) proton channels in Cox1 and 2 of *S. cerevisiae* complex IV in comparison to the bovine subunits (PDB: 1V54, white) (Adapted from Maréchal et al. 2011).

3.2.7. Deficiencies of heme and copper metabolism cause disease in humans

A number of proteins in both heme and copper metabolisms are conserved from yeast to humans. Human homologues of Cox10 and Cox15 exist and are able to complement the null mutants in yeast (Glerum and Tzagoloff, 1994; Mashkevich et al., 1997). Mutations of the human genes have been associated with severe infantile cardiomyopathy, tubulopathy and Leigh syndrome, a progressive neurodegenerative disorder (Antonicka et al., 2003a, 2003b; Tiranti et al., 1999).

SURF1 (Shy1 in yeast) is an inner membrane-associated protein proposed to have a double role in the assembly and hemylation of COX1. SURF1 does not complement the yeast null mutant; still the two proteins are proposed to share the same function, judging by the similar decrease of complex IV assembly and activity in mutated yeast and human systems (Barrientos et al., 2002a). Patient mutations in SURF1 are relatively frequent and are linked to the Leigh syndrome and complex IV deficiency (Tiranti et al., 1999).

Homologues of Cox11, Cox17, Cox19, Cox23, Sco1, Pet191, Cmc1 and Cmc2, involved in copper metabolism in yeast, have been identified in humans. Knockdown of human *COX17* affects the assembly of complex IV, and suggests that, differently from yeast, this protein could be more implicated in the copper insertion in Cox2 than in that of Cox1 (Oswald et al. 2009). Mutations in C2orf64, the human homologue of Pet191, are associated with a severe complex IV deficiency and lethal neonatal cardiomyopathy (Huigsloot et al., 2011).

3.3. The F_1F_o ATP synthase (complex V of the OXPHOS)

The F_1F_o ATP synthase (ATP synthase) is a highly conserved molecular machine able to synthesize (or hydrolyse) ATP from ADP and Pi, powered by the respiratory chain-generated proton gradient. In *S. cerevisiae*, the ATP synthase has seventeen subunits, organized in four structural units (Table 5), and forms functional dimers in the cristae of the inner membrane. Three subunits are encoded by the mtDNA: Atp6, Atp8 and Atp9. However, in mammals, Atp9 is encoded in the nucleus.

3.3.1. Structural units of ATP synthase

Thirteen subunits form the core of the enzyme: α (Atp1), β (Atp2), γ (Atp3), Atp4, Atp5 (Oligomycin Sensibility Conferring Protein, OSCP), Atp6, Atp8, Atp9, δ (Atp16), ε (Atp15), d (Atp7), f (Atp17), h (Atp14) (Velours and Arselin 2000). These proteins are associated in four functionally essential and widely conserved structural units (Lau et al. 2008, Fig. I-21). The matrix-exposed soluble catalytic head is composed of $\alpha_3\beta_3$ subunits and it bears the ATP biding sites, responsible for synthesis or hydrolysis of ATP (Boyer, 1997; Stock et al., 2000). The matrix exposed hexamer of the catalytic subunits is frequently referred to as the F_1 sector. The membrane embedded region of the enzyme is comprised of $Atp9_{10}$, Atp6, Atp8 and Atp17. The interface between Atp9 and Atp6 forms the channel through which protons are transferred from the IMS back to the matrix. The membrane region is also called the F_o sector (o for the the

Subunits of ATP synthase in yeast and humans		
	S. cerevisiae	*H. sapiens*
F_1	α_3 (Atp1)	α_3
	β_3 (Atp2)	β_3
Central stalk	γ (Atp3)	γ
	δ (Atp16)	δ
	ε (Atp15)	ε
F_o	Atp6	a
	Atp8	A6L
	$Atp9_{10}$	c_8
	f (Atp17)	f
Peripheral stalk	Atp4	b
	OSCP (Atp5)	OSCP
	d (Atp7)	d
	h (Atp14)	F6
Assembly and dimerization	e (Atp20)	e
	g (Atp21)	g
	i (Atp18)	-
	k (Atp19)	-

Table I-5 Subunit composition of *S. cerevisiae* and *H.sapiens* ATP synthase

enzyme's sensitivity to oligomycin, which binds to the membrane sector). Physical contacts between F_1 and F_o are enabled by two separate stalks: the central stalk, made up of the Atp3, Atp15 and Atp16 and the peripheral stalk, which consists of subunits Atp4, Atp7, Atp14 and Atp5.

In addition to the core subunits, four small subunits have been associated to the ATP synthase. Subunits i (Atp 18) and k (Atp 19) are involved in the stepwise assembly of the enzyme (Wagner et al., 2010), while subunits e (Atp20) and g (Atp21) were reported to be crucial for the dimerization of the enzyme (Arnold et al., 1998).

The structure and function of ATP synthase has been conserved through the evolution, as evidenced by the atomic structures of the F_1 sector/Atp9 ring from both yeast and bovine (Abrahams et al. 1994; Stock et al. 1999; Kabaleeswaran et al. 2006; Dautant et al. 2010), and functional complementation of yeast deletion mutants with rat and bovine subunits of the peripheral stalk (Prescott et al., 1995; Velours et al., 2001).

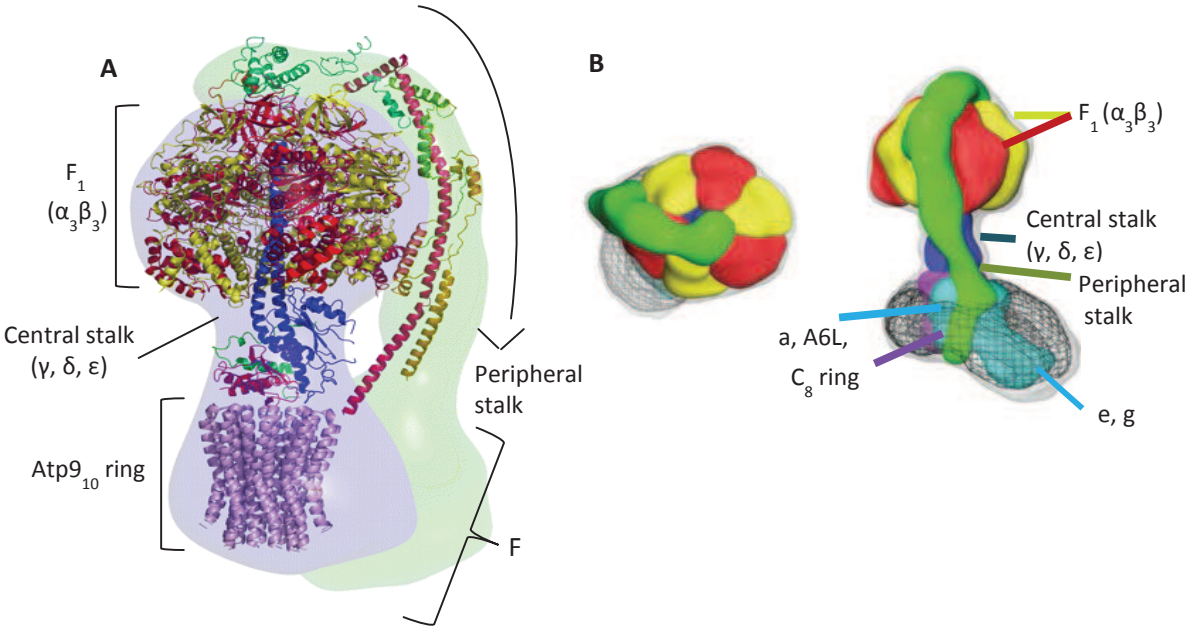


Figure I-21 Model of the whole structure of mitochondrial ATP synthase.
A) The region occupied by F_1Atp9_{10} and the central stalk from *S. cerevisiae* is blue; the peripheral stalk from bovine and the membrane subunits a, e, f, g, and A6L are green (from (Rees et al., 2009)). B) Structural units of the bovine enzyme shown as colored surfaces (from (Baker et al., 2012).

3.3.2. Assembly of ATP synthase

The assembly of the ATP synthase is a step-wise process that, according to the latest reports in *S. cerevisiae*, assembles from three different modules: the F_1, Atp9 ring and the Atp6/Atp8-containing complex (Rak et al. 2011, Fig I-22). Assembly factors have been described for each of the assembly steps, but considering the complexity of the enzyme their number seems rather small.

3.3.3. Biogenesis of F_1

Besides the general matrix chaperon, Hsp60 (Gray et al., 1990), two other matrix proteins, Atp11 and Atp12, participate in the correct assembly of the $\alpha_3\beta_3$ hexamer and protect

α and β from proteolytic degradation (Ackerman and Tzagoloff 1990; Wang et al. 2001). Even in the cases of deficient assembly of F_1, α and β are stabilized by Atp11 and Atp12 in the matrix,

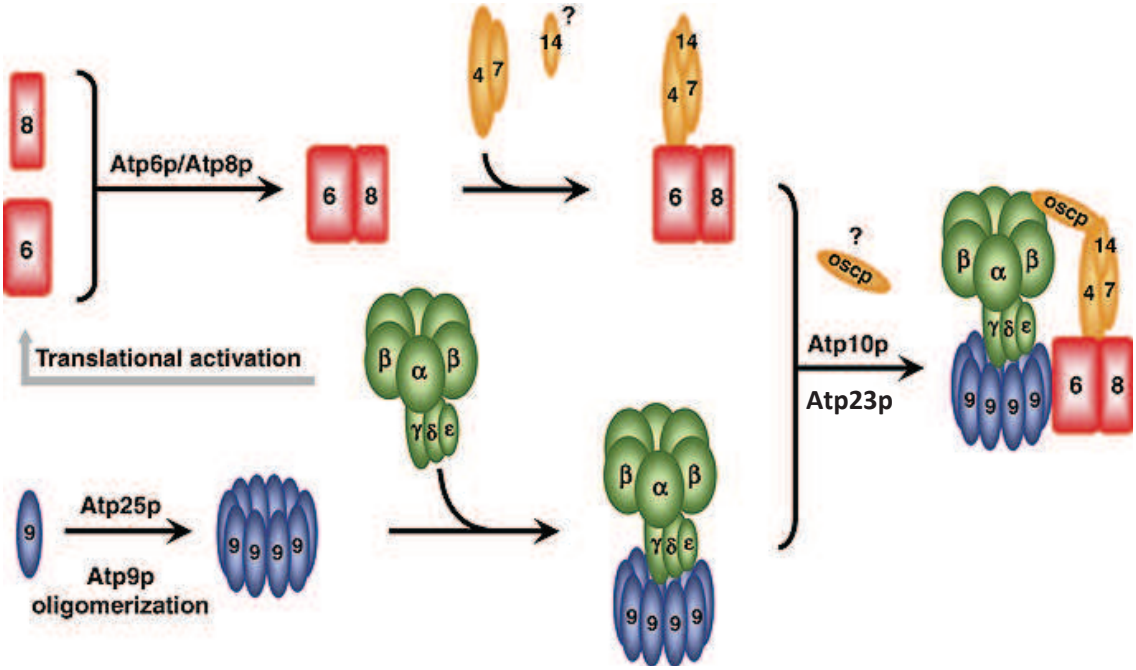


Figure I-22 Model for the assembly of the ATP synthase in *S. cerevisiae*.
The diagram shows the two separate pathways for assembling the immediate precursors of the ATP synthase. In this scheme, the ATP synthase is composed of at least three different modules, F_1, the Atp9 ring and the Atp6/Atp8/stator subcomplex. At present, the possibility that the stator is also a separate module, interacting with the Atp6/Atp8 subcomplex as a preformed unit, cannot be excluded. Activation of Atp6 and Atp8 translation by F_1 is denoted by the grey arrow. Atp25 is a chaperone which promotes the oligomerization of Atp9 (From Rak et al. 2011).

where they are found in protease-resistant inactive aggregates. Both Atp11 and Atp12 are conserved in humans. An additional protein, Fmc1, was reported to be necessary for the correct assembly of the catalytic head in heat stress conditions in *S. cerevisiae* (Lefebvre-Legendre, 2000). The F_1 subsequently associates with the central stalk and the Atp9 ring in the membrane.

3.3.4. The Atp9 ring

The Atp9 subunit is encoded by mitochondrial DNA and the assembly of Atp9 ring requires the function of the inner membrane protein Atp25. Atp25 is both a translational activator required for the stability of Atp9 mRNA and a chaperon of the oligomerization process

(Zeng et al., 2008). The oligomerization of Atp9 is a moderately slow process, which is followed by a rapid interaction with the assembled F_1/central stalk to form a late intermediate of assembly.

3.3.5. The Atp6/Atp8 module

Atp22 is a translational activator of Atp6, proposed to specifically interact with the 5'UTR region of the Atp6 mRNA (Zeng et al. 2007). Additionally, the translation of Atp6 and Atp8 was shown to be dependent on the presence of assembled, but not necessarily catalytically active F_1 (Rak and Tzagoloff 2009). After its synthesis, Atp6 rapidly interacts with Atp8 and it was also co-fractioned with several subunits of the peripheral stalk, such as Atp4 and Atp7. Atp10 was also found to be a part of the Atp6/Atp8 complex where it assists the folding and assembly of Atp6, together with the metalloprotease Atp23, which also cleaves the presequence of this subunit once it is assembled with the rest of the enzyme (Tzagoloff 2004; Zeng et al. 2007). The cleavage of the presequence is not essential for the assembly of Atp6 within ATP synthase, or for the activity of the mature enzyme.

The rate limiting association of F_1 with the Atp9 ring in the first place and the subsequent translational activation of the fast-forming Atp6/Atp8 module coordinate the assembly of ATP synthase to synchronize the formation of the proton conducting channel with the catalytic part of the enzyme, thus preventing proton leakage across the membrane.

3.3.6. Dimerization of ATP synthase and the morphology of the inner membrane

Monomeric forms of ATP synthase are already catalytically active; nevertheless, the enzyme associates in homodimers organized in rows along the curved rims of the cristae (Strauss et al., 2008; Fig. I-23). The subunits Atp4, Atp20 and Atp21 were all found to be essential for the dimer formation (Soubannier et al., 2002; Wittig et al., 2008), but not for contacts between different dimers in the cristae rows. Each dimer alone is capable of bending

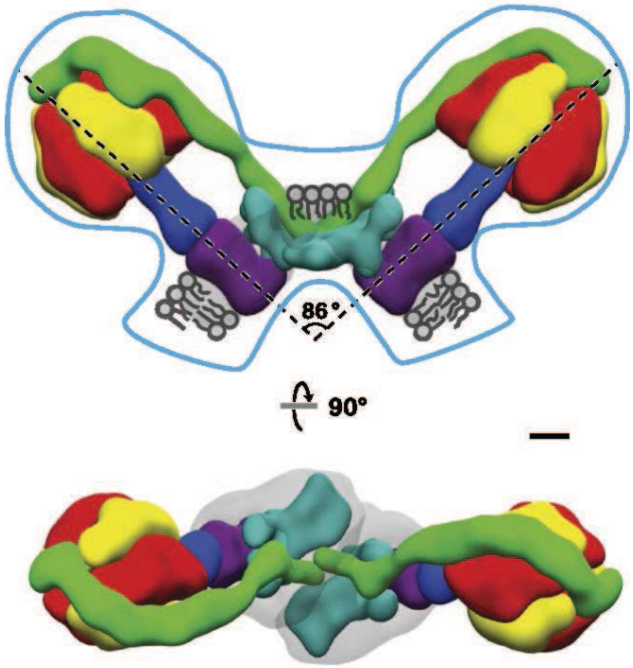


Figure I-23 Model of dimeric bovine ATP synthase.
Side and top views of a model of dimers of ATP synthase based on the observed arrangement of protein and micelle in the cryo-EM map as well as the overall size and shape of dimers observed in mitochondrial membranes (blue outline in *B*). The angle between the two monomers in the dimer would be ~86°, which is in good agreement with the ~80° observed by electron tomography. (Scale bar: 25 Å.). Color code as in Fig. I-21B. (Baker et al. 2012).

the membrane and they are disposed at variable distances from one another, suggesting an accessory protein-independent mechanism for row formation (Davies et al., 2012; Fig. I-24). The dimers are proposed to drive their own self-association in the membrane by means of random diffusion and minimization of the membrane elastic energy, allowing the quicker and more cost-effective remodeling of the inner membrane in adaptation to different metabolic conditions. The particular lipid composition of the inner membrane probably also plays a role in the organization of ATP synthase dimers, as cardiolipin was found to be tightly associated with the enzyme (Acehan et al., 2011), but also with complexes III and IV of the respiratory chain (Pfeiffer, 2003).

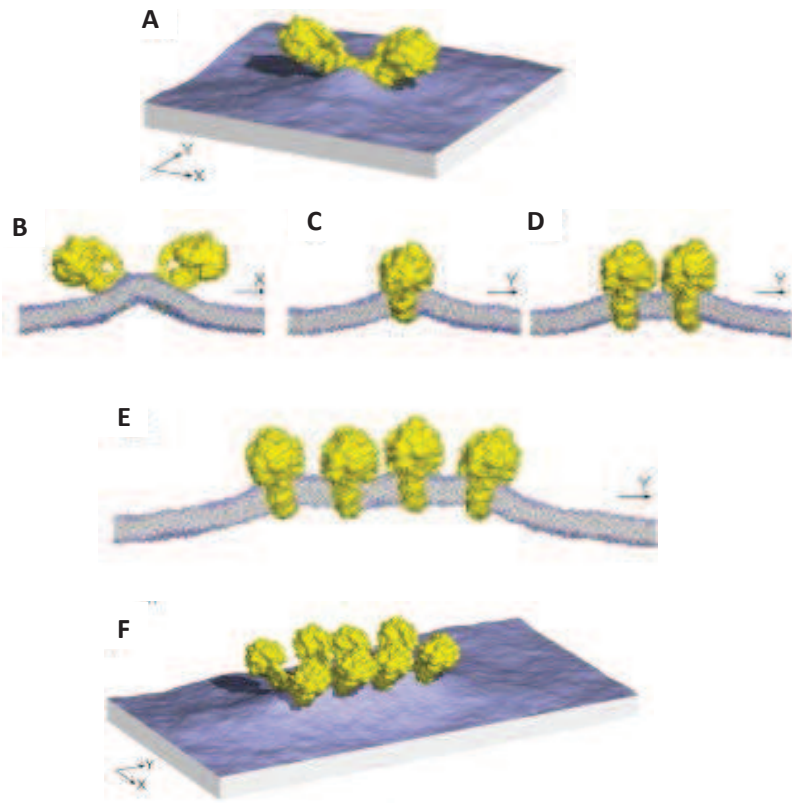


Figure I-24 Membrane curvature induced by ATP synthase dimers.
A) Perspective view of a simulated membrane patch with an ATP synthase dimer distorting the planar lipid bilayer. B, C) cross sections through the membrane patch in A) showing the curvature profile of the lipid bilayer in x and y direction. D) and E), curvature profiles as in C) for membranes with two or four ATP synthase dimers, side by side. Note how the membrane deformation in y direction is relieved when two or more dimers assemble into a row. F) perspective view of the row of four dimers shown in E). (Davies et al. 2012)

3.3.7. Catalysis of ATP synthase

ATP synthase functions through a rotary mechanism, originally proposed by Paul Boyer and intensively studied since (Gresser et al. 1982; Boyer 1997; Stock et al. 2000; Nakamoto et al. 2008). The subunits of the Atp9 ring can bind and translocate protons from the IMS to the matrix through a unidirectional rotation of the ring, driven by electrostatic forces. As the Atp9 ring and the catalytic $\alpha_3\beta_3$ head are connected through the central stalk, the rotation of the ring translates to the rotation of the γ subunit and drives conformational changes in the catalytic sector. The ATP binding sites of the β subunits cycle between three states during catalysis. In the "open state" ADP and Pi can bind to the active site, after which the proton translocation changes the conformation to the "loose state" and subsequently to the "tight state", during

which the ATP synthesis occurs. Another switch to the “open state” releases the formed ATP and can binds new molecules of ADP and Pi to perform a new round of synthesis. Approximately four protons are considered to be necessary to synthesize one molecule of ATP (Cross and Müller, 2004; Ferguson, 2010). Under certain metabolic conditions, the enzyme can function in reverse mode and pump protons from the matrix to the IMS at the expenses of ATP hydrolysis.

3.3.8. ATP synthase in human pathology

Isolated ATP synthase deficiency is rare among the known defects of the OXPHOS complexes; nevertheless several mutations in both structural subunits and assembly factors of the human enzyme have been reported. Most disease-related mutations have been found in mitochondrial encoded *ATP6*, but cases were reported for *ATP12* and a nuclear *TMEM70*, coding for a possible assembly factor of ATP synthase in mammals (Houštěk et al., 2006; Kara et al., 2012; De Meirleir, 2004; Torraco et al., 2012). Clinical symptoms of the diseases involve defined syndromes such as NARP (Neuropathy, Ataxia and Retinitis Pigmentosa) and MILS (Maternally Inherited Leigh Syndrome). Clinical outcomes are heterogeneous, possibly due to environmental or tissue specific factors and variations of mtDNA.

3.4. Supercomplex organization

The electron transfer was initially thought to occur during the random collision of respiratory complexes during their diffusion in the membrane. With the development of the native electrophoresis technique (Schägger and von Jagow 1991) it was clearly demonstrated that the complexes of the respiratory chain associate in higher-order structures, termed the supercomplexes (Schägger and Pfeiffer, 2000). Evidence exists for the formation of the supercomplexes III_2-IV_{1-2} in yeast (Fig. I-25) and I_1-III_2-IV_{1-4} in mammalian mitochondria, but the regulation of their formation is still not completely understood. Two proteins recently discovered in *S. cerevisiae* bind the supercomplex III-IV and participate in its stability; these are Rcf1 and Rcf2 (Respiratory super Complex Factor 1 and 2) (Chen et al., 2012; Strogolova et al.,

2012; Vukotic et al., 2012). Rcf1 has at least two homologs in humans, HIGD1A and HIGD2A (belonging to the Hypoxia Inducible Gene family of proteins). Human HIGD2A can partially compensate for the growth defect of *Δrcf1* yeast, while its knockdown in mice was shown to decrease the formation of supercomplexes containing complex IV.

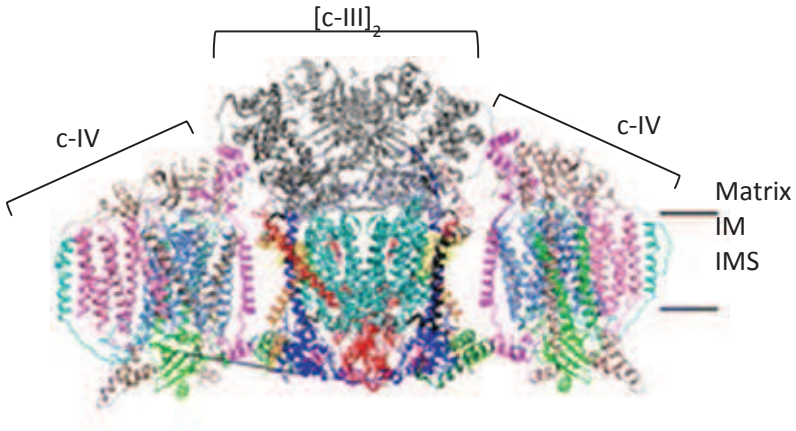


Figure I-25 Model of the III_2IV_2 supercomplex in *S. cerevisiae*.
Complex III (c-III): Cor1, dim gray; Cor2, dark gray; cytochrome b, light sea green; Rip1, red; cytochrome c_1, dark blue; Qcr6, green; Qcr7, light gray; Qcr8, black; Qcr9, orange; CL (CN3), yellow. Complex IV (c-IV): Cox1, cornflower blue; Cox2, bright green; Cox3, hot pink;Cox5, magenta; Cox13, cyan. All other subunits of Complex IV are in light brown. Horizontal lines in A indicate the position of phospholipid bilayer (Mileykovskaya et al., 2012).

Supercomplexes were proposed to stabilize the individual complexes, enhance the electron flow and regulate ROS production (Boekema and Braun, 2007; Dudkina et al., 2010). In the supercomplex III-IV of *S.cerevisiae* the sites of cytochrome *c* binding in the two complexes are brought in close contact, but the physiological relevance of this structure was questioned given the absence of a direct demonstration for substrate channeling (Trouillard et al. 2011).

In mammals, complexes I, III and IV assemble to form the so-called respirasome, which has been characterized as a respiratory active structure, capable of binding and presumably shuttling both ubiquinone and cytochrome *c* between the three complexes (Acín-Pérez et al., 2008; Althoff et al., 2011; Lapuente-Brun et al., 2013). Complex I was reported to act as a scaffold for the combined incorporation of complexes III and IV subunits, and its stability and activity depend on its correct assembly in the respirasome, and in particular with the complex III (Moreno-Lastres et al., 2012). However, a part of mammalian complexes III and IV also exist

in smaller supercomplexes or as individual complexes in the membrane, which raises further questions on the respirasome regulation and function.

3.5. Regulation of the OXPHOS system

3.5.1. Transcriptional regulation

In *S. cerevisiae*, it has been known for some time that the metabolic conditions influence the expression of mitochondrial proteins, both nuclear and mitochondria encoded. When yeast is using a fermentable carbon source, such as glucose, the OXPHOS system is not required for energy production, leading to the downregulation of transcription of many nuclear and all mitochondrial genes, a phenomenon known as the glucose repression (Carlson, 1999). Metabolic switch to fermentable carbon source rapidly de-represses the transcription of nuclear genes encoding mitochondrial proteins; the expression of mitochondrial genes follows and results in up to a twenty-fold increase in mtRNA abundance (Ulery et al., 1994). Mitochondrial RNA polymerase (Rpo41) was proposed to act as an *in vivo* sensor of intramitochondrial ATP concentration, and to accordingly regulate the transcription of mtDNA encoded genes (Amiott and Jaehning, 2006).

3.5.2. Channeling of mitochondrial mRNAs to the inner membrane

Stability and coordinated translation of mtRNAs may be considered as an additional point in regulation of the OXPHOS function. Mitochondrial genes are presumed to be transcribed near the inner membrane by Rpo41. The polymerase interacts physically with soluble matrix protein Nam1, which appears to chaperon mRNAs to the inner membrane surface, together with two membrane-bound proteins, Sls1 and Rmd9 (Bryan et al., 2002; Nouet et al., 2006; Rouillard et al., 1996; Wallis et al., 1994). At the inner membrane surface, the mRNAs can interact with their specific translational activators and the mitochondrial ribosomes (Fig. I-26). Nam1 was shown to interact with Pet309, a seven-pentatricopeptide

(PPR) domain- containing protein, capable of binding both the 5'-UTR of COX1 mRNA and the mitoribosome, as well as with Pet111 and Pet494, translational activators of COX2 and COX3 mRNA, respectively (Naithani et al., 2003; Tavares-Carreon et al., 2007). Physical interactions of specific translational activators of a given OXPHOS complex have already been described above; an additional layer of regulation is to be added to the picture as translational activators of different complexes have been found to physically interact, as in the case of Pet309 and Cbp1, activators of Cox1 and Cytb, respectively (Krause et al., 2004).

Mitochondrial ribosomes are tethered to the inner membrane via three proteins: Oxa1, Mba1 and Mdm38. Mdm38 is a bifunctional protein (it has role in mitochondrial K^+/H^+ exchange) which seems to have overlapping functions with Mba1, thought to bind the ribosome and align the ribosomal exit tunnel with Oxa1, which co-translationally inserts newly synthesized proteins into the inner membrane (Gruschke et al., 2010; Lupo et al., 2011; Ott et al., 2006; Szyrach et al., 2003).

3.5.3. Regulation of the respiratory chain through complex IV subunit switch

Under physiologic conditions, complex IV is considered to be rate limiting for the functioning of the respiratory chain and the central point for its regulation. In *S. cerevisiae*, two isoforms of Cox5, Cox5a and Cox5b, are expressed under high and low oxygen conditions, respectively (Burke et al., 1997). The two isoforms display 66% identity of sequence and differentially affect the activity of complex IV; enzyme containing Cox5b has higher turnover rates and higher rates of heme *a* oxidation (Waterland et al., 1991).

In vertebrates, several complex IV subunits have different isoforms, both inducible and expressed in tissue-specific manner (Yanamura et al. 1988; Hüttemann et al. 2001). As different isoforms change catalytic features of the complex, their ubiquitous tissue distribution usually reflects the oxidative capacity and workload of a given tissue (Vijayasarathy et al., 1998). The environment-induced subunit switch in the enzyme is one of the mechanisms that allow adaptation to changing metabolic conditions.

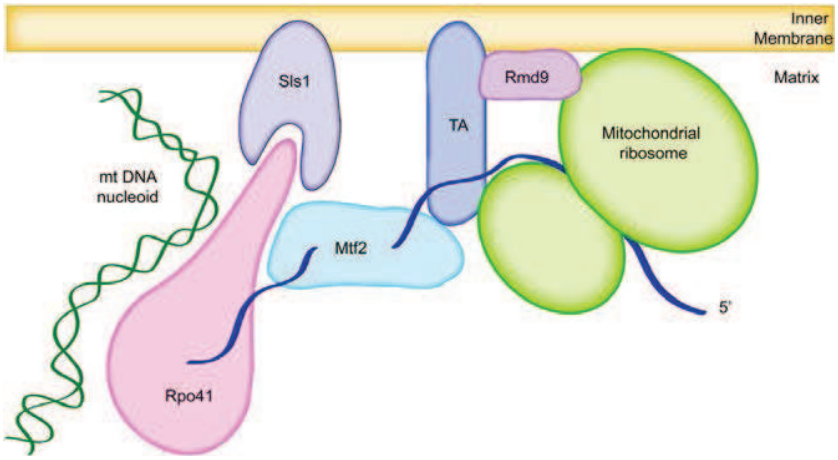


Figure I-26 Channeling of mitochondrialy-encoded mRNAs to the inner membrane.
mtRNAs are channelled from RNA polymerase (Rpo41) to membrane-bound ribosomes by Sls1, Mtf2, Rmd9, and mRNA-specific translational activators (TA). (The figure is not intended to suggest that mRNAs are translated while still emerging from RNA polymerase (Fox, 2012).

3.5.4. Phosphorylation and ATP/ADP ratio regulate the activity of OXPHOS complexes

Subunits of the OXPHOS system can be modified by phosphorylation which can change the properties of the resulting complexes. Although it is not precisely known which kinases are involved in the phosphorylation, numerous subunits of the bovine complex IV have been shown to be phosphorylated, including subunits I, II, IV, Va, Vb and VIa. In *S. cerevisiae*, the phosphorylation of the non-catalytic subunit of the complex III, Cor2, and the assembly factors Cbp6 and Bcs1 was reported, but with no detailed functional reports (Chi et al., 2007). Subunits Cox4 and Cox12 of complex IV, as well as seven subunits of the ATP synthase (Atp1, Atp2, Atp4, OSCP, Atp15, Atp16, Atp21) were also found to be phosphorylated, with different impact on both activity and assembly of the complexes (Kane et al., 2009; Reinders et al., 2007; Helling et al., 2012).

It has been shown in both yeast and mammals that ATP and ADP can bind to the complex IV, to the subunits Cox5 (IV in mammals) and Cox13 (VIa in mammals) (Beauvoit and Rigoulet, 2001; Napiwotzki and Kadenbach, 1998). Multiple binding sites, on both matrix and IMS side of the membrane have been reported for the two systems. High ADP content increases the activity of the enzyme, while high ATP results in its allosteric inhibition. However,

the inhibition of complex IV by ATP can be reversed, and one way of doing so is through the IVi1 to IVi2 (Cox5a-Cox5b) subunit switch, the latter not being subjected to the inhibition by ATP (Horvat et al. 2006).

ATP synthase is regulated by its substrate nucleotides; conformational rearrangements of Atp15, that responds to the increase of the proton-motive force and ADP concentration, promote the synthesis mode of action of the enzyme (Suzuki et al., 2003). When membrane potential is low, the affinity of the enzyme for phosphate decreases and prevention of excessive ATP hydrolysis is achieved through binding of Mg-ADP to the tight catalytic site, a phenomenon called the ADP inhibition (Drobinskaya et al., 1985). Another way of inhibiting ATP hydrolysis is through the ATP-dependent binding of the endogenous Inhibition Factor (IF1) to the F_1 sector (Pullman and Monroy, 1963), which masks the nucleotide sites in the F_1-IF1 complex. Upon membrane energization, the ATP synthase affinity for Pi dramatically increases, and this is considered to be necessary to prevent the competitive inhibition of ATP synthesis by high concentration of ATP (for review Weber and Senior, 2000, 2003).

Regulation of the respiratory chain function is a major theme of my PhD project. The assembly of respiratory complex III was taken as the starting point of the study, but the results obtained emphasize the importance of the interplay between the OXPHOS complexes and the role of the ATP/ADP ratio in the regulation of their functioning.

Ic – Genetic suppressor approach

Depending on their type, position and resulting phenotype, gene mutations can provide some information on the structure and function of the encoded protein product. However, uncovering the intricate interaction network of the protein requires going further in the examination of the altered phenotype. A powerful approach to investigate gene expression, function and interaction is the isolation and analysis of genetic suppressors (Forsburg, 2001; Prelich, 1999), extensively used with *S. cerevisiae*, due to ample genetic methods available.

These suppressors can be divided in three classes: intragenic, extragenic and high-copy number suppressors.

The first step in this type of genetic analysis is the isolation of "revertants" from the studied mutant – strains that have acquired an additional mutation which changes the original mutant phenotype by rendering it less severe or completely wild type-like. Secondary genetic analysis allows identifying the nature of suppressor mutations.

In the simplest scenario, the "true reversion", the mutant carries a single base change difference from the wild type and the intragenic suppressor mutation simply changes the sequence back to the wild type. Another type of intragenic suppression for non-functional proteins is the introduction of a second mutation elsewhere in the protein, which is able to correct the original defect. Local structural changes affecting the function of a protein may be compensated for in this way.

Extragenic suppressor is a mutation in a different gene from the one initially studied. This type of suppression can occur through a multitude of different pathways including modulators of activity, protein interaction, bypass, removal of a toxic protein product, dose effect and a number of so-called informational mechanisms (those altering the genetic apparatus through which genes are expressed), such as alterations in mRNA expression/stability, protein translation and turnover.

High-copy-number suppressors are genes that, when overexpressed, bypass the defect caused by the mutation in the initial gene. Screening for this type of suppressors requires transforming the mutant strain with a high-copy *S. cerevisiae* genomic library and searching for a wild type-like phenotype among transformants. Plasmids bore by the transformants of interest need to be tested to see if they contain a different gene from the one initially mutated, which, if so, could be the high-copy suppressor.

Different modifications (gain-of-function, loss-of-function, null, insertions, missense, nonsense mutations) will not usually be compensated for through the same pathways, hence the nature of the original mutant is crucial in determining the nature of the suppressor mutation. A missense mutation that alters the conformation of a protein may be suppressed by a mutation in the interacting protein, while nonsense mutations are frequently suppressed by

the informational suppressors. To compensate for a deletion mutant, another protein or pathway have to be modulated to bypass the need for the initial gene product that is no longer available.

Being familiar with the original mutant is therefore essential for setting up the suppressor hunt, but even with this kind of direction the information obtainable through this approach can be quite overwhelming; multiple suppression mechanisms can exist for a given mutation and the identification of a suppressor mutation is often just a tip of the iceberg when it comes to understanding the underlying biology of the interaction. However, the study of a gene identified as a suppressor can help to shed light on both the suppressor and the gene containing the initial mutation.

The analysis of genetic suppressors is hard work and can be somewhat compared to a leap in the dark, still the information obtained can have numerous projections and its further examination is extremely exciting and intellectually-stimulating.

This approach is in the foundation of my PhD project and it was crucial in providing the "raw material" for the research that has occupied me during the last three years. The gene of interest is *BCS1*, which codes for the late assembly factor of mitochondrial respiratory complex III, conserved from yeast to humans. Bcs1 is an ATPase, member of the conserved superfamily of AAA proteins (ATPases Associated with different cellular Activities) which comprises a number of mitochondrial proteins (Truscott et al., 2010). Structure–function analysis of the yeast Bcs1 by randomly generating a collection of respiratory-deficient point mutants was previously undertaken in the laboratory (Nouet et al., 2009). Three point mutations were selected for further characterization, based on their respiratory growth and position in the AAA domain, which was outside the well-studied canonical functional motifs of AAA proteins. Two mutations, *bcs1*-C289Y and F432I, were close to the nucleotide binding site, while *bcs1*-F342C was predicted to be located near the central pore formed in the putative Bcs1 hexamer (Fig. II-1A). Genetic suppressor hunt was performed for the three point mutants and the *bcs1* null mutant (Nouet et al., 2009). Complete loss of Bcs1 could not be bypassed while only intragenic suppressors were isolated for *bcs1*-C289Y and F432I. Several suppressors displayed mutations of the residue P226, located near the nucleotide entrance, suggesting that the nucleotide

binding may be impaired in *bcs1*-C289Y and F432I and could be overcome by a conformational change in this area. In the case of *bcs1*-F342C, mostly extragenic suppressors were isolated, which could suggest that the F342 residue was important for interactions with partner or substrate proteins (Fig. I-27B).

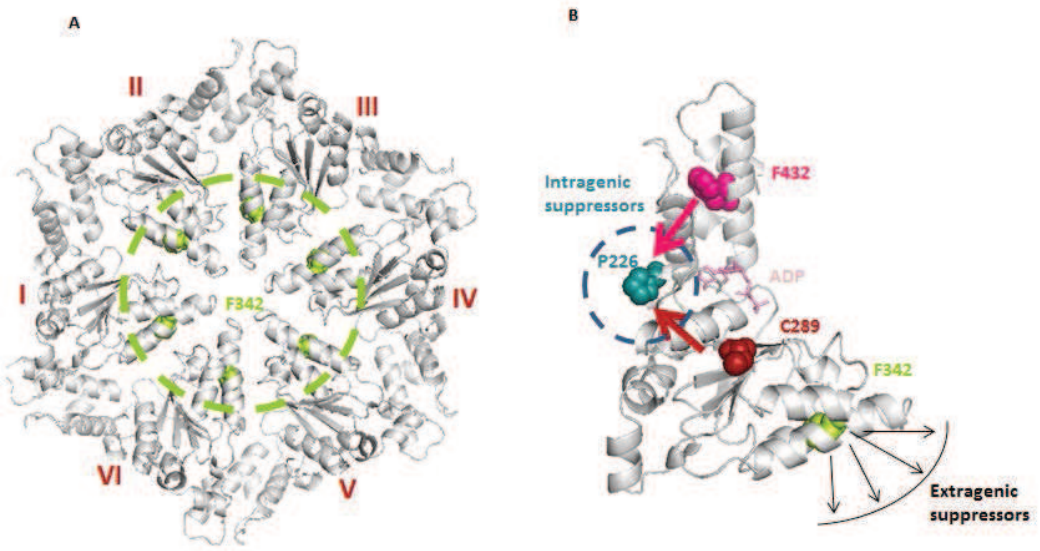


Figure I-27 Predictive structure of the AAA domain of *S.cerevisiae* Bcs1.
A) Residue F342 (green spheres) is predicted to be located near the central pore formed in Bcs1 homohexamer.
B) Residues P226, C289, F342, F3432 and ADP are shown on Bcs1 monomer

Identification of suppressors of *bcs1*-F342C and elucidation of the suppression mechanism were the first tasks in my PhD work. Finally, the identified suppressor mutants could be sorted in different groups, operating through different suppression mechanisms, one of which is uncovered in the article from part III. Suppressors from the remaining groups will be commented in the discussion that follows the article and the characterization of one of them will be presented separately in part IV.

Partners or substrates of Bcs1 were not found, but rather proteins able to indirectly modulate its activity. Although starting from the same point mutation the suppressors were diverse and pointed to the connection of different aspects of mitochondrial respiratory function, going from the assembly of the complexes of the respiratory chain, through the regulation of their activity to the curious features of the mitochondrial protein synthesis. This is what the initial leap in the dark is all about.

Results and Discussion

II – BCS1

1. Article (accepted for publication in Cell Metabolism)

The energetic state of mitochondria modulates complex III biogenesis through the ATP-dependent activity of Bcs1

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Running tittle: ATP-dependency of Bcs1 and complex III biogenesis.

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.

Highlights:

. Specific mutations in ATP synthase subunits compensate for Bcs1 deficiencies.

. The compensatory mutations decrease the hydrolytic activity of the ATP synthase.

. Increasing the ATP concentration compensates for the Bcs1 deficiency.

. ATP synthase mutations rescue a human disease-related mutation modeled in yeast.

Keywords: *Saccharomyces cerevisiae*; mitochondrial respiratory complex III; ATP synthase; AAA protein Bcs1; mitochondrial diseases; intramitochondrial adenine nucleotide pool

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 to IV) transfer electrons to the final acceptor, oxygen. This transfer is coupled to proton translocation across the inner membrane, 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 (Fig. 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 and 2000; Conte et al., 2010). 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, anchored to the inner membrane and protruding into the matrix. An internal signal within the N-terminal domain targets Bcs1 to mitochondria (Folsch et al., 1996). Bcs1 contains a Bcs1-specific domain and a highly conserved AAA-region typical of the AAA-protein family (ATPase Associated with diverses cellular Activities; Fig. 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, Hanson and Whiteheard, 2005). In mitochondria, the AAA proteins play a central role in the biogenesis and quality control of proteins (Gerdes et al., 2011).

Mutations in the human gene *BCS1L* (*BCS1*-like) are the most frequent nuclear mutations resulting in complex III-related 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; Visapaa et al., 2002; De Meirleir et al., 2003; Hinson et al., 2007; Fernandez Vizarra et al., 2007; Moran et al., 2010; Kotarsky et al., 2010; Leveen et al., 2010). 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; Fig. S1). According to the theoretical 3D-model of the yeast Bcs1 protein (Fig. 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 (Fig. 1D) and it did not affect the steady-state level and oligomerization of Bcs1 (Fig. 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 super complexes III/IV and on ATP synthase

oligomers. Under the 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 (Fig. 1E, 1F). High molecular weight complexes revealed with the Cyt1 and Cox2 antibodies were detected in the mutant *bcs1*-F342C. Two dimension 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% vs. wt, Fig. 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 (see for review, 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 giving some subcomplexes that co-migrate with pre-III. Finally, the complexes around 440 kDa that are detected with anti-Cox2 only 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 co-immunoprecipitated in the wt and the *bcs1*-F342C mutant, whereas Rip1 was only detected in the wt immunoprecipitate (Fig. 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 (Fig.1E). This enzyme was detected mainly as monomers (V) and dimers (V_2) both in wt and the mutants. The faint band between V and V_2 (denoted with a #) also reacted with antibodies against several other ATP synthase subunits (Fig. S2 and data not shown) 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 (Fig. 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 *Δbcs1* 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 (Fig. 1E, S2). Due to its low amount this complex could no longer be detected in a 2D experiment (Fig. 1F). Thus the *bcs1*-F342C mutation only seems to have a slight effect on the integrity of ATP synthase.

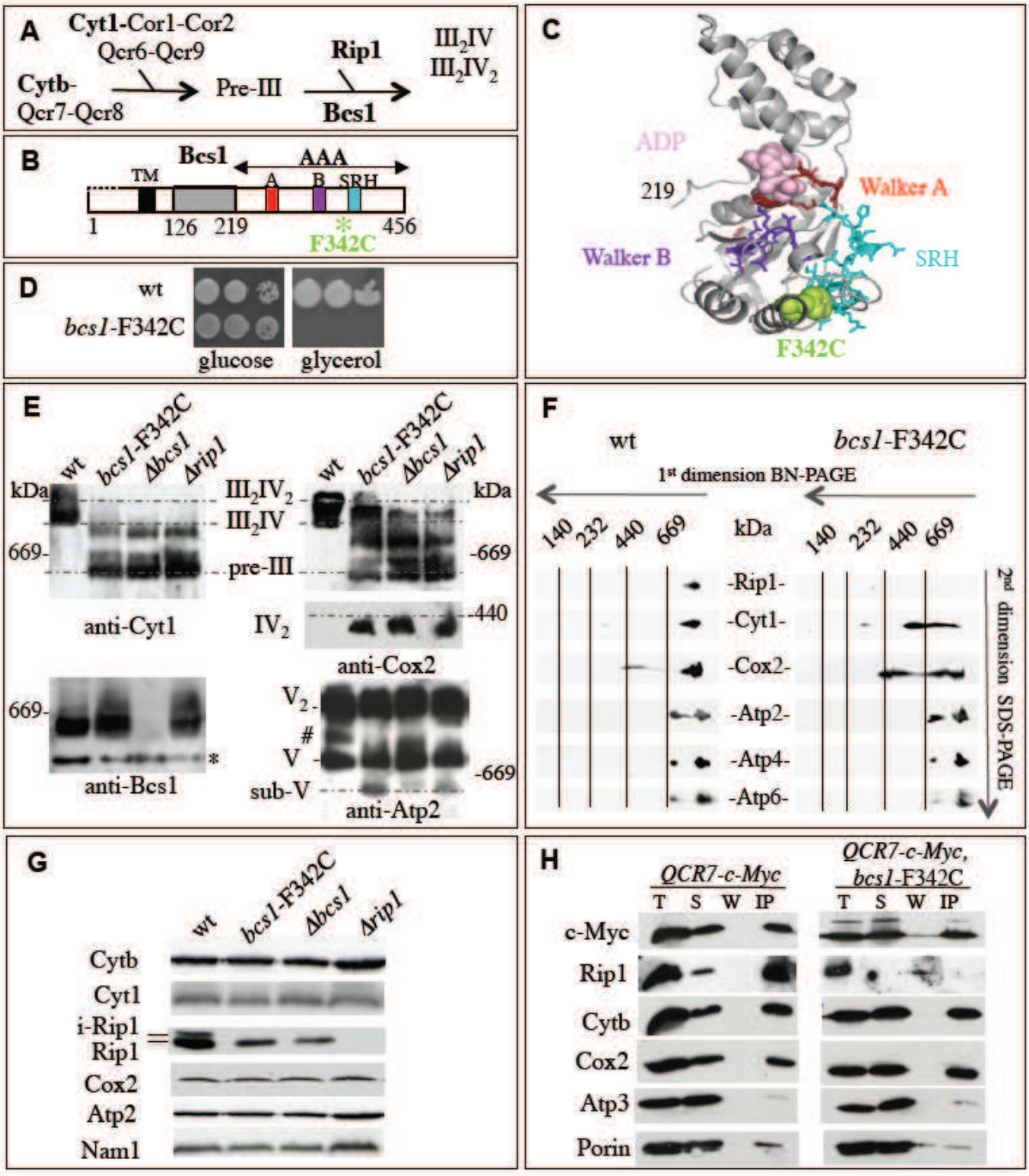


Figure 1: The *bcs1*-F342C mutation located in the AAA domain of Bcs1 affects the assembly of complex III and of supercomplexes III/IV.

(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 (grey) as well as the AAA domain with the positions of Walker A (red), B (purple), SRH (blue) motifs and of the amino acid F342 (green,*) are indicated. **(C)** Theoretical structural model of the AAA domain of the yeast Bcs1 (amino acids 219 to 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 four 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 of the bands present in low amount (#) and (sub-V) are indicated. *: aspecific band revealed by the anti-Bcs1 antibody. Positions of the protein molecular mass markers (669 and 440 kDa) are indicated. **(F)** Mitochondrial complexes from wt and *bcs1*-F342C were analyzed by a 1st dimension BN-PAGE followed by a 2nd 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 co-immunoprecipitation 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

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 (Fig. 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 (Fig. 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 (Fig. 2C) and ubiquinol-cytochrome *c* oxidoreductase activity measurements (Fig. 2D). As a result, the oxygen

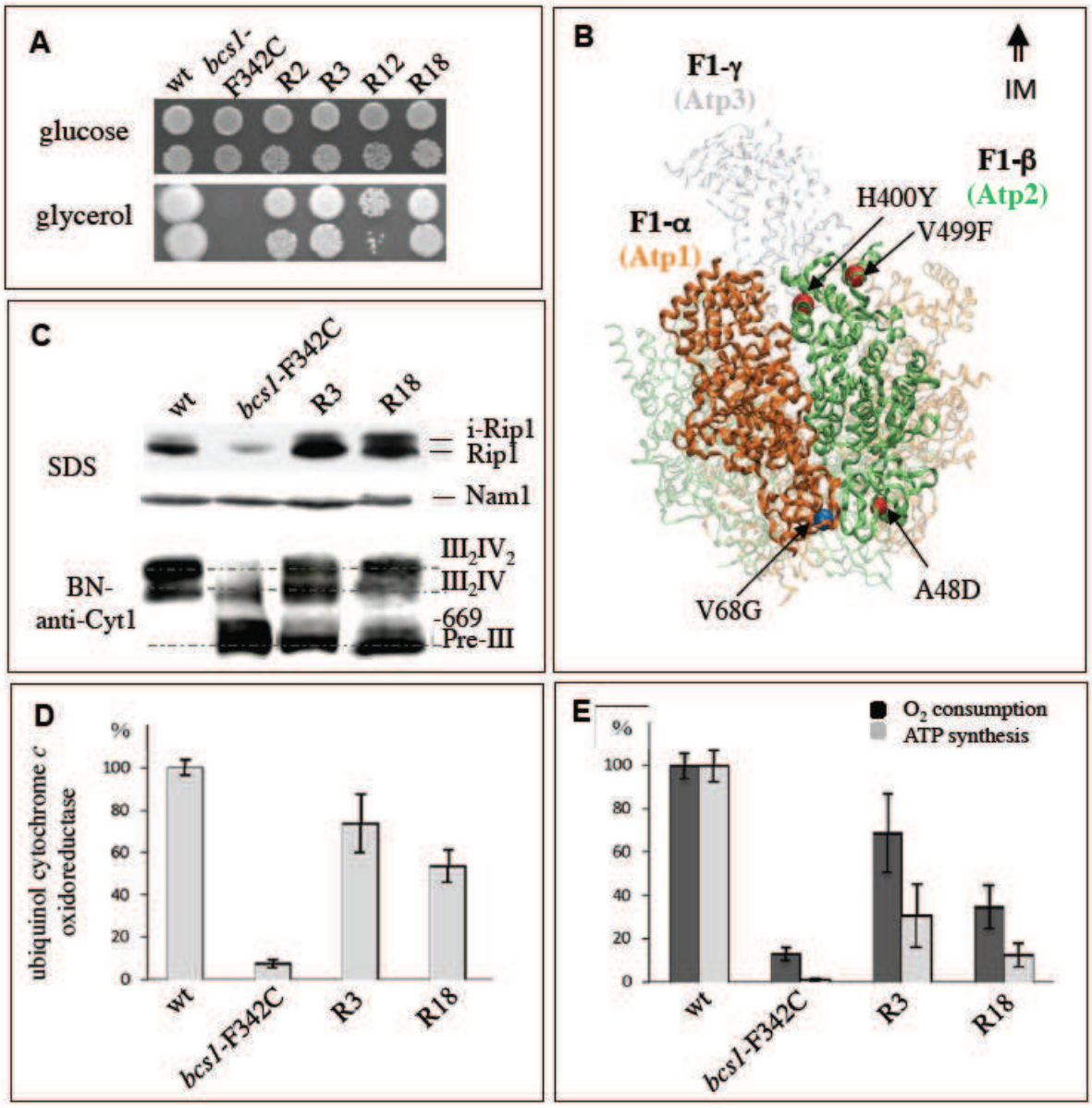


Figure 2: The *bcs1*-F342C mutation is compensated for by mutations in F_1 subunits of the mitochondrial ATP synthase.

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, R18, were grown for three days at 28°C. (**B)** The α-F_1, β-F_1 and γ□subunits(Atp1 in orange, Atp2 in green, Atp3 in grey) 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- and BN-PAGE and immunoblotted with antibodies against Rip1 and Nam1 (SDS) or Cyt1 (BN) (See legend of Fig. 1F). (**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 (1800 nmol of reduced cytochrome *c*/min/mg of proteins). Data represent the mean of three independent experiments and error bars are standard deviation. (**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 Experimental Procedures and Table S2). Both the O_2 consumption and the ATP synthesis are represented as a percentage of the wild-type measurements.

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 (Fig. 2E and Table S2A). 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, 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 (Fig. 3A and Table S2B). 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 (Fig. 3B). However, despite the reduced content in F_1, the steady levels of the Atp1 and Atp2 were essentially unaffected (Fig. 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

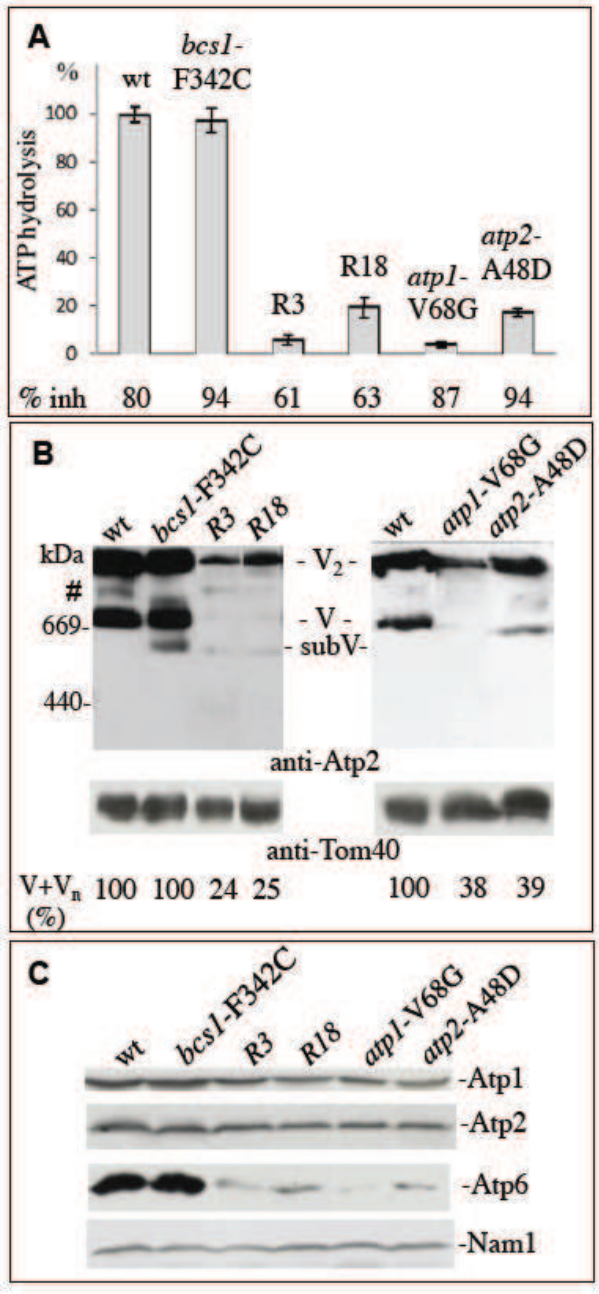


Figure 3. The *atp1*-V68G and *atp2*-A48D mutations affect the assembly of ATP synthase and lead to a strong decrease in the hydrolytic activity of F_1.
Mitochondria were purified from wt, *bcs1*-F342C, R3 (*bcs1*-F342C *atp1*-V68G), R18 (*bcs1*-F342C *atp2*-A48D), *atp1*-V68G and *atp2*-A48D. **(A)** Three independent ATP hydrolysis assays were performed on frozen/thawed mitochondria in absence of osmotic protection and in presence of saturating amounts of ATP. Specific enzyme activities are represented as a percentage of the wild-type activity (2135 nmPi/min/mg of protein). The percentage of inhibition of ATP hydrolysis by oligomycin is indicated. See Table S2B for complete data. **(B)** BN-PAGE were immunoblotted with antibodies against Atp2 and Tom40, a protein of the mitochondrial outer membrane as loading control. (**C)** SDS-PAGE were immunoblotted with antibodies against Atp1, Atp2, Atp6 and the loading control Nam1.

strongly enriched in Atp1 and Atp2 proteins were indeed observed on electronic micrographs of *atp1*-V68G and *atp2*-A48D cells (Fig. 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 (Fig. 3C).

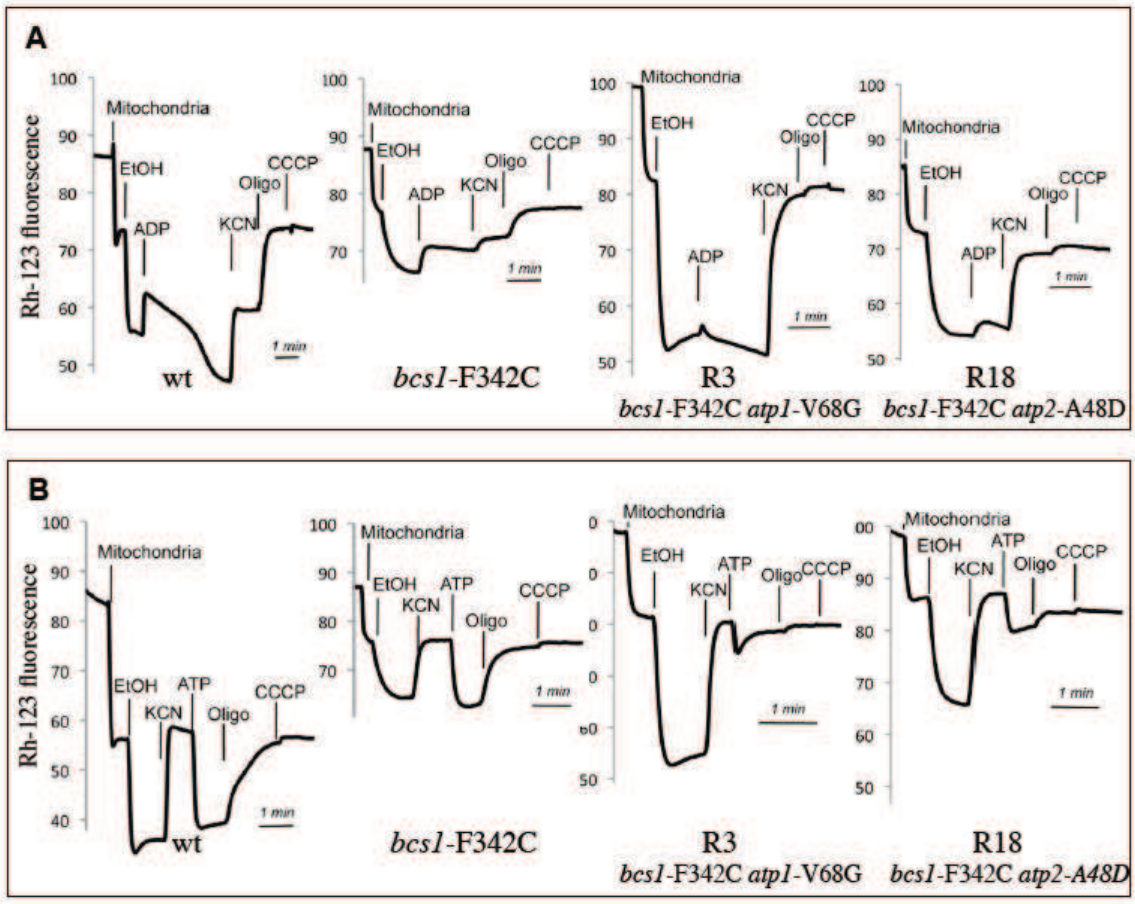


Figure 4. The *atp1*-V68G and *atp2*-A48D mutations lower the potential of the mitochondrial inner membrane by ATP.

Membrane potential analyses were performed on fresh osmotically-protected mitochondria from wt, *bcs1*-F342C, R3 and R18 using Rhodamine 123 (Rh-123) whose fluorescence decay is proportional to the mitochondrial membrane potential. 50 µM ADP (**A)** or 1mM ATP **(B)** were 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, 3 µM CCCP. KCN, an inhibitor of complex IV, was used to collapse the membrane potential induced by the respiratory chain, oligomycin to block the ATP synthase and CCCP to dissipate the proton gradient across the inner membrane. Experiments have been repeated three times.

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 (Fig 4A), we tested the effect of the addition of a small amount of ADP, which induces a $\Delta\psi$ 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 (Fig. 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 *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, Retinitis Pigmentosa) or MILS (Maternally Inherited Leigh Syndrome) (for review, 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; Fig. 5A). The *atp6*-L183P and *atp6*-L247P partially compromise the assembly/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 (Fig. 5B). In the *atp6*-

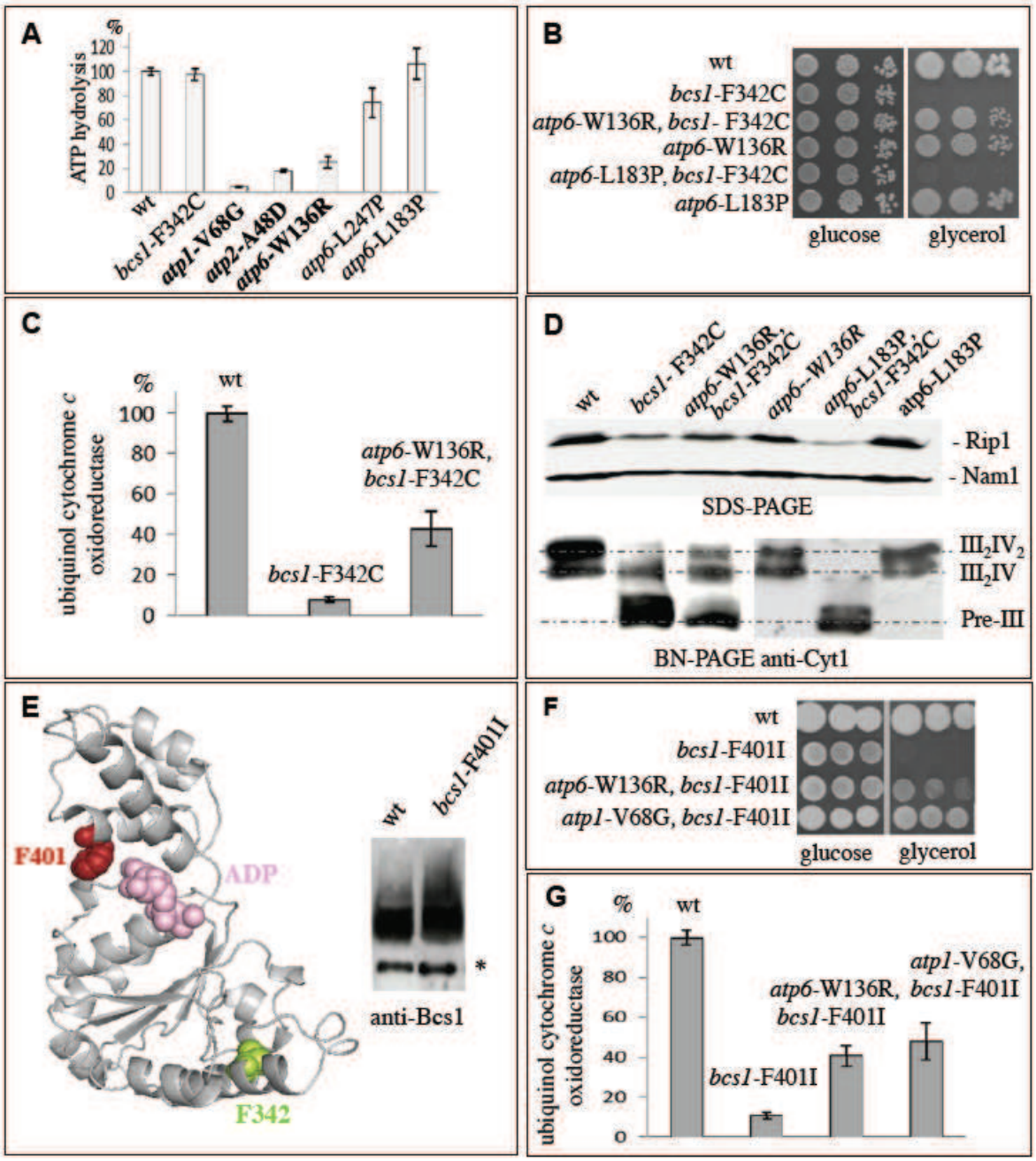


Figure 5: Mutations in subunits of F_1 and F_O rescue *bcs1* mutations one of which is a human disease-related mutation modeled in yeast.

(A) Comparison of ATP hydrolysis of the *atp1*-V68G, *atp2*-V48D and of the three *atp6* mutants. See legend of Fig. 3A and of Table S2B. Mutants that compensate for *bcs1-F342C* are in bold. **(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 three days at 28°C. **(C)** Complex III activity, see Fig. 2D. **(D)** SDS- and BN-PAGE analysis of mitochondrial proteins as in Fig. 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 Fig 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 four days at 28°C. **(G)** Complex III activity, see Fig. 2D.

W136R *bcs1*-F342C double mutant, complex III activity and the insertion of this complex into supercomplexes were partially recovered while they were not in the *atp6*-L183P *bcs1*-F342C (Fig. 5C, 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 (Fig S1). According to theoretical 3D-models of the human and yeast Bcs1 proteins, this mutation is located near the ATP binding site of Bcs1 (Fig 5E). The *bcs1*-F401I mutation led to a stringent respiratory growth deficiency (Fig. 5F) and very severely compromised the activity of complex III (Fig. 5G). As with the *bcs1*-F342C mutation, the steady state levels and oligomerization of Bcs1 were not affected in the *bcs1*-F401I mutant (Fig. 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 (Fig. 5F) and showed an improved complex III activity (Fig. 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 hexa-histidine tag fused to their C-terminus (Fig. S4 and data not shown). The tag had no influence on the chaperon activity of Bcs1, and both proteins kept their capacity to form oligomers both *in vivo* and after purification from mitochondrial digitonin-extracts (Fig. 6A and data not shown). 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 two- to three-fold lower than that of the wt protein (Fig. 6B). However, at higher ATP concentrations, 10 or 20 mM, no significant difference was observed between the mutant and the wt (Fig. 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 and 2000; Conte et al., 2010; 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, which 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 maintain the integrity of respiratory chain supercomplexes. Combined defects of OXPHOS complexes were also reported in *BCS1L* deficient patients (Fernandez-Vizarra et al., 2007; Moran et al., 2010).

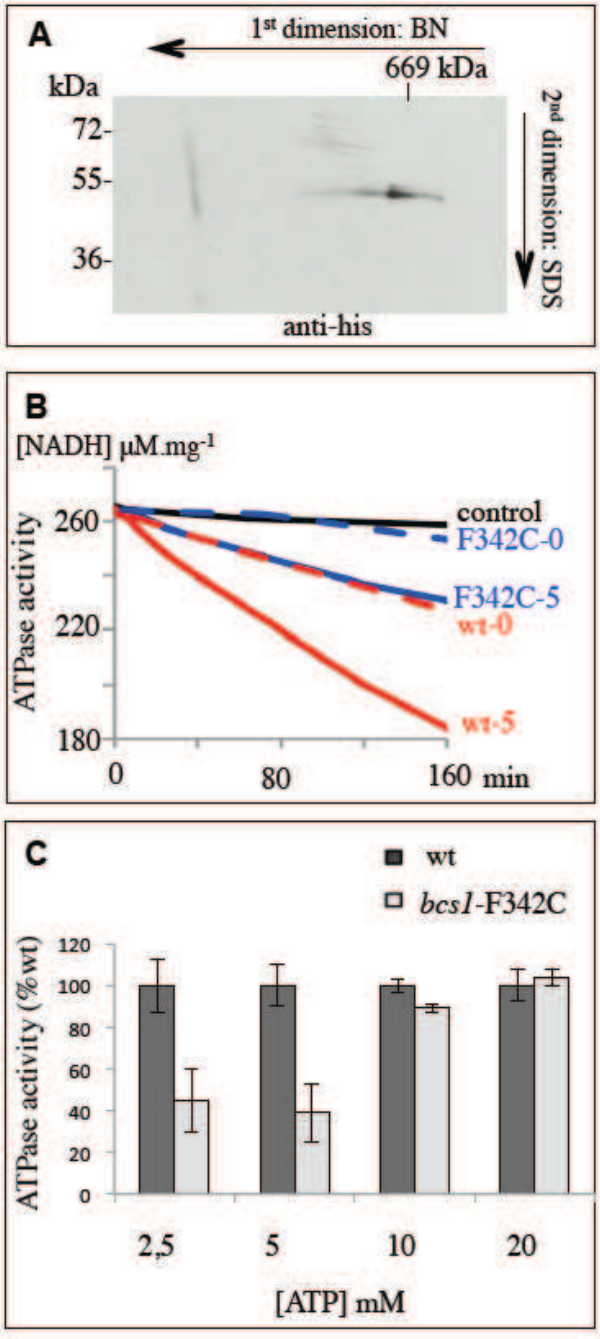


Figure 6: *In vitro* ATPase activity of purified wt and mutated Bcs1.
(A) Ni-NTA purified Bcs1 was analyzed by 2D BN-PAGE/SDS-PAGE and immunoblotted with anti-His antibody. Positions of size markers are indicated. **(B)** The *in vitro* ATPase activity of Ni-NTA partially purified wt and mutated Bcs1 proteins were 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 to 20mM) are represented as mean values of three independent experiments with standard deviations as error bars.

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, which 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/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 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, 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 Whiteheard, 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 (Fig. S5). 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 2.5-5mM, 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 re-establishment 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 Fig. 7). When yeast cells rely on oxidative phosphorylation the level of ATP inside mitochondria is high and exchanged against

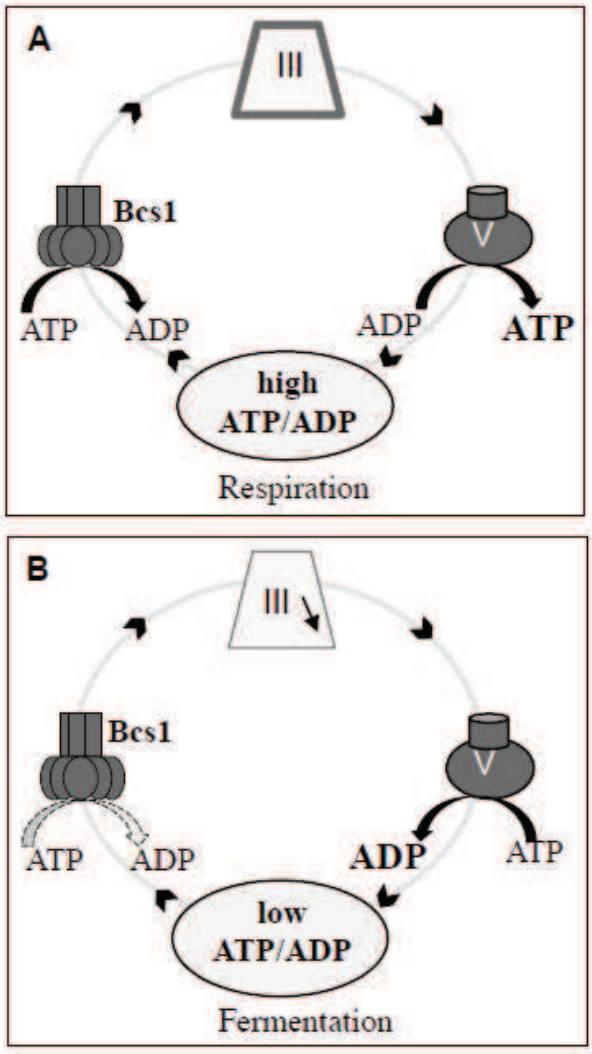


Figure 7. Schema for the modulation of complex III biogenesis through the ATP dependent activity of Bcs1.
The ATP/ADP ratio is known to be much lower in fermentative (glucose) than in respiratory (ethanol) conditions (Beauvoit *et al.,* 1993), see discussion.

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 and the glycolytic ATP is imported into mitochondria by the ADP/ATP translocator and there is no 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. 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 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 would be to our knowledge the first example where ATP influences 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 be also fruitful in the case of Bcs1-based disorders.

Supplementary figures and tables



Figure S1. Multiple alignment of the AAA domain of Bcs1 proteins
The positions of the conserved motifs of the AAA proteins (Walker A, Walker B, SRH) and of the two amino acids mutated in the *bcs1*-F342C (green) and *bcs1*-F401I (orange) are indicated. M.m: *Mus musculus*; R.n: *Rattus norvegicus*; H.s: *Homo sapiens*; P.t: *Pan troglodytes*, B.t: *Bos taurus;* X. l: *Xenopus laevis;* S.c: *Saccharomyces cerevisiae*

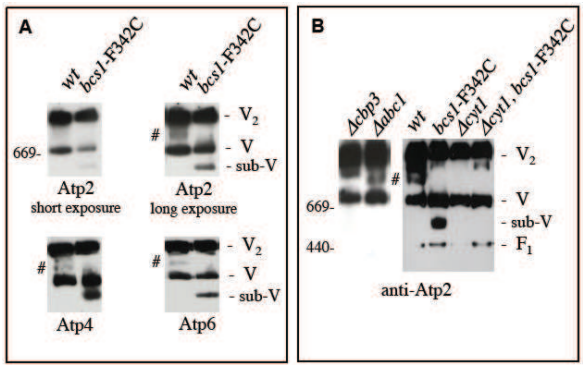


Figure S2. A sub-V complex is present at a low level in the *bcs1*-F342C mutant
Mitochondrial complexes from wt and *bcs1*-F342C (Panel A) as well as from wt, *bcs1*-F342C, three other single mutants affecting complex III: *Δcbp3, Δabc1, Δcyt1* and the double mutant *Δcyt1 bcs1*-F342C (Panel B) were analyzed by BN-PAGE and immunoblotted with antibodies against Atp2, Atp4 or Atp6. Protein molecular mass marker (669 and 440 kDa) as well as the positions of monomers (V), dimers (V_2) of ATP synthase and of the bands present at a low level # and sub-V, are indicated. Two exposures of the same immunoblot with anti-Atp2 are

presented in Panel A: sub-V and # are only visible after the long exposure. Cbp3 is essential for the insertion of Cytb while the absence of Abc1 (Coq8) leads to an inactive complex III.

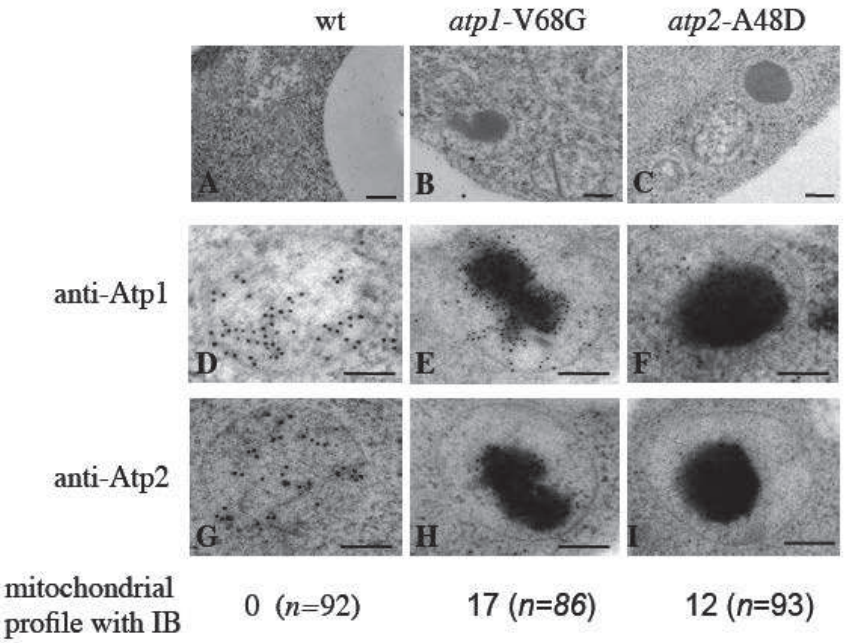


Figure S3. Ultrastructural and immuno-cytochemical analyses.
(A-C) Ultrastructure micrographs of araldite 80 nm-thick sections of cells from wt **(A**), *atp1*-V68G (**B**) and *atp2*-A48D (**C)**. Bars, 200 nm. **(D–I**) LR Gold labeling of 80 nm-thick sections of cells with primary antibodies anti-Atp1 (**D-F**) or anti-Atp2 (**G-I**) and 10 nm gold-conjugated secondary antibodies. Bars, 100 nm. The number of mitochondrial profiles with inclusion bodies (IB) are indicated with the total number of images analyzed (n).

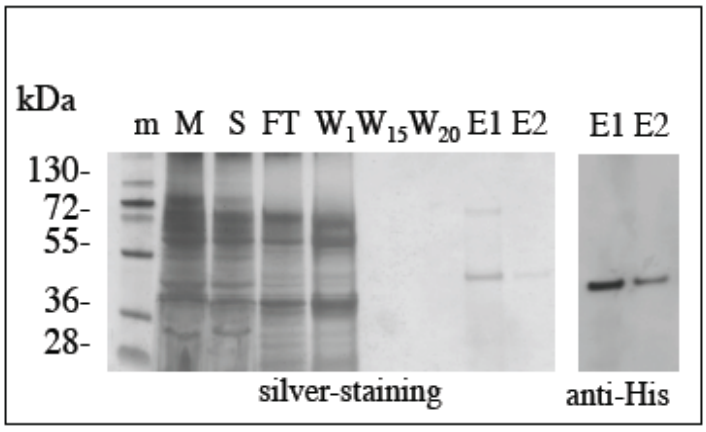


Figure S4. Purification of Bcs1-His by Ni-NTA chromatography (See Experimental Procedures)
SDS-PAGE analysis of the fractions obtained after the Ni-NTA chromatography of the hexahistidine tagged variants of Bcs1: the proteins were revealed by silver staining (left); Bcs1 was detected by immunoblotting with anti-His antibodies (right). Positions of size markers (m) are indicated. M: mitochondria; S: soluble fraction applied to Ni-NTA beads; FT: flow-through; W: washing; E: eluate.

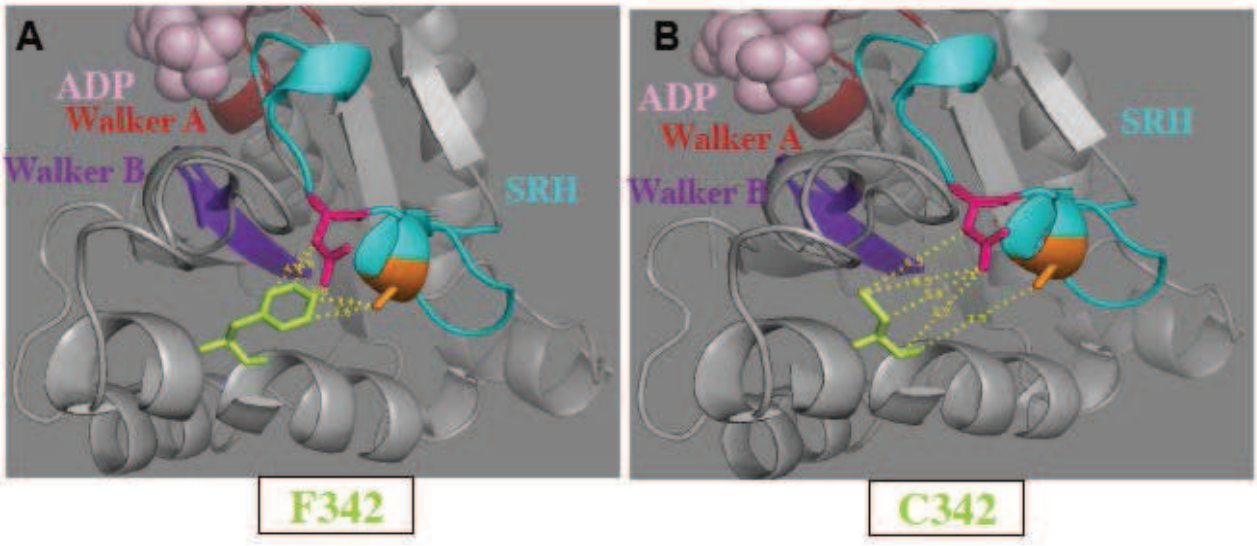


Figure S5. Proximity of residue 342 and motif SRH on the theoretical 3D-model of the AAA domain of Bcs1. The residues 342 (F, panel **A** or C, panel **B**) are depicted in green. Two of the conserved residues of the SRH motif, D371 and A373, are depicted in fuschia and orange, respectively. Distances between the residues are indicated in yellow: F342-D371 = 4,4-4,6 Å; F342-A373 = 3,4-3,6 Å ; C342-D371 = 6,1-6,3 Å ; C342-A373 = 7,2-7,9 Å. The figure was generated with the Pymol v1.3 software (http://pymol.sourceforge.net).

Strains	Nuclear genotype	Mitochondrial genotype	References
Δbcs1	*bcs1 ::URA3*	rho+	Nouet *et al*., 2009
F342C	*bcs1*-F342C	rho+	Nouet *et al*., 2009
F401I	*bcs1*-F401I	rho+	This work
R2	*bcs1*-F342C *atp2*-H400Y	rho+	This work
R3	*bcs1*-F342C *atp1*-V68G	rho+	This work
R12	*bcs1*-F342C *atp2*-V499F	rho+	This work
R18	*bcs1*-F342C *atp2*-A48D	rho+	This work
JB18-2A	*bcs1*-F401I *atp1*-V68G	rho+	This work
CP74	*atp2*-H400Y	rho+	This work
CP90	*atp1*-V68G	rho+	This work
CP72	*atp2*-V499F	rho+	This work
BR9	*atp2*-A48D	rho+	This work

JB8	*atp1*-V68G *atp2*-A48D	rho+	This work
Δcyt1	*cyt1 ::LEU2*	rho+	Hamel *et al*., 1998
Δrip1	*rip1 ::LEU2*	rho+	This work
Δqcr9	*qcr9 ::URA3*	rho+	Saint-Georges *et al*., 2001
Δabc1	*abc1 ::URA3*	rho+	Bousquet *et al.,* 1991
Δcbp3	*cbp3 ::G418*	rho+	Euroscarf collection
QCR7-cmyc	*QCR7-cmyc HIS3*	rho+	This work
DM2	*QCR7-cmyc HIS3 bcs1*-F342C	rho+	This work
F401I/60	*bcs1*-F401I	*rho°*	This work
F342C/60	*bcs1*-F342C	*rho°*	This work
BCS1-6HIS	*BCS1*-6HIS	rho+	This work
F342C-6HIS	*bcs1*-F342C-6HIS	rho+	This work
CJO8*	*bcs1*-F342C	*atp6*-W136R	This work
CJO20*	*bcs1*-F401I	*atp6*-W136R	This work
CJO9*	*bcs1*-F342C	*atp6*-L247R	This work
CJO10*	*bcs1*-F342C	*atp6*-L183P	This work
JC/RKY39*		*atp6*-W136R	This work
JC/RKY38*		*atp6*-L247R	This work
JC/RKY38*		*atp6*-L247R	This work

Table S1. List of strains.
The strains are all isonuclear to the control wild type strain CW252 *MAT*α, *ade2, ura3, his3, trp1, leu2, can1-100* (Saint-Georges *et al*., 2001) except the three last JC strains which have the nuclear background of JC8 *MATα, leu1, kar1, can1-1*. The *kar1* mutation allows the introduction of mutated mitochondrial genomes into *rho°* strains by cytoduction. All the strains have the same intron-less mitochondrial genome except Bcs1-108/60 and Bcs1-F401/60 that have no mitochondrial genome (*rho°)* and the strains noted (*) that carry a mitochondrial *atp6* mutation. To check the presence of the mitochondrial genome, the cells were crossed with tester strains devoid of mitochondrial genome and the growth of diploids on glycerol medium was analyzed.

A Strains	+ NADH (σ)	+ADP-State3 (σ)	ADP-State 4 (σ)	+ CCCP (σ)
wt	124 (11)	195 (11)	120 (10)	301 (47)
bcs1-F342C	27 (12)	26 (5)	23 (3)	32 (11)
R3	97 (18)	134 (34)	115 (32)	336 (88)
R18	46 (19)	68 (19)	56 (24)	106 (23)
atp1-V68G	115 (10)	150 (8)	118 (15)	420 (59)
atp2-A48D	188 (68)	290 (82)	192 (48)	678 (211)

B Strains	Total ATPase	O^R ATPase	O^S ATPase	O^S/total % inhib.	O^S ATPase % wt (σ)	ATP synt. (σ)	ATP synt. % wt
wt	2135	286	1849	87	100 (3)	608 (44)	100
bcs1-F342C	1920	117	1803	94	98 (5)	7 (1)	1
R3	190	74	116	61	6 (2)	186 (88)	31
R18	587	215	372	63	20 (4)	79 (32)	13
atp1-V68G	105	21	84	80	5 (1)	81 (11)	13
atp2-A48D	353	22	331	94	18 (1)	312 (96)	51

Tables S2A and S2B. O_2 consumption, ATP hydrolysis and synthesis activities.
State 3: O_2 consumption in presence of an excess of external ADP. State 4: basal respiration (see Experimental Procedures). O^R ATPase: oligomycin-resistant ATPase activity ; O^S ATPase: oligomycin-sensitive ATPase activity. The O_2 consumption (nM O_2 min^{-1} mg $prot^{-1}$), ATP hydrolysis (nM Pi.min^{-1} mg $prot^{-1}$) and ATP synthesis (nM ATP min^{-1} mg $prot^{-1}$) were measured on purified mitochondria from the six strains as described in Experimental Procedures. Additions were 4mM NADH, 150 µM ADP, 4 µM CCCP, 3 µg/ml oligomycin. The values reported are the means of triplicate assays; the standard-deviations (σ) are indicated.

Experimental procedures

Strains, media

S. cerevisiae strains are listed in Table S1. The non-fermentable 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 sub-clones 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 were isolated, carrying *ATP1* or *ATP2*. Sequencing of these two genes revealed that R3 does carry a mutation in *ATP1* and R2, R12 and R18, mutations in *ATP2.*

Gene deletion, site-directed mutagenesis, epitope tagging

The genes were deleted in the wt strain (CW252): the ORFs were replaced by the *URA3*, *LEU2* or *KanR* markers (see Table S1). The *bcs1*-F401I mutant was constructed by site-directed mutagenesis with the Stratagene QuickChange^{TM} 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, 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- BN-PAGE were electro-transferred and immunodetection was carried out using the chemiluminescent method from Pierce. Polyclonal antibodies against Cyt1, Bcs1, Cytb, Nam1 were raised in the laboratory and were used at a 1/30000 for Cyt1 and 1/5000 for the others. The polyclonal Anti-Rip1 (1/3000) is from N. Fisher (Liverpool, UK); the polyclonal antibodies against the subunits of ATP synthase (1/10000) are from J. Velours (Bordeaux, F); the monoclonal anti-Cox2 (1/5000) is from Molecular Probes; the monoclonal anti-c-Myc (1/20000) is from JM Galan (Paris, F); the polyclonal anti-SDH (1/500) is from B. Guiard (Gif sur Yvette, F), anti-Tom40 from C Meisinger (Freiburg, G).

Co-immunoprecipitation experiments

Mitochondria were solubilised in 50mM Tris HCl pH 7.4, 100mM NaCl, 1% digitonine for 30 min at 4°C and centrifugated 15 min at 100000g. 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 presence of saturating amounts of decylubiquinol and the cytochrome *c* oxidase (complex IV) activity 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.

Mitochondrial respiration, membrane potential, ATP synthesis and hydrolysis measurements

Oxygen consumption rates were measured with a Clark electrode in respiration buffer (0.65M mannitol, 0.36 mM EGTA, 5 mM Tris-phosphate, 10 mM Tris-maleate, pH 6.8). Mitochondrial respiration was measured with NADH whose electrons enter the respiratory chain through the external NADH-ubiquinone oxidoreductase, (i) at state 4 (basal respiration or non-phosphorylating conditions), (ii) in the presence of an excess of external ADP (state 3, phosphorylating conditions), or (iii) in the presence of the membrane potential uncoupler CCCP where respiration is maximal. 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 sprectrophotometer. 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 2M KOH/0.3M MOPS. The luciferin/luciferase assay (ThermoLabsystems) was used to determine ATP concentrations. The specific ATPase activity at pH 8.4 of non-osmotically 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.

10 mg of mitochondrial proteins were 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) washed with buffer “A”. After an overnight incubation at 4 °C, the flow-through was collected by centrifugation at 1000 g for 1 min at 4°C. Beads were washed with 40 volumes of buffer “A” by centrifugation at 1000 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 were 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 concentration 0, 2.5, 5, 10 or 20 mM). ATP concentrations of 0-5mM 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.

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References

Abrahams, J.P., Leslie, A.G., Lutter, R., and Walker, J.E. (1994). Structure at 2.8 A resolution of F1-ATPase from bovine heart mitochondria. Nature *370*, 621-628.

Ackerman, S.H., and Tzagoloff, A. (1990). Identification of two nuclear genes (ATP11, ATP12) required for assembly of the yeast F1-ATPase. Proc Natl Acad Sci U S A *87*, 4986-4990.

Ackerman, S.H., and Tzagoloff, A. (2005). Function, structure and biogenesis of mitochondrial ATP synthase. Prog Nucleic Acid Res Mol Biol *80*, 95-133.

Augustin, S., Gerdes, F., Lee, S., Tsai, F.T.F., Langer, T., Tatsuta, T. (2009). An intersubunit signaling network coordinates ATP hydrolysis by *m*-AAA proteases. Mol Cell *35*, 574-585.

Beauvoit, B., Rigoulet, M., Bunoust, O., Raffard, G., Canioni, P., and Guerin, B. (1993). Interactions between glucose metabolism and oxidative phosphorylations on respiratory-competent *Saccharomyces cerevisiae* cells. Eur J Biochem *214*, 163-172.

Beauvoit, B., and Rigoulet, M. (2001). Regulation of cytochrome *c* oxidase by adenylic nucleotides. Is oxidative phosphorylation feedback regulated by its end-product? IUBMB Life *52*, 143-152.

Bousquet, I., Dujardin, G., and Slonimski, P.P. (1991). *ABC1* a novel yeast nuclear gene has a dual function in mitochondria: it supresses a cytochrome *b* translational defect and is essential for the electron transfer in the *bc1* complex. EMBO J. *10*, 2023-2031.

Conte, L., Trumpower, B.L., and Zara, V. (2010). Bcs1p can rescue a large and productive cytochrome *bc1* complex assembly intermediate in the inner membrane of yeast mitochondria. Biochim Biophys Acta *1813*, 91-101.

Couplan, E., Aiyar, R.S., Kucharczyk, R., Kabala, A., Ezkurdia, N., Gagneur, J., St Onge, R.P., Salin, B., Soubigou, F., Le Cann, M.*, et al.* (2011) A yeast-based assay identifies drugs active against human mitochondrial disorders. Proc Natl Acad Sci U S A *108*, 11989-11994.

Cruciat, C.M., Brunner, S., Baumann, F., Neupert, W., and Stuart, R.A. (2000). The cytochrome *bc1* and cytochrome *c* oxidase complexes associate to form a single supracomplex in yeast mitochondria. J Biol Chem *275*, 18093-18098.

Cruciat, C.M., Hell, K., Folsch, H., Neupert, W., and Stuart, R.A. (1999). Bcs1p, an AAA-family member, is a chaperone for the assembly of the cytochrome *bc1* complex. EMBO J *18*, 5226-5233.

Cui, T.Z., Smith, P.M., Fox, J.L., Khalimonchuk, O., and Winge, D.R. (2012). Late-stage maturation of the Rieske Fe/S protein: Mzm1 stabilizes Rip1 but does not facilitate its translocation by the AAA ATPase Bcs1. Mol Cell Biol *32*, 4400-4409.

de Lonlay, P., Valnot, I., Barrientos, A., Gorbatyuk, M., Tzagoloff, A., Taanman, J.W., Benayoun, E., Chretien, D., Kadhom, N., Lombes, A.*, et al.* (2001). A mutant mitochondrial respiratory chain assembly protein causes complex III deficiency in patients with tubulopathy, encephalopathy and liver failure. Nat Genet *29*, 57-60.

De Meirleir, L., Seneca, S., Damis, E., Sepulchre, B., Hoorens, A., Gerlo, E., Garcia Silva, M.T., Hernandez, E.M., Lissens, W., and Van Coster, R. (2003). Clinical and diagnostic characteristics of complex III deficiency due to mutations in the *BCS1L* gene. Am J Med Genet A *121A*, 126-131.

Dudkina, N.V., Kudryashev, M., Stahlberg, H., and Boekema, E.J. (2011). Interaction of complexes I, III, and IV within the bovine respirasome by single particle cryoelectron tomography. Proc Natl Acad Sci U S A *108*, 15196-15200.

Fernandez-Vizarra, E., Bugiani, M., Goffrini, P., Carrara, F., Farina, L., Procopio, E., Donati, A., Uziel, G., Ferrero, I., and Zeviani, M. (2007). Impaired complex III assembly associated with *BCS1L* gene mutations in isolated mitochondrial encephalopathy. Hum Mol Genet *16*, 1241-1252.

Folsch, H., Guiard, B., Neupert, W., and Stuart, R.A. (1996). Internal targeting signal of the Bcs1 protein: a novel mechanism of import into mitochondria. EMBO J *15*, 479-487.

Gerdes, F., Tatsuta, T., and Langer, T. (2011). Mitochondrial AAA proteases--towards a molecular understanding of membrane-bound proteolytic machines. Biochim Biophys Acta *1823*, 49-55.

Hamel, P., Lemaire, C., Bonnefoy, N., Brivet-Chevillotte, P., and Dujardin, G. (1998). Mutations in the membrane anchor of yeast cytochrome *c1* compensate for the absence of Oxa1p and generate carbonate-extractable forms of cytochrome *c1*. Genetics *150*, 601-611.

Hanson, P.I., and Whiteheart, S.W. (2005). AAA+ proteins: have engine, will work. Nat Rev Mol Cell Biol *6*, 519-529.

Heinemeyer, J., Braun, H.P., Boekema, E.J., and Kouril, R. (2007). A structural model of the cytochrome *c* reductase/oxidase supercomplex from yeast mitochondria. J Biol Chem *282*, 12240-12248.

Hinson, J.T., Fantin, V.R., Schonberger, J., Breivik, N., Siem, G., McDonough, B., Sharma, P., Keogh, I., Godinho, R., Santos, F.*, et al.* (2007). Missense mutations in the *BCS1L* gene as a cause of the Bjornstad syndrome. N Engl J Med *356*, 809-819.

Houstek, J., Pickova, A., Vojtiskova, A., Mracek, T., Pecina, P., and Jesina, P. (2006). Mitochondrial diseases and genetic defects of ATP synthase. Biochim Biophys Acta *1757*, 1400-1405.

Hunte, C., Koepke, J., Lange, C., Rossmanith, T., and Michel, H. (2000). Structure at 2.3 A resolution of the cytochrome *bc(1)* complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment. Structure *8*, 669-684.

Iwata, S., Lee, J.W., Okada, K., Lee, J.K., Iwata, M., Rasmussen, B., Link, T.A., Ramaswamy, S., and Jap, B.K. (1998). Complete structure of the 11-subunit bovine mitochondrial cytochrome *bc1* complex. Science *281*, 64-71.

Karata, K., Inagawa, T., Wilkinson, A.J., Tatsuta, T., and Ogura, T. (1999). Dissecting the role of a conserved motif (the second region of homology) in the AAA family of ATPases. J Biol Chem *274*, 26225-26232.

Kotarsky, H., Karikoski, R., Morgelin, M., Marjavaara, S., Bergman, P., Zhang, D.L., Smet, J., van Coster, R., and Fellman, V. Characterization of complex III deficiency and liver dysfunction in GRACILE syndrome caused by a BCS1L mutation. Mitochondrion *10*, 497-509.

Kucharczyk, R., Ezkurdia, N., Couplan, E., Procaccio, V., Ackerman, S.H., Blondel, M., and di Rago, J.P. (2010). Consequences of the pathogenic T9176C mutation of human mitochondrial DNA on yeast mitochondrial ATP synthase. Biochim Biophys Acta *1797*, 1105-1112.

Kucharczyk, R., Giraud, M.F., Brethes, D., Ezkurdia, N., Salin, B., Velours, J., Camougrand, N., Haraux, F., and di Rago, J.P. (2012). A pathogenic human mtDNA mutation (T8851C) results in aberrant catalytic properties of ATP synthase and increased autophagy in *Saccharomyces cerevisiae*.

Kucharczyk, R., Rak, M., and di Rago, J.P. (2009). Biochemical consequences in yeast of the human mitochondrial DNA 8993T>C mutation in the *ATPase6* gene found in NARP/MILS patients. Biochim Biophys Acta *1793*, 817-824.

Lefebvre-Legendre, L., Salin, B., Schaeffer, J., Brethes, D., Dautant, A., Ackerman, S.H., and di Rago, J.P. (2005). Failure to assemble the alpha 3 beta 3 subcomplex of the ATP synthase leads to accumulation of the alpha and beta subunits within inclusion bodies and the loss of mitochondrial cristae in *Saccharomyces cerevisiae*. J Biol Chem *280*, 18386-18392.

Lemaire, C., and Dujardin, G. (2008). Preparation of respiratory chain complexes from *Saccharomyces cerevisiae* wild-type and mutant mitochondria : activity measurement and subunit composition analysis. Methods Mol Biol *432*, 65-81.

Leveen, P., Kotarsky, H., Morgelin, M., Karikoski, R., Elmer, E., and Fellman, V. (2010). The GRACILE mutation introduced into Bcs1l causes postnatal complex III deficiency: a viable mouse model for mitochondrial hepatopathy. Hepatology *53*, 437-447.

Longtine, M.S., McKenzie III, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and midification in *Saccharomyces cerevisiae*. Yeast *14*, 953-961.

Moran, M., Marin-Buera, L., Gil-Borlado, M.C., Rivera, H., Blazquez, A., Seneca, S., Vazquez-Lopez, M., Arenas, J., Martin, M.A., and Ugalde, C. (2010). Cellular pathophysiological consequences of *BCS1L* mutations in mitochondrial complex III enzyme deficiency. Hum Mutat *31*, 930-941.

Nobrega, F.G., Nobrega, M.P., and Tzagoloff, A. (1992). *BCS1*, a novel gene required for the expression of functional Rieske iron-sulfur protein in *Saccharomyces cerevisiae*. EMBO J *11*, 3821-3829.

Nouet, C., Truan, G., Mathieu, L., and Dujardin, G. (2009). Functional analysis of yeast *bcs1* mutants highlights the role of Bcs1p-specific amino acids in the AAA domain. J Mol Biol *388*, 252-261.

Rak, M., Zeng, X., Brière,J-J, Tzagoloff, A. (2009). Assembly of Fo in *Saccharomyces cerevisiae.* Biochim Biophys Acta 1793, 108-116

Ramzan, R., Staniek, K., Kadenbach, B., and Vogt, S. (2010). Mitochondrial respiration and membrane potential are regulated by the allosteric ATP-inhibition of cytochrome *c* oxidase. Biochim Biophys Acta *1797*, 1672-1680.

Saint-Georges, Y., Hamel, P., Lemaire, C., and Dujardin, G. (2001). Role of positively charged transmembrane segments in the insertion and assembly of mitochondrial inner-membrane proteins. Proc Natl Acad Sci U S A *98*, 13814-13819.

Schagger, H., and Pfeiffer, K. (2000). Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J *19*, 1777-1783.

Smith, P.M., Fox, J.L., and Winge, D.R. (2012). Biogenesis of the cytochrome *bc(1)* complex and role of assembly factors. Biochim Biophys Acta *1817*, 276-286.

Venard, R., Brethes, D., Giraud, M.F., Vaillier, J., Velours, J., and Haraux, F. (2003). Investigation of the role and mechanism of IF1 and STF1 proteins, twin inhibitory peptides which interact with the yeast mitochondrial ATP synthase. Biochemistry *42*, 7626-7636.

Visapaa, I., Fellman, V., Vesa, J., Dasvarma, A., Hutton, J.L., Kumar, V., Payne, G.S., Makarow, M., Van Coster, R., Taylor, R.W*., et al.* (2002). GRACILE syndrome, a lethal metabolic disorder with iron overload, is caused by a point mutation in *BCS1L*. Am J Hum Genet *71*, 863-876.
Wagener, N., Ackermann, M., Funes, S., and Neupert, W. (2011). A pathway of protein translocation in mitochondria mediated by the AAA-ATPase Bcs1. Mol Cell *44*, 191-202.
Zara, V., Conte, L., and Trumpower, B.L. (2009). Biogenesis of the yeast cytochrome *bc1* complex. Biochim Biophys Acta *1793*, 89-96.

2. Conclusion on part II

In the preceding article we proposed a novel regulation mechanism for the respiratory chain, occurring in the late assembly of the complex III, *via* the ATP-dependency of its assembly factor Bcs1. The ATP-mediated regulation is commonly found in living organisms, but this type of regulation in the assembly process has not been previously reported. It could be so because such a regulation is supposed to be much slower, possibly less efficient that a far more direct regulation of enzymatic activity, and/or more rare that the latter. Still, in the light of our results, it may be significantly fast if operated in the late assembly of multimeric enzymes. Is it possible that we missed a common regulatory mechanism or is the Bcs1 case just an exception? It is difficult to say with only one example. Either way, a precise understanding of Bcs1 function, interactions and regulation should be very helpful in addressing this matter.

2.1. Bcs1-nucleotide interactions

Although this point can still be questioned, we are currently assuming, given the experimental evidence both referenced and shown in our paper, that functional Bcs1 is a homohexamer. Crystalizing the quaternary structure of Bcs1 will be one of the essential steps in further studies, since without it, various aspects of its nature stay quite mysterious.

In the classification of AAA proteins, Bcs1 belongs to the extended AAA group, which contains proteins such as Pex1/6 (peroxisome biogenesis), katanin (microtubule disassembly), Cdc48 (mitotic spindle disassembly, ubiquitin proteasome-dependent processing), FtsH (protein defolding and degradation) and Rubisco activase (activation of enzymatic activity) (Snider and Houry, 2008). As highlighted by this group, the AAA family seems to be extraordinarily diversified when it comes to substrate recognition, interacting proteins, cellular compartmentalization and processes. Accordingly, different models have been proposed for the mechanisms of nucleotide binding and exchange for different hexameric AAA ATPases (Augustin et al., 2009; Briggs et al., 2008; Hersch et al., 2005; Smith et al., 2011). In principle, these hexamers may either function in a concerted manner, meaning that all subunits bind, hydrolyze, and then release nucleotides simultaneously, or in a nonconcerted manner in which the different subunits within the ring bind and hydrolyze nucleotides at distinct times. In all cases different subunits are required to communicate and regulate each other's behavior. Subunits within the ring can either be vacant or bind one of the two nucleotides, ATP or ADP. Each condition is thought to affect the conformation and function of the neighboring subunits so that ATP hydrolysis occurs in a nonconcerted or sequential manner around the hexameric ring. However, a stochastic hydrolysis was found to be possible, at least for some AAA proteins (Martin et al., 2005). Which mechanism is adopted by Bcs1 is not known.

Depending on the mechanism of nucleotide hydrolysis/exchange the ATP/ADP ratio can differently modulate the activity of a given protein. ATP and ADP may competitively bind to the same binding site (Beauvoit and Rigoulet, 2001; Jiang and Ninfa, 2007; Prodromou et al., 1997), and given that at least six binding site are predicted for the Bcs1 oligomer, different combinations of site occupancy could produce variable cooperative interactions between the nucleotides. In this case the ATP/ADP ratio would directly modulate the activity of Bcs1 through the differential binding of the two nucleotide species.

Another point to consider is that for the same enzyme, at different concentrations, ATP can be either substrate or inhibitor, as in the case of phosphofructokinase1 (PFK) (Hofmann and Kopperschla¨ger, 1982; Strater et al., 2010). PFK catalyzes the rate limiting ATP-dependent phosphorylation of fructose 6-phosphate to form fructose 1,6-bisphosphate and ADP. Low

ATP/ADP (/AMP) ratio activates PFK, while at higher concentrations ATP acts as an allosteric inhibitor of the enzyme. In the ATP hydrolysis assay we performed in the article the ATP consumption reached its peak at 10mM ATP and subsequently decreased at 20mM, which designed the hyperbolic curve that might suggest an allosteric inhibition (Fig. II-1).

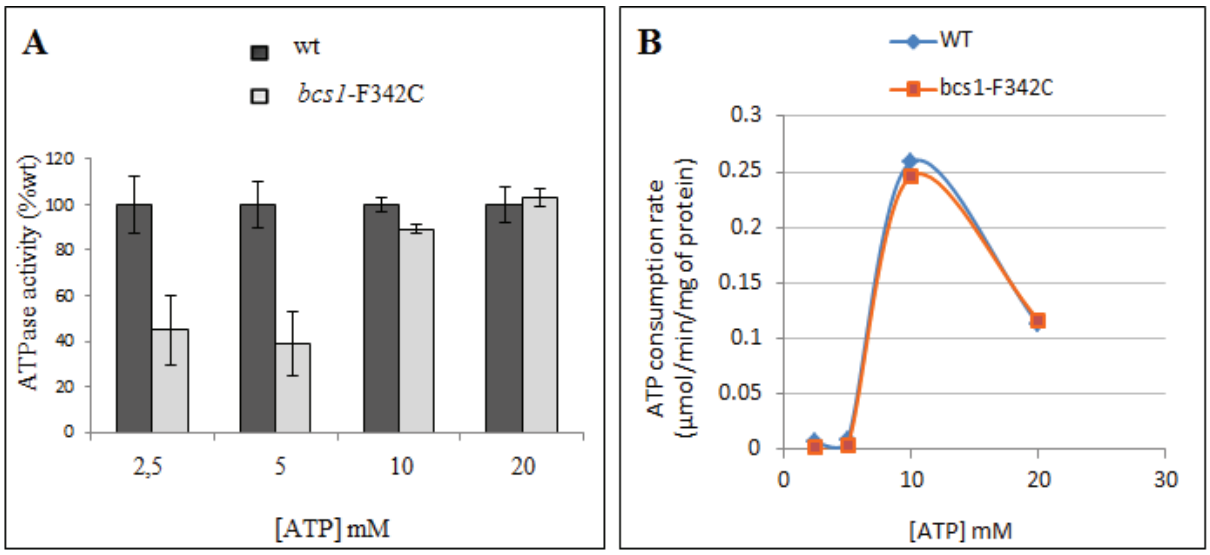


Figure II-1 Kinetics for ATP of Bcs1 purified from mitochondria of *S. cerevisiae*.
A) ATP consumption rates of the mutated *versus* wt Bcs1 at different ATP concentrations in the assay (2,5 to 20mM) are represented as % of the wt value. Histograms are mean values of three independent experiments with standard deviations as error bars. B) ATP consumption rates of the mutated *versus* wt Bcs1 at different ATP concentrations in the assay (2,5 to 20mM) are represented as µmol ATP hydrolysed/min/mg of mitochondrial protein

Determination of the physiologic intracellular concentration of ATP is an important point for this matter, but it seems somewhat difficult to define. The intracellular concentrations of nucleotides are reported to be variable in different organisms, cell types, cellular compartments, metabolic conditions and experimental contexts, with generally reasonable range of 0.5-10mM (Allue et al., 1996; Beis and Newsholme, 1975; Gribble et al., 2000; Jouaville et al., 1998; Metelkin et al., 2009). According to this, the physiological ATP concentrations may effectively be able to activate Bcs1, but whether the ATP could be compartmentalized in the matrix up to the "inhibitory" concentrations above 10mM is questionable. The aim of the experiment presented was not to understand the precise nature of the Bcs1-nucleotide

interaction and further test are required to rule in favor or against the allosteric inhibition of Bcs1 by ATP.

Moreover, other regulatory mechanisms may exist for Bcs1, as both human and yeast proteins have been reported to be phosphorylated (Chi et al., 2007), www.phosphosite.org). Several research areas would benefit from experiments aiming to dissect the protein's mechanism of activity; it would help to better understand the AAA protein family, the regulatory relationships existing between the pool of adenine nucleotides and the OXPHOS system and could provide clues in dealing with Bcs1-linked pathologies in human patients.

2.2. Bcs1-mediated translocation of Rip1

Another interesting point arising from studies of Bcs1 is its role as a translocase of Rip1 in the inner membrane. Rip1 follows a curious path from its synthesis in cytoplasm towards its electron-transferring activity in the complex III. Firstly, Rip1 is targeted to mitochondria through its N-terminal sorting sequence which is cleaved by the MPP protease during the import. Interestingly, the protein contains one internal hydrophobic transmembrane segment but it does not act as a stop-transfer signal in the membrane; instead, it allows the complete transfer of Rip1 to the matrix (Hartl et al., 1986). In the matrix Rip1 is processed again after its insertion in the complex III, by the Oct1 protease, which cleaves an additional eight residues to yield a mature protein. However, this step is not required for the insertion or activity of Rip1 in the complex III (Nett et al., 1997). Since the biogenesis of iron sulfur clusters takes place in the matrix (Kispal et al., 1999; Lill, 2009), and the C-terminal domain of Rip1, which contains the ISC, protrudes in the IMS, it has been assumed that import of Rip1 in the matrix was necessary for it to acquire the cluster. Rip1 is proposed to reach its final topology after it has been translocated by Bcs1 across the inner membrane, in its folded cofactor-containing state (Wagener and Neupert, 2012). Bcs1 was shown to interact with Rip1 in ATP-dependent manner, and different parts of Rip1 were reported to have different functions in the translocation process; the hydrophobic transmembrane stretch induces ATP hydrolysis by Bcs1 and the lateral release of Rip1 in the membrane while the C-terminal is required initially for the correct

interaction of the two proteins. However, since Rip1 is able to assemble into the complex III in the absence of its ISC (Graham and Trumpower, 1991), the formation of the cluster and its addition to Rip1 have not been followed in the study by Wagener and colleagues.

Interestingly, the C-terminal of Rip1 is a site of another interaction, with the Mzm1 protein, which seems to stabilize Rip1 in the matrix (Atkinson et al., 2011). Mzm1 contains a poorly conserved, but functional LYR motif, which can be found in proteins involved in biogenesis of the Fe/S clusters (Wiedemann et al., 2006). The LYR motif of Mzm1 seems to be required for the interaction with the C-terminal domain of Rip1, but there are no experimental data supporting the requirement for the assembled ISC in this interaction. Mzm1 is more generally proposed to protect Rip1 from proteolytic degradation, regardless of ISC content, and possibly keep it in a translocation-competent conformation (Cui et al., 2012).

Although further direct experimental evidence would be necessary to define whether Bcs1 translocates folded ISC-containing Rip1 from the matrix this idea is rather interesting and has an evolutionary aspect to it. Wagener and colleagues discussed that, in mitochondria, Bcs1 would replace the Twin-Arginine Translocation (Tat) system, required for the translocation of homologs of Rip1 in bacteria and chloroplasts (Aldridge et al., 2008). Tat system is able to translocate folded proteins, often containing a cofactor, and to do so it relies on the proton motive force, but not the nucleotide hydrolysis (for review on Tat system see Palmer and Berks, 2012; Robinson et al., 2011). Curiously, the Tat system has not been conserved in mitochondria, just as Bcs1 has never been found in bacteria, which could comfort the idea of the replacement of Tat by Bcs1, at least for Rip1 membrane insertion.

In general, translocation of folded proteins across tight polarized membranes is complicated, and only few examples of such transfer have been described, notably during the peroxisomal protein import and possibly in the ERAD (ER-Associated Degradation) translocation machinery (Girzalsky et al., 2009; Schliebs et al., 2010). Although details of the translocation mechanism and the precise subunit composition of the translocation pores are still elusive, it is interesting to note that both pathways rely on ATP hydrolysis by proteins of the AAA family (Pex1-Pex6 and Cdc48). The AAA proteins do not seem to directly form, but rather interact with the translocation pores, and the mechanical force derived from the ATP hydrolysis is proposed

to be coupled to the translocation of cargo proteins across the membrane (Schliebs et al., 2010). The AAA proteins have already proven to be extremely versatile when it comes to different cellular activities, and whether a part of these proteins could have another specialization in the transport of folded/cofactor-containing proteins across cellular membranes is certainly worth exploring in future projects.

2.3. Intragenic suppressors of bcs1-F342C

bcs1-S314R was the only intragenic suppressor mutation of *bcs1*-F32C isolated in the screen. The mutated residue is located in close proximity of the Walker B motif on the predictive structure of the AAA domain (Fig. II-2), which is important for nucleotide hydrolysis (Hanson and Whiteheart, 2005). Interestingly, the S314 residue is conserved in humans and it was found mutated in patients with a complex III deficiency (mutation S277N in BCS1L, (de Lonlay et al., 2001). However, when tested in yeast by the same authors, the substitution S314N (as the suppressor S314R) maintained normal respiratory phenotype. The original mutant *bcs1*-F342C induces a local structural change in the AAA domain that affects the activity of the protein and this structural change is likely counter balanced by the S314R modification in the same domain. Besides these observations, the intragenic suppressor was not studied further.

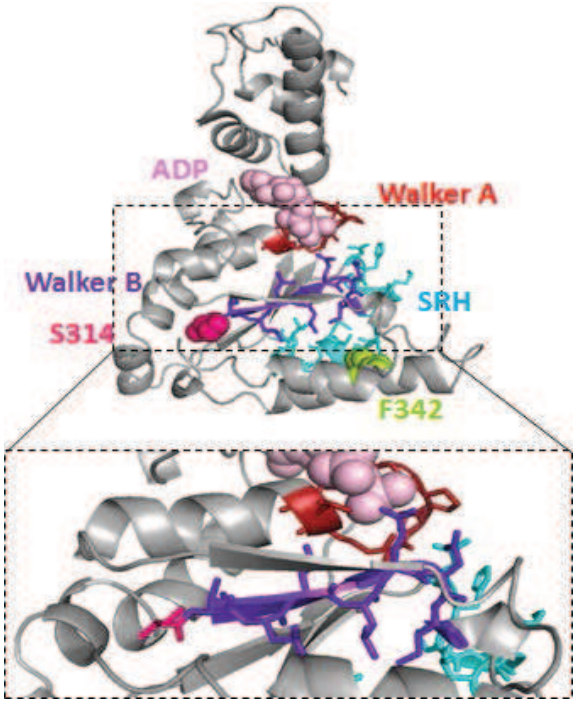


Figure II-2 The intragenic suppressor of bcs1-F342C
Positions of S314, F342 and ADP are shown as colored spheres; motifs Walker A, B and SRH are shown as colored sticks. S314 is shown as colored stick (fuscia) in the bottom image to show its proximity to the Walker B motif.

2.4. Extragenic suppressors of bcs1-F342C can be divided in two distinct groups

All remaining suppressor mutations were recessive and extragenic. We crossed different suppressor mutants with one another and observed that some double mutants had maintained their respiratory competence while others presented severe respiratory phenotype, as the double mutant *atp1*-V68G/*atp2*-A48D, presented in the article. These results suggested the presence of two different groups of suppressors, each containing mutants that genetically interact with one another, but not with the members of the other group. Transformation of respiratory deficient double mutant strains with *S. cerevisiae* genomic bank allowed the identification of genes carrying the suppressor mutations (see Material and Methods in the article). The first group contained mutations in *ATP1* and *ATP2* genes, described in the article. The second group contained four mutations in three different genes, *YME1* (*yme1*-D340H and *yme1*-G325A), *RRF1* (*rrf1*-N214*) and *ATP23* (*atp23*-R96*) (Fig. II-3).

Yme1 is an AAA ATPase of the inner membrane which exposes its AAA domain in the IMS. The protein is mostly implicated in degradation of misfolded inner membrane proteins which have IMS-protruding domains (Leonhard et al. 1996; Weber et al. 1996), but its function seems to be at least partly redundant since *Δyme1* strains are respiratory competent in yeast. Suppressor mutations are situated in the AAA domain of the protein. We have constructed the mutant of the proteolytic site (*yme1*-E541Q) and the complete deletion (*Δyme1*) and determined that both constructions were able to suppress *bcs1*-F342C, indicating that loss of the proteolytic activity of Yme1 was probably required for the compensation of *bcs1*-F342C (Fig. II-3A).

Rrf1 is a conserved mitochondrial ribosome recycling factor for which few reports are present. Silencing of the ortholog of Rrf1 in HeLa cells, mtRRF, was shown to be lethal (Rorbach et al., 2008). In yeast, deletion of Rrf1 was reported to be respiratory deficient, with an extremely high percentage of mtDNA instability (Teyssier et al., 2003), which has made it unsuitable for further experimental investigation. However, it has been reported that *Δrrf1* yeast strains may show variable phenotypes depending on the intron content of their

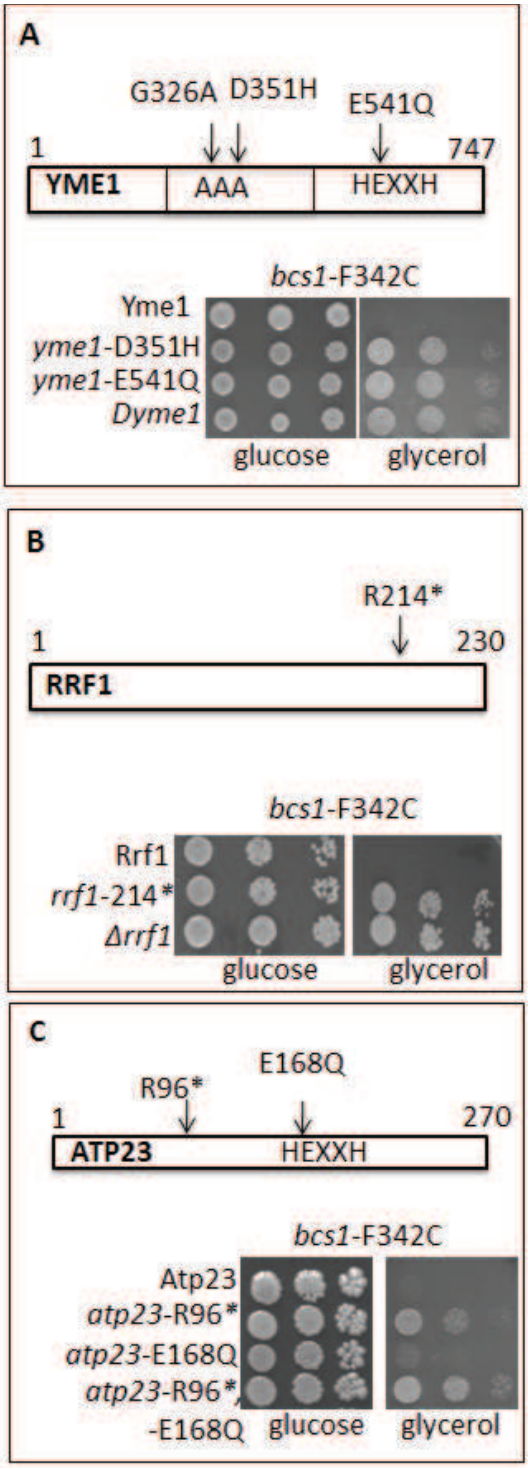


Figure II-3 Extragenic mutations in *YME1*, *ATP23* and *RRF1* are suppressors of *bcs1*-F342C
A), B), C) - Positions of the suppressor mutations in the schematic primary structures of Yme1, Rrf1 and Atp23 are indicated, as well as the proteolytic (HEXXH) and the AAA domains. Dilution series of indicated strains were spotted on glucose- and glycerol-containing plates and incubated for 3 days.

mitochondrial genomes (Rorbach et al., 2008). This was confirmed in our study, and we were able to obtain a stable, respiratory competent *Δrrf1* strain in an intronless mitochondrial background. Moreover, both the non-sense mutant *rrf1*-N214* and *Δrrf1* suppressed *bcs1*-F342C (Fig. II-3B). Since the mitochondrial ribosome recycling process is still poorly understood in eukaryotes, we have characterized the *Δrrf1* mutant in a separate project that will be presented in the part III.

Atp23 is a metaloprotease of the IMS, tightly associated with the inner membrane and required for the processing and assembly of Atp6 within the ATP synthase (Osman et al. 2007; Zeng et al. 2007). The suppressor mutation in *ATP23* causes the formation of a stop codon in the first half of the protein (96/270 amino acids), which could suggest that a complete loss-of-function of Atp23 is required for the suppression. However, *Δatp23* is respiratory deficient and does not suppress *bcs1*-F342C, implicating that the premature stop codon formed in the *atp23*-R96* mutant is likely to be read-through; maintain of very low amounts of the protein is possibly required for the suppression. Since Atp23 has both a chaperon and a proteolytic function, we have constructed the mutant of the proteolytic site, E168Q. This

mutant alone did not suppress *bcs1*-F342C, but the double *atp23*-R96*, E168Q mutant did, showing that the impairment of the proteolytic activity alone was not sufficient for the suppression (Fig. II-3C).

We have tested the read-through hypothesis. The efficiency of translational read-through events is increased by the yeast prion-like factor (psi+), whose presence in strains of *S. cerevisiae* depends on the chaperon protein Hsp104 (Chernoff et al., 1995; Helsen and Glover, 2012). We deleted *HSP104* in *atp23*-R96* background and observed that it significantly reduced the respiratory competence of the original mutant (Fig. II-4A). However, the *atp23*-R96*, *Δhsp104* mutant was still able to suppress *bcs1*-F342C (not shown), suggesting that the

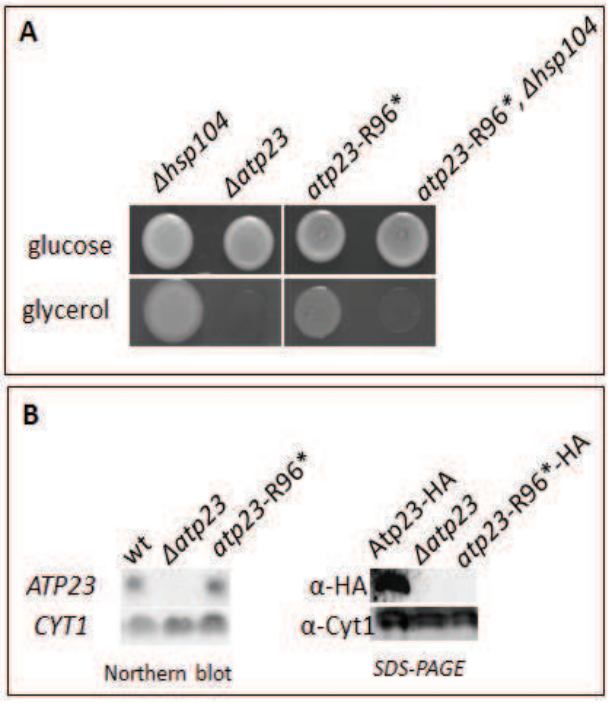


Figure II-4 Translational read-through of the non-sense mutation *atp23*-R96*
A) The HSP104 gene was deleted in the wt and the *atp23*-R96* mutant. The single and double mutants were grown on glucose and glycerol-containing media for 3 days. B) Total RNAs were extracted and hybridized with the *ATP23* and *CYT1* probes (left). Mitochondrial proteins were extracted from indicated strains, resolved by SDS-PAGE and immunoblotted with antibodies against Cyt1 and HA (right).

accumulation of very low amounts of Atp23 are necessary for the suppression to occur. Nothern blot analysis has shown that *ATP23* mRNA accumulates in the *atp23*-R96* mutant, but the low amount of protein that may accumulate in this context was not detectable by western blot (Fig II-4B).

All identified suppressors were able to restore wild type-like levels of Rip1 in the *bcs1*-F342C mutant (Fig. II-5A), but the effects on the assembly and the activity of ATP synthase differed between them. The assembly of ATP synthase was compromised in *atp23* and *rrf1* mutants, and this was reflected in the extremely low levels of ATP hydrolysis by the coupled ATP synthase (F_1+F_o) (Fig II-5B, 5C). But, due to the over-accumulation of the catalytic F_1 sector they displayed wild type-like levels of total ATP hydrolysis. The *Δyme1* mutant showed no significant effect on the assembly or the activity of ATP synthase (Fig II-5B, 5C).

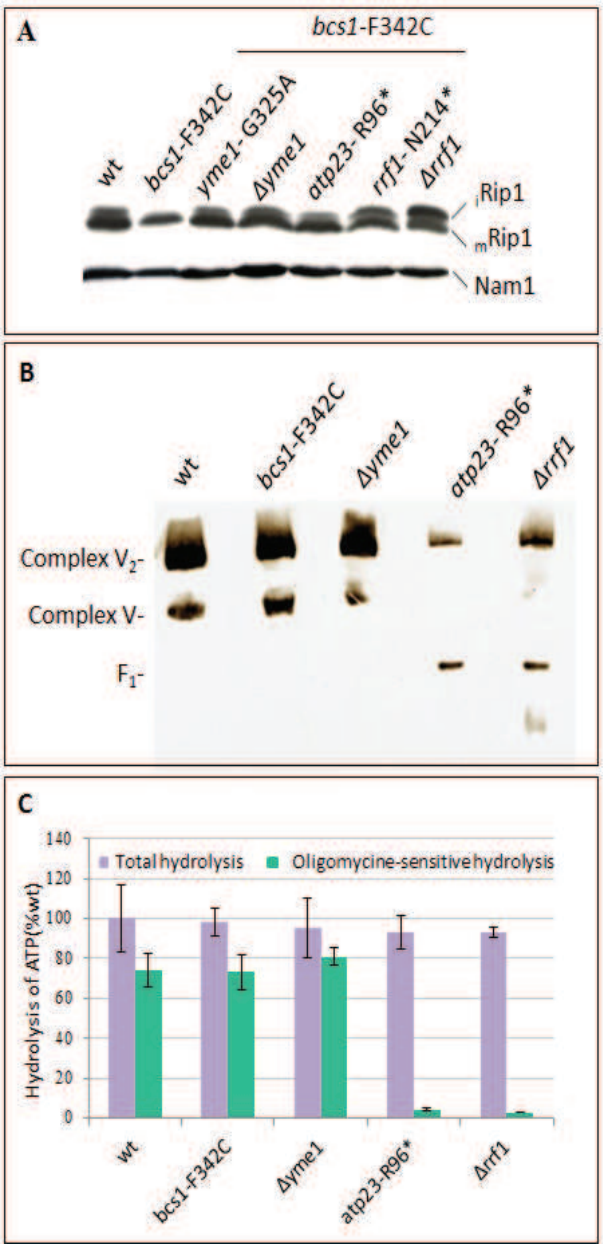


Figure II-5 Suppressor mutations in *YME1*, *RRF1* and *ATP23* differently affect the ATP synthase.
A) Mitochondrial proteins from indicated strains were resolved by SDS-PAGE and immunoblotted with antibodies against Rip1 and Nam1. B) Mitochondrial proteins from indicated strains were resolved by BN-PAGE and immunoblotted with antibody against Atp1. Positions of dimers (V_2), monomers (V) and F_1 are indicated. C) Total and oligomycin-sensitive hydrolysis were measured in presence of saturating amounts of ATP in mitochondria purified from indicated strains.

These results are only the beginning of the investigation on this group of suppressors and additional tests will be conducted in the future. Although no working hypothesis elaborate enough can be proposed at the moment, the characterization of these mutants point to a different suppression mechanism from the one uncovered for the other group of extragenic suppressors, for which the markedly reduced levels of total ATP synthase-mediated ATP hydrolysis were central to the suppression mechanism. Together, these results further highlight the power of the genetic suppressor approach, its ability to branch out and connect diverse domains of cellular biology and uncover unpredictable networks of protein functions and interactions.

III – RRF1

1. Project

As previously shown, deletion of Rrf1, the mitochondrial ribosome recycling factor, was found to suppress the respiratory defect of *bcs1*-F342C. Until now, the effect of the absence of Rrf1 on yeast mitochondrial translation could not be thoroughly studied as it led to the rapid loss of the mitochondrial DNA (mtDNA). However, in an intronless mtDNA background, we have obtained a *Δrrf1* mutant with stable mtDNA content and analyzed its effect on the biogenesis of the three OXPHOS complexes of dual genetic origin.

The ribosome recycling factor Rrf1 plays a role in the coupling of Cox1 synthesis to its assembly in *S.cerevisiae*

INTRODUCTION

Protein synthesis is divided in four stages. In the initiation stage, the two ribosomal subunits associate with the mRNA, the start codon is positioned in the ribosomal P site and it is recognized by a tRNA loaded with formylmethionine. In the elongation stage, the mRNA is read by the ribosome and the corresponding peptide is synthesized as the reading proceeds. When the mRNA stop codon arrives at the ribosomal A site, the synthesis of the peptide terminates and it is released from the ribosome. In the final recycling stage the two subunits of the ribosome, mRNA and the deacylated tRNA are disassembled (Fig. II-1A). Each stage requires general translation factors that are called initiation, elongation, release and recycling factors that are well conserved in living organisms.

In bacteria, concerted action of the ribosome recycling factor RRF and the elongation factor-G (EF-G) dissociates the two ribosomal subunits, through a mechanism that has been intensively studied (Hirashima and Kaji 1973; Czworkowski et al., 1994; AEvarsson et al., 1994; Rodnina et al., 1999; Ito et al., 2002; Zavialov et al., 2005; Gao et al., 2005; Barat et al., 2007;

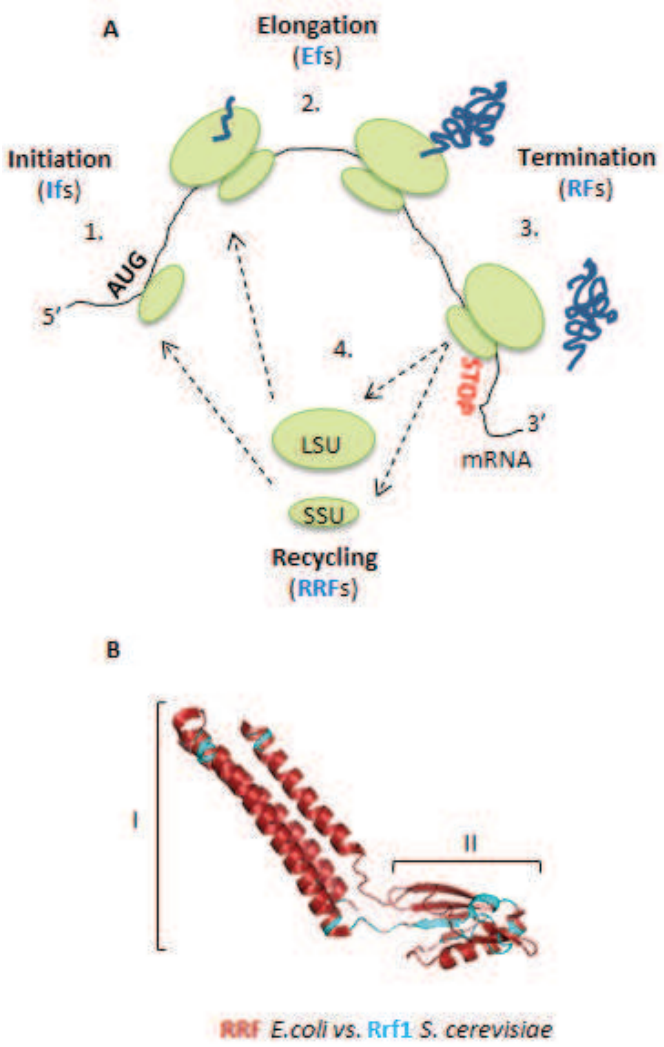


Figure III-1 The mitochondrial ribosome recycling factor Rrf1

A) Schematic representation of the four stages of protein synthesis. IFs, EFs, RFs and RRFs are initiation, elongation, release and recycling factors, respectively. LSU and SSU are the large and small ribosomal subunits, respectively. B) Predictive 3-dimensional structure of the *S. cerevisiae* Rrf1 (shown as turquoise cartoon) was generated with Phyre software (Kelley and Sternberg, 2009) and then aligned with RRF from *E.coli* (shown as red cartoon, PDB code 1ek8) in Pymol v1.3. Domains I and II of RRF are indicated.

Pai et al., 2008; Yokoyama et al., 2012). After the newly synthesized peptide has been released, the two ribosomal subunits, deacylated tRNA and mRNA stay bound in the so-called post-termination complex (PoTC). RRF binds to the PoTC and induces a conformational change loosening the interactions between the two ribosomal subunits. The subsequent binding of EF-G and GTP hydrolysis shift the tRNA towards the exit site and change the position of RRF, leading to the dissociation of the two subunits of the ribosome. Finally, binding of the initiation factor IF3 to the small ribosomal subunit prevents the subunits from re-associating and promotes the initiation of the next round of protein synthesis. RRF is an essential protein (Janosi et al., 1994; 1998) whose structure is known in several bacterial species (Selmer et al., 1999; Toyoda et al., 2000; Kim et al., 2000; Yoshida et al., 2001; Nakano et al., 2003; Saikrishnan et al., 2005). A highly flexible linker region connects domains I and II of RRF, and allows them to assume the different orientations, necessary for ribosome splitting (Selmer et al., 1999; Yoshida et al., 2001).

In mitochondria, a small number of proteins is encoded and synthesized on mitochondrial ribosomes, which differ not only from prokaryotic and cytoplasmic ribosomes

but present subtle differences between mitoribosomes of different organisms as well (Pietromonaco et al. 1991; Graack and Wittmann-Liebold 1998; Agrawal et al. 2011). Mitochondrial ribosome recycling relies on at least two proteins in *Saccharomyces cerevisiae*, the ribosome recycling factor Rrf1 (homolog to the bacterial RRF, mtRRF1 in mammals) and Mef2 (homolog to EF-G in bacteria, EF-$G2_{mt}$ in mammals). A crystal structure does not exist for *S. cerevisiae* Rrf1, but a homology search for its tertiary protein structure identifies the bacterial ribosome recycling factor (RRF) as its closest match. Alignment with the RRF structure from *E. coli* is shown in (Fig. III-1B) . The primary sequence identity between the two proteins is 23%. However, 76% of the tertiary structure of Rrf1 was modeled on the *E. coli* template with 100% confidence. EF-G has two orthologs in mammal mitochondria, EF-$G1_{mt}$, involved in translational elongation and EF-$G2_{mt}$ that, functions in ribosome recycling (Tsuboi et al., 2009). Contrary to the bacterial system, GTP hydrolysis does not seem to be necessary for ribosome disassembly. Binding of GTP to EF-$G2_{mt}$ is required for ribosome splitting while its hydrolysis seems to be necessary only afterwards, to release EF-$G2_{mt}$ (and possibly mtRRF as well) from the large ribosomal subunit (Tsuboi et al., 2009). mtRRF was shown to interact with the ribosome in human cell lines, where its depletion was reported to be lethal (Rorbach et al., 2008). In *S. cerevisiae*, deletions of both Rrf1 and Mef2 were reported to cause respiratory deficiency associated with the loss of mitochondrial DNA (Callegari et al., 2011; Teyssier et al., 2003).

Seven hydrophobic subunits of the OXPHOS complexes, and one protein of the small ribosomal subunit are encoded by the yeast mitochondrial DNA (mtDNA) and synthesized on mitochondrial ribosomes in *S. cerevisiae:* Cytb, a subunit of complex III, three subunits of complex IV (Cox1, 2, 3) and three subunits of ATP synthase (Atp6, 8, 9). Translation of these mitochondrial mRNAs is promoted by messenger-specific translational activators, which interact with both their target RNAs and the ribosome (reviewed in Herrmann et al. 2013). mtDNA-encoded proteins are co-translationally inserted in the inner membrane by the Oxa1 export machinery (Bonnefoy et al., 2009; Szyrach et al., 2003), tightly linking the translation to the assembly process. Regulatory feedback loops adjusting the rate of protein synthesis to the assembly were first described in chloroplasts (Choquet et al., 2000; Wostrikoff and Stern, 2007). In yeast mitochondria, regulatory loops of this sort were reported for the assembly of the

respiratory complexes III and IV and the ATP synthase (Gruschke et al. 2011; Barrientos et al. 2004; Perez-Martinez et al. 2009; Rak and Tzagoloff 2009). One of the examples is the regulatory loop involving Cytb. It relies on the complex Cbp3/Cbp6, which binds the ribosome, promotes the translation of *CYTB* mRNA and assists the initial assembly of the newly synthesized Cytb (Gruschke et al., 2011).

The best understood regulatory feedback loop in yeast mitochondria is the one involving Cox1 and the translational activator Mss51 (Barrientos et al., 2004; Decoster et al., 1990; Fontanesi et al., 2009; Khalimonchuk et al., 2009; Manthey and McEwen, 1995; McStay et al., 2013a; Mick et al., 2010; Perez-Martinez et al., 2003, 2009; Pierrel et al., 2007; Soto et al., 2012; Fig III-2). Mss51 with Pet309 and the heat shock protein Ssc1 binds to the ribosome and the *COX1* mRNA to promote translation (HMW). Then, the newly synthesized Cox1 interacts with assembly factors Cox14 and Coa3, which recruit Mss51 and Ssc1 to Cox1 by a still unknown mechanism. The Mss51-SSc1-Cox1-containing complexes ($Mss51^{A}$) are regarded as early assembly intermediates of Cox1, which sequester Mss51, preventing it to re-stimulate the translation of *COX1* mRNA. When the assembly of Cox1 proceeds to downstream stages with the binding of

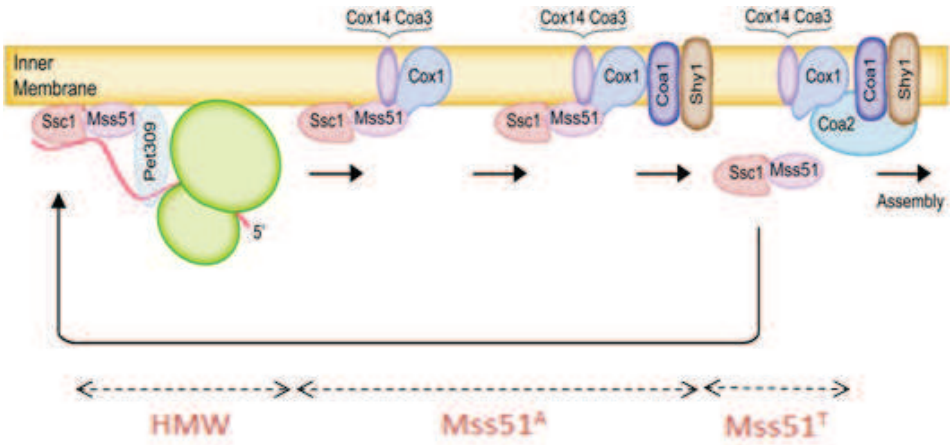


Figure III-2 Schematic representation of the feedback control of Cox1 synthesis by Mss51.
Mitochondria translation of the *COX1* mRNA (in red) is activated by the mRNA-specific activators Pet309 and Mss51-Ssc1. Synthesis of a new Cox1 polypeptide nucleates an early assembly intermediate containing Mss51-Ssc1 and the assembly factors Cox14 and Coa3. As additional assembly factors associate with newly synthesized Cox1, Mss51 is released from the assembly intermediates and becomes available to initiate additional Cox1 synthesis. (from Fox 2012). Mss51 is found in three complexes: in HMW with the ribosomes, in the intermediate assembly complex ($Mss51^{A}$) and in the translation-active reserve with only Ssc1 ($Mss51^{T}$).

the assembly factors Coa1, Shy and Coa2, Mss51-Ssc1 complex is released and is again available for translational activation ($Mss51^{T}$). If, however, the correct assembly of complex IV is hindered, Mss51-Ssc1 stay in the sequestered state and the translation of *COX1* is reduced. In this way, the cycling of Mss51 between translation and assembly is thought to adjust the amount Cox1 synthesis to its effective assembly within the complex IV.

In this work, a *Δrrf1* mutant with stable mtDNA content has been studied and shown to differentially impact the three OXPHOS complexes with mtDNA-encoded subunits. Interestingly, an increased synthesis of two subunits, Cox1 and Cytb, was observed while the synthesis of the other mtDNA-encoded subunits was reduced, as expected in the absence of a general translation factor. We have shown that the increased synthesis of Cox1 and Cytb is respectively dependent on the translational activators Mss51 and Cbp6. Moreover, the absence of Rrf1 impairs the Cox1 regulatory loop and the association of Mss51 with Cox1-containing assembly intermediates. We propose that the general translation factor Rrf1 might play a role *via* the specific translation activator Mss51, in the early steps of regulatory feedback loop that adjusts the synthesis of Cox1 to its assembly in the complex IV.

RESULTS

Strain	Nuclear and Mitochondrial Genotypes	Reference
CW252	MATα, ade2, ura3, his3, trp1, leu2, can1-100, rho+, Δi	Saint-Georges et al. 2001
Δrrf11	MATα, ade2, ura3, his3, trp1, leu2, can1-100, rrf1::KanR, rho+, Δi	This work
BR2 - 6B	MATa, ade2, ura3, his3, leu2, lys2, can1-100, rrf1::KanR, rho+, Δi	This work
IK43-1	MATα, ade2, ura3, his3, trp1, leu2, can1-100, cbp6::KanR, rho+, Δi	Kühl et al. 2012
Δmss51	MATα, ade2,his3, leu2, trp1, can1-100, mss51::URA3, rho+, Δi	This work
C6R1-8B	MATα, ade2, ura3, his3, leu2, can1-100, cbp6::KanR, rrf1::KanR, rho+, Δi	This work
dMR-6D	MATα, ade2,his3, leu2, lys2, can1-100, mss51::URA3, rrf1::KanR,rho+, Δi	This work
$MSS51^{MYC}$	MATα, ade2, ura3, trp1, leu2, can1-100, MSS51-c-Myc, rho+, Δi	This work
$Rrf1^{3HA}$	MATα, ade2, ura3, trp1, leu2, can1-100, RRF1-3HA, rho+, Δi	This work
dRMC-9D	MATα, ade2, ura3, his3, trp1, leu2, can1-100, MSS51-c-Myc, rrf1::KanR,rho+, Δi	This work
dRMC-5A	MATa, ade2, ura3, his3, leu2, lys2, can1-100, MSS51-c-Myc, rrf1::KanR,rho+, Δi	This work
MCRH-4C	MATα, ade2, ura3, trp1, leu2, can1-100, MSS51-c-Myc, RRF1-3HA, rho+, Δi	This work
Δcox14	MAT a, ura3, his3, leu2, met15, cox14::KanR, rho+, i (BY4147)	Euroscarf
CR1-3C	MATa, ade2, ura3, his3, trp1, leu2, can1-100, cox14::KanR, rrf1::KanR, rho+, Δi	This work

Table III-1 List of strains

Deletion of the yeast mitochondrial recycling factor *RRF1* in a mitochondrial intron-less strain leads to a partial respiratory growth defect

RRF1 was previously reported to be essential for the respiratory competence of *S. cerevisiae*, its deletion causing massive loss of mtDNA that encodes essential subunits of the OXPHOS complexes (Teyssier et al., 2003). However, decreasing the mitochondrial intron content was shown to increase the stability of mtDNA in the *Δrrf1* mutant (Rorbach et al., 2008)and data not shown). We have deleted *RRF1* in the intron-less mtDNA background and tested the phenotype of *Δrrf1* mutant on respiratory media (Fig III-3A). The yeast cells presented a moderate respiratory growth defect when grown on non-fermentable medium at 28°C, which was exacerbated at 37°C. We have raised a polyclonal antibody against Rrf1 from *S. cerevisiae* and tested the accumulation of the protein in the wt and *Δrrf1* strains (Fig III-3B). A band corresponding to the expected 26 kDa was revealed in the wt but not in the mutant. The percent of rho^0 colonies in *Δrrf1* mutant grown for 10 generations was 15%, indicating that mitochondrial DNA content was stable enough to further study the effect of the mutation on the biogenesis of OXPHOS complexes.

The *Δrrf1* mutation impairs the assembly and activity of the respiratory complex IV and ATP synthase

Since the partial respiratory defect in *Δrrf1* mutant could not be solely explained by mtDNA loss, we analyzed the three OXPHOS complexes containing mtDNA-encoded subunits by a combination of biochemical methods.

The steady state levels of the two catalytic subunits of complex III, the mtDNA-encoded Cytb and nuclear DNA-encoded (nDNA) Rip1, were similar in the *Δrrf1* mutant and the wt strain (Fig. III-3C). Measurements of the maximal activity of the complex III showed no difference between the mutant and wt (Fig. III-3D). Mitochondrial proteins were solubilized in n-dodecylmaltoside and subjected to a Blue Native gel electrophoresis (BNGE) to analyze their supramolecular structure. Similar amounts of complex III dimers (III_2) were detected in the mutant and wt (Fig. III-3E). Thus, the absence of Rrf1 has no effect on complex III assembly and activity.

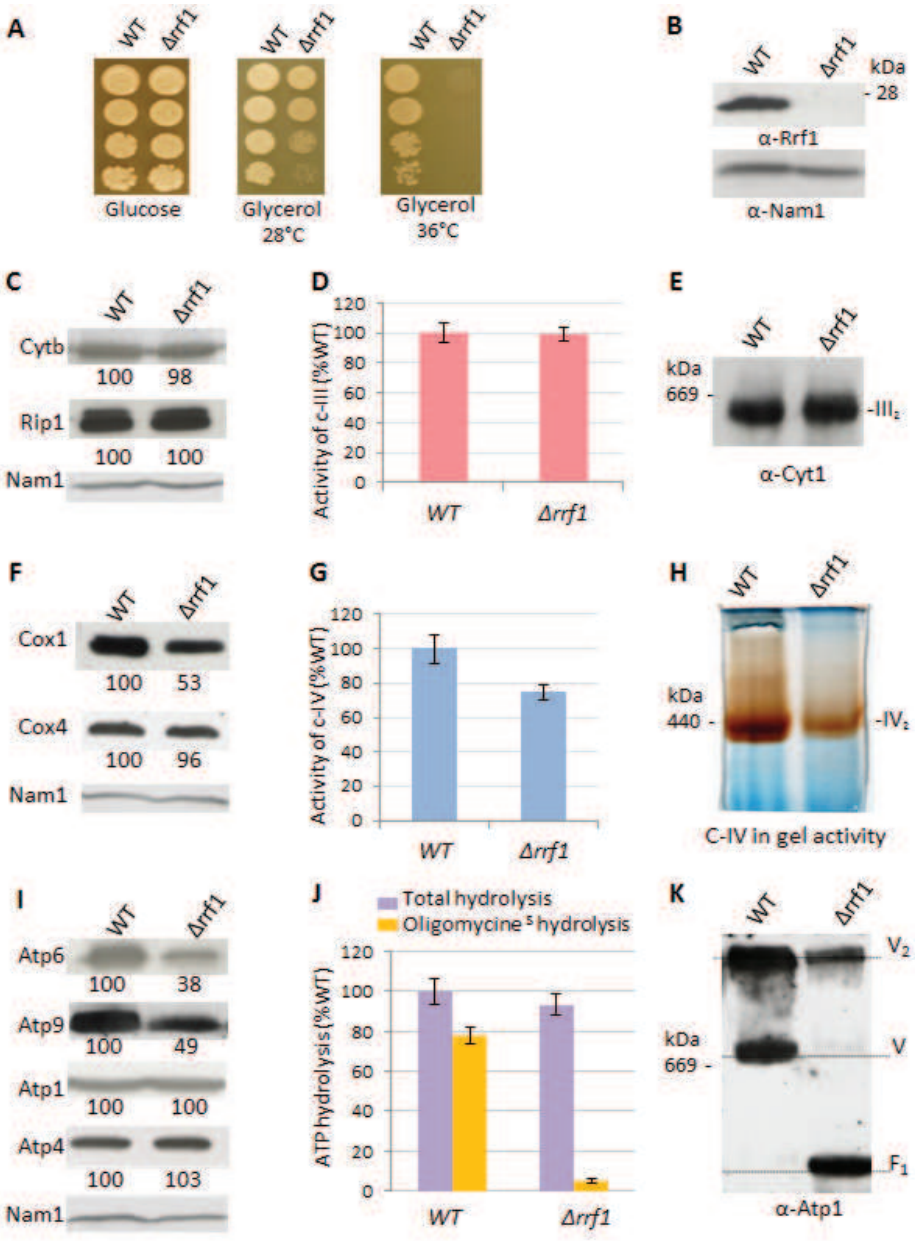


Figure III-3 Effect of the *Δrrf1* mutation on the assembly and activities of OXPHOS complexes.
A) Dilution series of cells from wt and *Δrrf1* were spotted on media containing glucose (fermentable) or glycerol (respiratory) and incubated for 3 days at 28 or 36 °C. B) Mitochondrial proteins were purified from wt and *Δrrf1*, resolved in SDS-PAGE and immunoblotted with antibodies raised against Rrf1 (see Material and Methods) and Nam1 as loading control. A to K: Mitochondria were purified from wt and *Δrrf1*. A, F, I) Mitochondrial proteins were resolved by SDS-PAGE and immunoblotted with antibodies against Cytb, Rip1, Cox1, Cox4, Atp6, Atp9, Atp1, Atp1, Atp4 and Nam1 as loading control. D, G) The ubiquinol cytochrome *c* oxidoreductase activity (complex III activity, D) and the cytochrome *c* oxidase activity (complex IV activity, G) were measured on purified mitochondria. Data represent the mean of three independent experiments and error bars are standard deviation. E, H) Mitochondrial complexes were solubilized in 1% n-dodecylmaltoside, analyzed by BN-PAGE and immunoblotted with antibodiy against Cyt1 (E) or stained for complex IV activity (H). Position of the complex III dimers (III_2), complex IV dimers (IV_2) and size marker are indicated. J) Three independent ATP hydrolysis assays were performed on frozen/thawed mitochondria in absence of osmotic protection and in presence of saturating amounts of ATP.

Standard deviation is indicated. K) Mitochondrial complexes were solubilized in 1% digitonine, analyzed by BN-PAGE and immunoblotted with antibody against Atp1. Positions of the dimers V_2, monomers V, free F_1 and size marker are indicated.

The accumulation of mtDNA-encoded catalytic subunit Cox1 in the *Δrrf1* mutant was decreased by 50% while the steady state level of nDNA-encoded Cox4 was not affected (Fig. III-3F). Activity measurements on isolated mitochondria showed that the maximal activity of complex IV in the *Δrrf1* mutant was 70% of the wt activity (Fig. III-3G). In BNGE analysis, complex IV was resolved as a functional dimer (IV_2) that could be stained in an in-gel activity assay (Fig. III-3H). Quantification of active complex IV dimers showed a signal decreased by 50% in *Δrrf1* mutant when compared to the wt, suggesting an impairment of both the activity and assembly of this complex.

The mtDNA-encoded subunits of ATP synthase, Atp6 and Atp9, were both substantially decreased in the *Δrrf1* mutant, to 38% and 49% of the wt amount, respectively. In contrast, no significant difference was observed in the accumulation of nDNA-encoded Atp1 and Atp4. (Fig. III-3I). ATP synthase is composed of the catalytic matrix-exposed F_1 sector and the membrane-integral proton channel-containing F_o sector, physically linked by the central and peripheral stalks (Velours and Arselin 2000; Lau et al. 2008). Atp6 and Atp9 are part of the F_o, while Atp1 and Atp4 belong to the F_1 and the peripheral stalk, respectively. F_1 sector bears the ATP binding sites and can synthesize or hydrolyze ATP (Boyer, 1997). When the enzyme is correctly coupled, the F_1-mediated hydrolysis of ATP is inhibited in presence of oligomycin, which binds to the F_o sector. However, if F_o and F_1 fail to properly assemble, oligomycin can no longer inhibit the ATP hydrolysis by soluble F_1. Thus, measurements of the ATP hydrolysis by the enzyme can provide insight in both its activity and assembly state. As shown in (Fig. III-2J), total hydrolytic activity of the *Δrrf1* mutant was similar to the wt, while its oligomycin sensitive hydrolysis was dramatically decreased indicating a defective coupling between F_1 and F_o. This was indeed confirmed by BNGE analysis that showed a markedly reduced assembly of F_o and F_1 into dimers and monomers (V_2, V) and an abundant accumulation of free F_1 in *Δrrf1* (Fig. III-2K). In conclusion, the absence of the mitochondrial recycling factor Rrf1 displays a differential impact on the assembly and activity of the three OXPHOS complexes, going from no effect on complex III, to a clear decrease in complex IV and a severe defect in ATP synthase.

The absence of Rrf1 impacts the translation and stability of mitochondrial mRNAs.

In order to determine if the differential impact of *Δrrf1* on the three OXPHOS complexes was due to differences in the translation of their mtDNA-encoded subunits, we have studied the effect of the absence of Rrf1 on mitochondrial protein synthesis. We performed *in vivo* ^{35}S radioactive labeling of newly synthesized mtDNA-encoded proteins (Fig. III-4A). The synthesis of the three mtDNA encoded subunits of ATP synthase (Atp6, 8, 9), and of two (out of three) mtDNA encoded subunits of complex IV (Cox2 and Cox3) were decreased, as expected in the absence of a general translation factor. But surprisingly, the synthesis of Cox1 and Cytb were increased two fold by comparison to wt (Fig. III-4A, 4B). Transformation of the *Δrrf1* cells with a plasmid carrying the *S. cerevisiae RRF1* (yRRF1) gene restored a wt synthesis of the seven subunits (Fig. III-4C,4D). Interestingly, the same restoration was observed by transforming the *Δrrf1* cells with a plasmid expressing the human ortholog *mtRRF*. (Fig. III-4C, 4D).

We then asked if the impairment of translation seen in the *Δrrf1* strain could have an upstream impact on the stability of mitochondrial mRNAs. We performed a Northern blot analysis and observed that mRNAs encoding proteins whose synthesis was decreased in the *Δrrf1* strain (Atp6/8, Atp9) also showed decreased accumulation (Fig. III-4E). On the contrary, the mRNAs of the two over-synthesized proteins, Cox1 and Cytb1, were stable and even over-accumulated slightly when compared to the wt strain. Thus, there is a correlation between the level of the synthesis of mtDNA encoded subunits and the steady state level of their corresponding mRNAs in the *Δrrf1* mutant

Increased syntheses of Cox1 and Cytb in the *Δrrf1* mutant are dependent of their specific translational activators

Synthesis of Cytb and Cox1 are controlled *via* their respective translational activators, Cbp6 and Mss51, that adjust the levels of their synthesis to the assembly into complexes III and IV (Gruschke et al., 2011; Perez-Martinez et al., 2009) To gain insight into the role of these translational activators in the increase of the syntheses of Cytb and Cox1 in the *Δrrf1* mutant we constructed double mutants combining the *Δrrf1* deletion with deletions of *MSS51* or *CBP6*. *In vivo* radioactive labeling has shown that the absence of each translational activator impaired

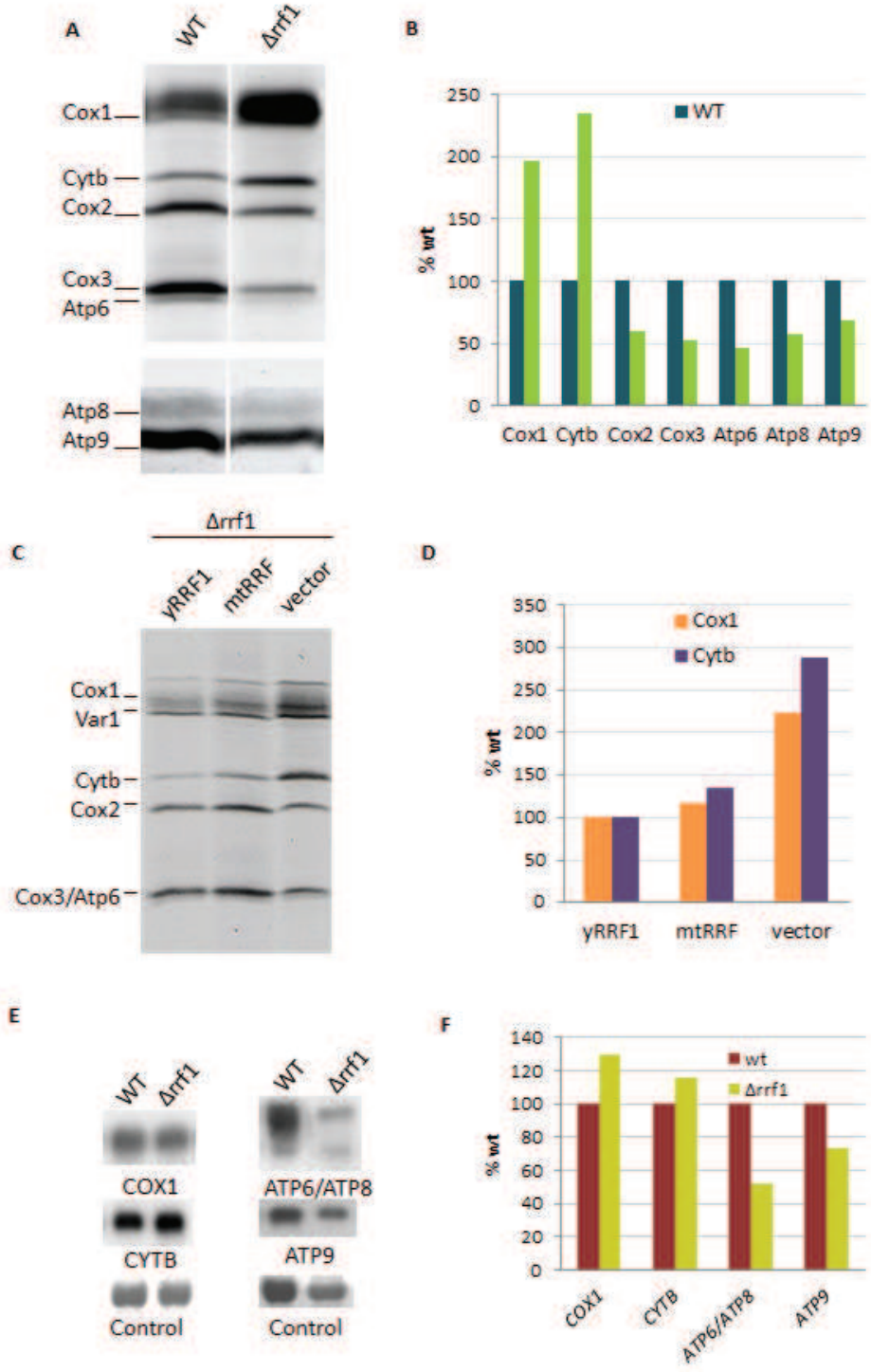


Figure III-4 Effect of the *Δrrf1* mutation on the translation and stability of mitochondrial mRNAs.
A) In vivo labeling of mitochondrial translation products was performed as indicated in Material and Methods. Wt and *Δrrf1* cells were labelled for 15min and the proteins were analysed by SDS-PAGE and autoradiography. B) Quantifications of labelled mitochondrial proteins from A).wt (blue) and *Δrrf1* (green). C) *Δrrf1* cells were transformed with either an empty vector or the same vector carrying *RRF1* (*S. cerevisiae*) or *mtRRF* (*H. sapiens*) and *in vivo* labelled as in A). D) Quantifications of labelled Cox1 (orange) and Cytb (violet) from C. E) Indicated mitochondrial mRNAs were extracted from wt and *Δrrf1* strains as described in Material and Methods and analyzed by Northern Blot with various probes. F) Quantification of mRNAs from E). rRNAs stained with methylene blue are the control.

only the translation of its target mRNA (Fig. III-5A, 5B). The level of Cytb in the double mutant *Δrrf1 Δcbp6* or of Cox1 in *Δrrf1 Δmss51* was strongly decreased in both wt and the single mutant *Δrrf1,* and was lower than in the respective single mutants. Thus, the increase of synthesis of Cytb or Cox1 in *Δrrf1* appeared to be Cbp6- or Mss51-dependent, respectively.

We then constructed diploid strains all carrying a homozygous *Δrrf1/Δrrf1* deletion as well as either heterozygous or homozygous deletions of *CBP6* (*Δcbp6/CBP6 or Δcbp6/Δcbp6)*. *In vivo* radioactive labeling has shown that he amount of Cytb was higher in the heterozygous strain (*Δcbp6/CBP6)* than in the homozygous strain (*Δcbp6/Δcbp6*), showing that it is Cbp6 dose-dependent (Fig. III-5C, 5D). Deletion of a single dose of *MSS51* completely impairs the synthesis of Cox1 (Fig. III-5A, 5B); however, increasing its dose by transformation with a multicopy plasmid carrying *MSS51* increases the synthesis of Cox1 (Fig. III-5E, 5F), in accordance with previously reported data (Barrientos et al. 2002). A similar effect on the synthesis of Cox1 was also described in the absence of Cox14, in which Cox1 is synthesized in high amounts but is prone to unspecific aggregation and degradation, impairing the function of complex IV (Barrientos et al. 2004; Mick et al. 2010; McStay et al. 2013b). We constructed the double mutant *Δrrf1 Δcox14* and observed that the synthesis of all mt-DNA encoded proteins was strongly decreased and that of Cox1 was nearly completely abolished (Fig. III-5E, 5F). In conclusion, both syntheses of Cox1 and Cytb appeared regulated by their specific translational activators in the *Δrrf1* mutant. Interestingly, the synthesis of Cox1 is increased in the *Δrrf1* mutant, despite the reduced level of complex IV assembly, suggesting that the coupling between translation and assemblyis impaired in the absence of the recycling factor Rrf1.

Absence of Rrf1 changes the equilibrium between the Mss51-containing complexes involved in translation and assembly

Given the large amount of information available for the Mss51-mediated Cox1 regulatory loop, we have decided to further study the interactions between Rrf1 and Mss51.
Mss51 has been detected in different complexes in native gel analyses (Fontanesi et al., 2009; Khalimonchuk et al., 2009; Mick et al., 2010; McStay et al. 2013a). In the complexes of highest

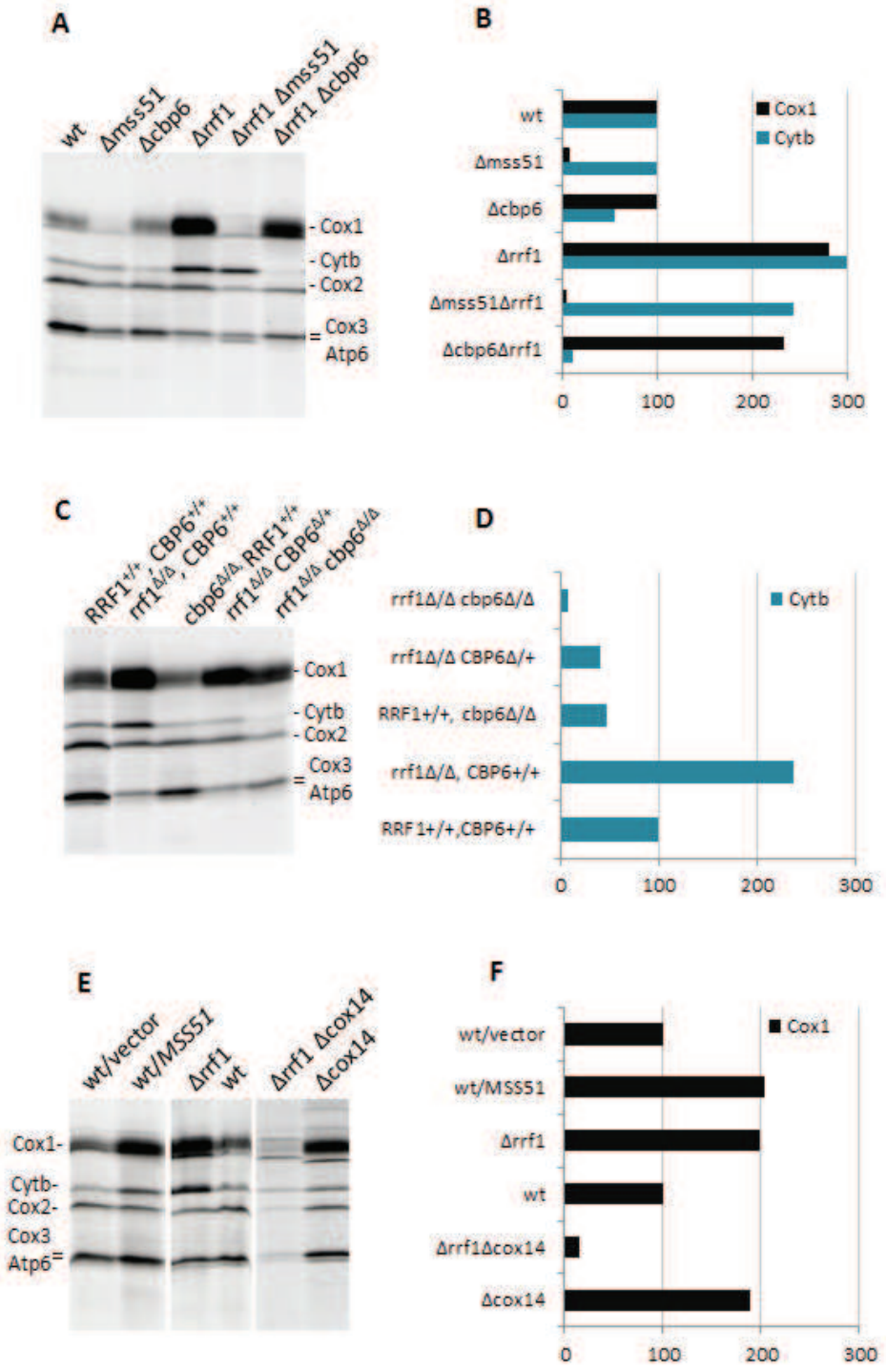


Figure III-5 Role of the specific translational activators in a *Δrrf1* context.
A), C), E) *In vivo* labelling of mitochondrial proteins from indicated strains was performed as in IV-4A (see text for details). B), D) and F) represent quantifications of Cox1 (black) and Cytb (blue) from A), C) and E), respectively.

molecular weight, Mss51 was suggested to bind the translational apparatus (HMW). Complexes varying from 250-450 kDa correspond to the Mss51-Ssc1-Cox1 containing assembly intermediates ($Mss51^{A}$). Finally, the low molecular weight complexes (120-180 kDa) are described as a translation-active reserve of Mss51 ($Mss51^{T}$) (see model in Fig. III-2).

In order to test if and how the absence of Rrf1 could affect the behavior of Mss51-containing complexes, we have constructed a c-Myc-tagged version of Mss51. We have analyzed mitochondrial proteins from the $MSS51^{MYC}$ and $MSS51^{MYC}\Delta rrf1$ strains. As shown on (Fig. III-6A), the absence of Rrf1 has no effect on the steady state levels of Mss51 and Ssc1, while the accumulation of Cox1 was decreased by 50% in $MSS51^{MYC}\Delta rrf1$ as expected (see Fig III-3D). Mitochondrial proteins were then solubilized and subjected to BNGE analyses (Fig. III-6B). In the $MSS51^{MYC}$ strain, Mss51 was essentially detected in 250-450 kDa assembly intermediates ($Mss51^{A}$) as described in (Fontanesi et al. 2009; Mick et al. 2010). In $MSS51^{MYC}\Delta rrf1$ strain, Mss51-containing complexes of lower molecular weight also accumulated. In particular, substantial amount of a 140 kDa complex was detected, which likely corresponds to the translation-active Mss51 ($Mss51^{T}$). In addition, a low amount of a high molecular weight tail, likely corresponding to HMW complexes, was also detected in the mutant.

Thus the absence of RRf1 does not affect the steady state level of Mss51 but leads to a shift in the dynamic equilibrium of Mss51-containing complexes towards the translation- active reserve of Mss51.

Interaction between Rrf1 and Mss51 might be ribosome-mediated

The shift in the dynamic equilibrium of Mss51-containing complexes towards the translation-active complexes might suggest that Rrf1 directly interacted with Mss51 in the translation process. To address this question, we performed a series of co-immunoprecipitation experiments from strains exhibiting tagged versions of Mss51 (c-Myc, $MSS51^{MYC}$) and Rrf1 (HA, $RRF1^{HA}$) as well as from the strain $MSS51^{MYC}\Delta rrf1$. Mitochondrial proteins solubilized with 1% digitonine were immunoprecipitated with HA- or c-Myc-agarose beads and the immunoprecipitates analyzed by SDS-PAGE with various antibodies (Fig III-7). When Rrf1 was

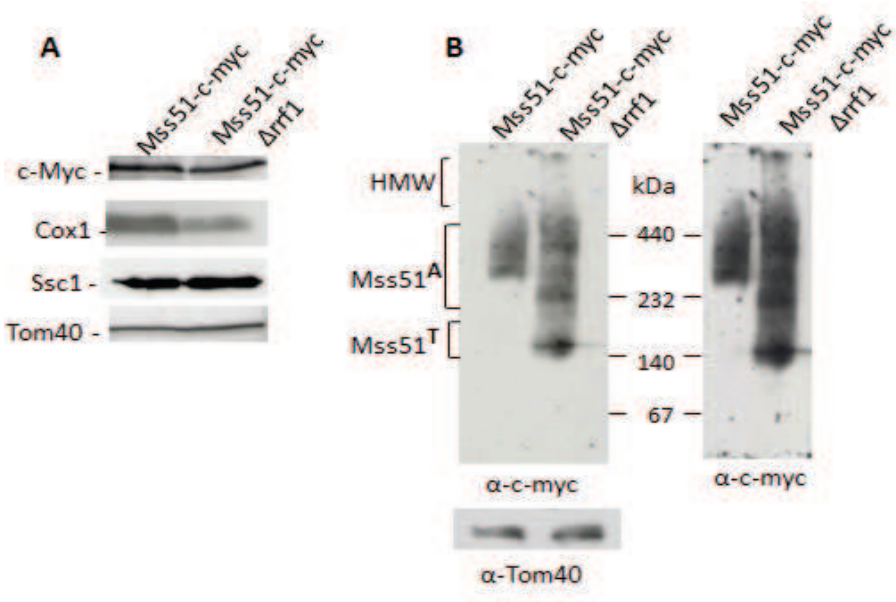


Figure III-6 Supramolecular organisation of Mss51-Ssc1 complex in the *Δrrf1* mutant.
A) Mitochondrial proteins from *MSS51*^{MYC} and *MSS51*^{MYC}*Δrrf1* were analysed by SDS-PAGE and immunoblotted with antibodies against c-Myc, Ssc1, Cox1, Tom40 as loading control. B) Mitochondrial complexes were solubilized in 1% digitonine, analysed by BNGE and immunoblotted with antibodies against c-Myc and Tom40 as a loading control. Positions of ribosome-associated Mss51 (HMW), Mss51 complexes involved in the assembly of Cox1 (Mss51^{A}) and Mss51-Ssc1 complex linked to translation of Cox1 (Mss51^{T}) are indicated according to (Fontanesi et al., 2009; Mick et al., 2010) as well as the size marker. Two different film expositions are shown.

absorbed on HA-agarose beads, small but repeatedly detected fractions of three ribosomal proteins, Mrp20, Mrpl4 and Mrp51, were co-immunoprecipitated with Rrf1 while Mss51-cmyc, Ssc1 or Cox1 were not. When Mss51 was absorbed on c-Myc-agarose beads, Ssc1 and Cox1 and small fractions of the three ribosomal proteins were co-immunoprecipitated with Mss51 while Rrf1-HA was not. The absence of Rrf1 did not modify the interactions of Mss51 with Ssc1, Cox1 and the ribosomal proteins.

In conclusion, no direct interaction between Rrf1 and Mss51 was observed in these co-immunoprecipitation experiments but both were found to interact with the ribosome, leaving the possibility of an indirect, ribosome-mediated interaction between the two proteins.

DISCUSSION

The major obstacle in studying the mitochondrial translation in *S. cerevisiae* is the fact that deletions of many factors involved in this process cause high instability and loss of mtDNA

(Contamine and Picard, 2000). The two general translation factors controlling ribosome recycling, Rrf1 and the GTPase Mef2, were both reported to cause loss of mtDNA when deleted

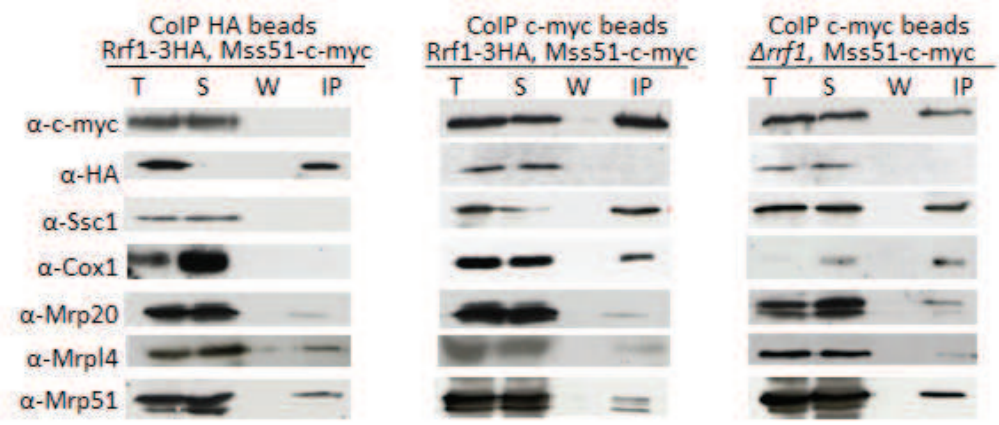


Figure III-7 Coimmunoprecipitation of Rrf1 or Mss51 with ribosomal proteins.
Mitochondrial proteins purified from the *MSS51*^{MYC}*RRF1*^{3HA} and *MSS51*^{MYC}*Δrrf1* strains were subjected to co-immunoprecipitation experiments using c-Myc or HA antibodies coupled to agarose beads as indicated. The fractions were analysed by SDS-PAGE and immunoblotted with antibodies against c-Myc, HA, Cox1, Ssc1, Mrp20, Mrpl4 and Mrp51. T: total; S: supernatant; W: washing; IP: immunoprecipitate.

in yeast (Callegari et al., 2011; Teyssier et al., 2003). However, we have here characterized a *Δrrf1* mutant constructed in an intron-less mitochondrial genome that only causes a weak instability of the mtDNA.

This *Δrrf1* mutant exhibits a partial respiratory defect and presents differential effects on the synthesis of different mtDNA-encoded subunits and their assembly in the corresponding complexes. Synthesis of ATP synthase subunits was decreased as well as that of Cox2 and Cox3, subunits of complex IV. Interestingly, the decrease in the translation of these OXPHOS subunits was reflected in the decreased accumulation of their mRNAs, indicating that the absence of a general translation factor can affect the stability of mitochondrial mRNAs.

A two-fold increase in the synthesis of Cox1 and Cytb was observed despite the fact that their downstream accumulation was either decreased for Cox1 in complex IV or wt-like for Cytb in complex III. The assembly and activity of respiratory complex III showed no significant difference when compared to the wild type while those of complex IV and ATP synthase were

decreased in the *Δrrf1* mutant. The core of complex IV is made up by Cox1, Cox2 and Cox3 and their syntheses are coupled to facilitate the subsequent assembly process (Naithani et al. 2003; Fiori et al. 2005). Syntheses of Cox2 and Cox3 are decreased in absence of Rrf1, which could explain the finally reduced accumulation of Cox1 within complex IV despite the initial boost in Cox1 synthesis.

However, the translation of mitochondrialy-encoded mRNAs is regulated *via* messenger-specific translational activators, and for both Cox1 (Mss51) and Cytb (Cbp6) these activators were found to adjust the translation in function of their subsequent assembly in respiratory complexes (Perez-Martinez et al. 2003; Barrientos et al. 2004; Gruschke et al. 2011). On the contrary in the *Δrrf1* mutant, the translation rate of Cox1 and Cytb is increased suggesting that this adjustment is lost and that Rrf1 is required for the regulatory loop between synthesis and assembly. Interestingly enough, the rate of Cox1 synthesis is also high in the absence of Cox14 or Coa3 (Barrientos et al., 2004; Mick et al. 2010; McStay et al. 2013b), but most of it is degraded and the complex IV is not assembled. In absence of Cox14, the interaction of Cox1 with Mss51 is unstable and Mss51 readily dissociates to re-stimulate futile Cox1 synthesis (Barrientos et al. 2004; Perez-Martinez et al. 2009). Double deletion of *COX14* and *RRF1* shows a very severe phenotype, with levels of newly synthesized Cox1 being nearly undetectable, suggesting a role for Rrf1 in the stabilization of newly synthesized Cox1. However, this function of Rrf1 is probably indirect, as no interaction of Rrf1 with Cox1 was detected.

We have shown the importance of the amount of Mss51 or Cbp6 for the increased synthesis of Cox1 or Cytb, suggesting that more Mss51 and Cbp6 are available for translation in absence of Rrf1. Knowing that Mss51 is present in different protein complexes either to promote the translation of the *COX1* mRNA ($Mss51^{T}$) or to allow a post-translational step in complex IV assembly ($Mss51^{A}$) (Fontanesi et al. 2009; Mick et al. 2010), it was tempting to propose that the absence of Rrf1 would lead to the trapping of Mss51 within the translation complex. Indeed, BNGE analyses of *Δrrf1* mutant show a different pattern of distribution of Mss51-containing complexes. In the control strain we essentially detected Mss51 in the assembly complexes, while in the *Δrrf1* strain Mss51 was distributed between the assembly- and translation-competent complexes. It is important to note that in the mutant strain a high

molecular weight tail, in which Mss51 was proposed to be attached to the ribosomes, was also detected, possibly indicating a stalling of Mss51 on the translational apparatus. Thus, there is an apparent shift of Mss51-containing complexes towards translation-competent state in the *Δrrf1* mutant.

We have shown that a small fraction of Rrf1 or Mss51 co-immunoprecipitates with ribosomal proteins from both the large and small subunits, but no direct interaction between Mss51 and Rrrf1 was detected. These experiments have shown the already known interactions of Mss51 with Cox1 and Ssc1, that also bind to mitoribosomes (Fontanesi et al., 2009; Mick et al., 2010; Westermann et al., 1996). It has been previously reported that mutations in the ribosomal 15S rRNA could interfere with the action of Mss51 in *S. cerevisiae* (Decoster et al., 1990), and that the binding of the bacterial homolog RRF alone to the ribosome induces important conformational changes (Barat et al., 2007; Gao et al., 2005; Yokoyama et al., 2012). Thus, the binding of Rrf1 to the mitochondrial ribosome might induce conformational change to the ribosomes contributing to the release of Mss51-Ssc1 from the translational apparatus and their cycling from the translation-competent complexes to the assembly complexes. In absence of Rrf1, Mss51 could be stalled on the ribosome and the transfer from translation- to assembly-competent state could be defective, leading to an increase in the pool of free Ms51-Ssc1, ready to promote the translation of Cox1 (Fig. III-8). Alternatively, it cannot be excluded that it is the ribosome dissociation *per se* that could release Mss51-Ssc1 from the ribosome, rather than just the binding of Rrf1. However, the deletion of Rrf1 exhibits a partial respiratory deficiency showing that the ribosome recycling is not completely blocked in its absence, probably due to other translation factors as the GTPase Mef2 (Callegari et al., 2011) or the initiation factor If3, proposed to actively participate in ribosome disassembling in mammal mitochondria (Christian and Spremulli 2009).

Finally, a number of proteins involved in early steps of Cox1 translational regulation have human orthologs and further elucidation of this process in yeast could be instrumental for the analyses of the human system. Severe pathological mutations have been reported for the human ortholog of Cox14 (Szklarczyk et al., 2012; Weraarpachai et al., 2012). The ortholog of Mss51 (NP_001019764.1) has not yet been thoroughly characterized, but there are reports

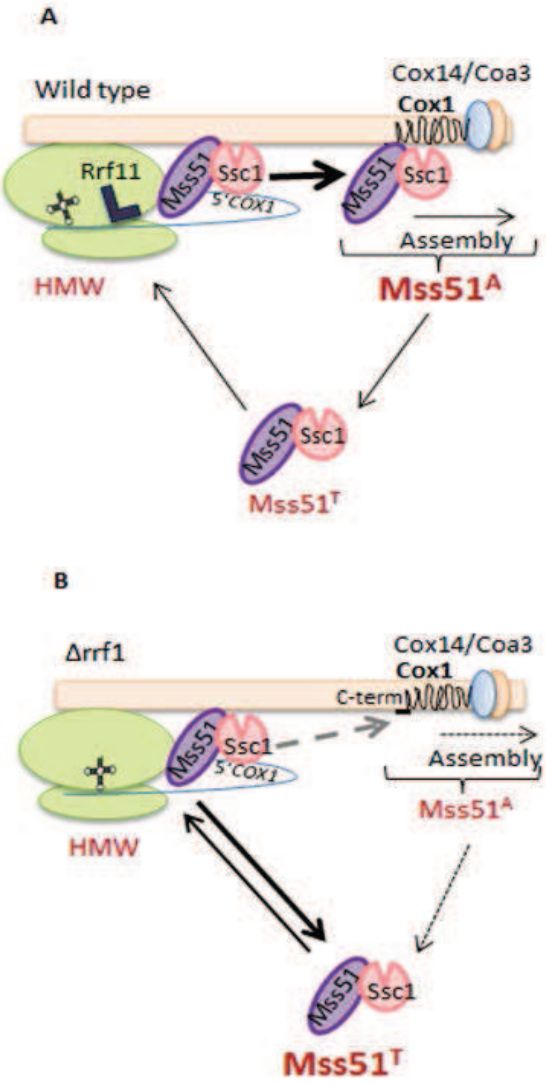


Figure III-8 Working model for the involvement of Rrf1 in the first steps of the Cox1 regulatory loop.
Representative proteins involved in first steps of Cox1 assembly are indicated. IMS, IM are the intermembrane space and the inner membrane, respectively. Mss51-Ssc1 bind to the 5'UTR of *COX1* mRNA and promote the synthesis of Cox1. Cox14-Coa3 are required to protect and stabilize the nascent Cox1. A) Upon completion of the synthesis, Rrf1 binds the ribosome and changes its conformation, triggering the dissociation of the two ribosomal subunits and the release of Mss51-Ssc1, which can bind to the C-terminal of Cox1 (Shingu-Vazquez et al., 2010). B) Ribosome recycling proceeds slowly in the absence of Rrf1, Mss51-Ssc1 is not trapped by the newly synthetized Cox1 protein. The accumulation of the translation active complex containing Mss51-Ssc1 leads to the increase in *COX1* translation.

suggesting it could be linked to neurological disorders (Liu et al., 2011). Both Rrf1 and Mef2 are also conserved in humans (Rorbach et al., 2008; Tsuboi et al., 2009) and the process of ribosome recycling still needs to be further detailed. Among diseases of the mitochondrial respiratory chain the deficiency of complex IV in human patients is frequently encountered (detailed references at http://omim.org/entry/220110) and investigations of the underlying assembly and regulatory processes, in both humans and model organisms, can only be precious for therapeutic development.

2. *Δrrf1* and the suppression mechanism

Although the mechanism by which the absence of *RRF1* is able to compensate for the respiratory defect of *bcs1*-F342C has not been thoroughly studied at this point, the characterization of the *Δrrf1* mutant might provide some insight into this matter. We have shown that the total hydrolytic activity of ATP synthase is unaffected in *Δrrf1* strain (see Fig. II-

5), which suggests that the mechanism of suppression is likely to be different from the one described for *atp* mutations of the F_1 sector. One of the prominent effects of the *Δrrf1* mutant is the increase in *COX1* and *CYTB* translation, which may be involved in the suppression mechanism. Experiments aiming at modulating the rate of Cox1 and Cytb syntheses are currently been set up in the laboratory. They might allow us to better understand if and how this type of modulation affects the compensation of *bcs1*-F342C by the deletion of *RRF1*.

MATERIALS AND METHODS

Yeast trains, media and genetic methods

All used strains are listed in table 1. The non-fermentable media contain 2% glycerol and the fermentable media contain either 2% glucose or 2% galactose with 0.1% glucose. For measurements of rho^0 production, cells were grown in liquid medium with 2% glycerol, inoculated in a fresh medium with 2% galactose, grown for 10 generations and streaked on selective media for rho^0 percentage count. For growth tests on plates, 1 OD_{600} of cells were harvested and serial 10-fold dilutions were spotted on plates containing glucose or glycerol as carbon source and incubated for 4 days. Tetrad dissection was performed using a Singer MSM micromanipulator. To check the presence of the mitochondrial genome, the cells were crossed with tester strains devoid of mitochondrial genome and the growth of diploids on glycerol medium was analyzed.

Complementation tests

Yeast strain bearing KanR-disrupted *RRF1* gene (BR2-6B) was crossed with the wild type strain CW252, diploids were isolated with Singer MSM micromanipulator and transformed with either an empty yeast vector or the same vector expressing *RRF1* ORF from *S. cerevisiae* or human *mtRRF*. The vector bearing the human gene was constructed as previously indicated (Rorbach et al., 2008). Sporulation of transformed diploid strains was induced and the spores carrying both the KanR deletion cassette and the plasmid marker were selected after dissection. Cells

were grown overnight in selective rich medium and mitochondria-encoded proteins were *in vivo* radiolabeled.

Gene deletion, epitope tagging

Deletions and tagging of genes were performed by transformation of strains with PCR-amplified URA3, KanR, 3HA or Myc cassettes, including site-specific 5' extensions for homologous recombination (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.

Co-immunoprecipitation experiments

Mitochondria were solubilised in 50mM Tris HCl pH 7.4, 100mM NaCl, 1% digitonine for 30 min at 4°C and centrifugated 15 min at 100000g. The supernatants were incubated with polyclonal anti-c-Myc or anti-HA 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.

RNA extraction and RNA hybridization

RNAs were purified from exponentially grown cells and their amount was estimated by spectrophotometric measurements at 260nm. RNAs were separated on 1.2% agarose formaldehyde gels and transferred on Hybond-C extra membrane (Amersham, Buckinghamshire, UK). Prehybridization and hybridization were done at 42°C in 50% formamide and Denhardt. Radiolabeled probes used for RNA detection, were generated by random priming (random primer DNA labeling system, Invitrogen, San Diego) from PCR-amplified primers of Cox1 (1.6 kb), Cytb (0.65 kb), ATP6/8 (0.7 kb) and ATP9 (0.25 kb). RNAs were quantified by using rRNAs as standard, after staining the membrane with methylene blue (Herrin and Schmidt, 1988).

Mitochondria purification, SDS-PAGE, BN-PAGE

Mitochondria were isolated as in Lemaire and Dujardin (2008) from cells grown overnight at 28°C in galactose medium. Denaturating 12-15% SDS-PAGE was used to separate mitochondrial proteins. For BN-PAGE, mitochondria were solubilized in digitonin (1%) or n-dodecylmaloside (1%) and the complexes were separated on 4-15% polyacrylamide gradient gels (Lemaire and Dujardin, 2008; Schägger and Pfeiffer, 2000). SDS- and BN-PAGE were electro-transferred and immunodetection was carried out using the chemiluminescent method from Pierce, except for radio-labeled samples that were detected by autoradiography. Polyclonal antibodies against Cyt1, Nam1 and Rrf1 were raised in the laboratory. The polyclonal anti-Mrp20, Mrpl4, Rip1, ATP synthase subunits, Tom40 and Ssc1 were gifts from M.Ott (Stockholm, SE), T.D. Fox (Ithaca, USA), N. Fisher (Liverpool, UK), J. Velours (Bordeaux, FR) and N. Pfanner (Freiburg, DE), respectively. The monoclonal anti-HA and c-Myc were gifts from R. Schweyen (Wien, A) and JM Galan (Paris, FR), respectively. The monoclonal anti-Cox1 and anti-Cox4 are from Molecular Probes.

Polyclonal α-Rrf1 antibody production

The ORF of RRF1 (with exclusion of the predicted mitochondrial targeting sequence) was PCR-amplified and cloned into the Nde1 and Xho1 sites of Pet24b *E.coli* expression vector, flanked by 6His tag. Pet24b-RRF1-6His was amplified in XL2Blue and used to transform BL21codon+ *E. coli* cells. Rrf1-6His protein was produced in IPTG-induced BL21codon+ cells, purified on NiNTA resin and injected into rabbits. Crude sera from immunized rabbits were recovered.

Activity measurements for complexes III, IV and ATP synthase

The activities of complexes III and IV 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 presence of saturating amounts of decylubiquinol and the cytochrome *c* oxidase (complex IV) activity 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. The specific ATPase activity at pH 8.4 of non-osmotically protected mitochondria (100 µg of proteins) was measured at 28°C in

the presence of saturating amounts of ATP with or without oligomycin (Lemaire and Dujardin, 2008). In-gel activity assay of complex IV was performed after resolving mitochondrial complexes on BN-PAGE and incubating the gel stripes in the activity buffer as previously described (Nijtmans et al. 2002).

***In vivo* radioactive labeling of mitochondrial proteins**

In vivo labeling of mitochondrial translation products was performed in cells grown overnight in rich medium with 2% glucose used as a carbon source. 0.6 OD_{600} of cells was harvested washed once and then resuspended in 500µl of reaction buffer (0.5M KP_i, pH 6, 2% galactose) and incubated for 15min at 28°C. Cycloheximide was added at the final concentration of 0.6 mg/ml and after 10min incubation at 28°C the labeling reaction was started by the addition of 70µCi [^{35}S]-mehionine + cysteine. The labeling was stopped after 10-20 min of incubation by putting the cells on ice, centrifuging and freezing the pellets in liquid nitrogen. Proteins were extracted by alkaline treatment, precipitated with 25% TCA and resolved by 17.5% SDS-PAGE.

IV - From yeast to man

1. *Genotype to phenotype correlations in mitochondrial disorders*

The usefulness of *S. cerevisiae* as a model organism for investigations of diverse basic processes in mitochondrial biogenesis and physiology has been well documented for many years. But, what we have learned from this unicellular eukaryote can cross the borders of fundamental biology and be of extraordinary use for applied research, in particular for the field of human mitochondrial disorders. These diseases affect the OXPHOS system and are often described as being rare, while they are in fact much more common than previously thought among the inherited metabolic diseases (Schaefer et al., 2004). Moreover, mutations of mtDNA may be involved in complex metabolic disorders, such as diabetes, cancer and neurodegenerative diseases (Ciccone et al., 2013; Morán et al., 2012; Poulton et al., 2002; Wallace, 2012), although it is still debated whether they should be considered the cause or the consequence of these pathologies.

Requirements for energy and other aspects of mitochondrial function can significantly change in different cell types and tissues and diagnosis may be hindered by the fact that different tissues of the same patient may not display the same effect on the OXPHOS system. Mutations of numerous nuclear and mitochondrial genes are involved in mitochondrial dysfunction, and in the latter case heterogeneity plays an important role in determining the expression and severity of a disease as well. Mutations in genes encoding subunits or proteins directly involved in the assembly of a given complex often result in an isolated deficiency of that complex, while the combined deficiencies of several complexes arise from mutations in genes encoding proteins involved in maintenance, replication and transcription of mtDNA, protein translation, import, fusion and fission (for review (Smits et al., 2010).

During my PhD, I have studied Bcs1, an assembly factor of OXPHOS complex III and some other proteins that appeared to modulate this assembly process, particularly the mitochondrial ribosomal recycling factor Rrf1. The function of both Bcs1 and Rrf1 appears to be

at least partially conserved from yeast to humans and I hope that the results obtained in yeast will have some medical interest in the longer term.

2. Yeast as a model for mutations in BCS1L

Mutations in *BCS1L* cause the most severe complex III deficiencies. Disease-related mutations in *BCS1L* found to date are either homozygous or compound heterozygous, leading to extremely different clinical pictures, as previously discussed (Blázquez et al., 2009; Fernandez-Vizarra et al., 2007; Gil-Borlado et al., 2009; Hinson et al., 2007; de Lonlay et al., 2001; De Meirleir et al., 2003; Visapää et al., 2002). Looking at the primary structure of *BCS1L,* disease-related mutations have been found in all domains of the protein and there is no clear correlation between their position and the severity of the clinical outcome (Fig. IV-1). As *BCS1L* can partially rescue the respiratory deficiency of the yeast *Δbcs1* mutant, complementation of this mutant with human gene carrying disease mutations have been previously performed (Hinson et al., 2007; de Lonlay et al., 2001). The ability of these mutations to complement the yeast mutant is usually consistent with the severity of the phenotype in patients. However, since the rescue of the yeast respiratory growth by *BCS1L* is only partial, subtle differences of phenotype might be overlooked. A different approach is to construct the equivalents of human *BCS1L* disease-related mutations in yeast, in both haploid and diploid strains. One of the studied examples is the F368I mutation, found in a patient with a complex III deficiency (Fernandez-Vizarra et al., 2007; Fig. IV-2). The equivalent mutation in *S. cerevisiae* is F401I*,* presented in the third part, which impairs the respiratory growth of yeast cells on respiratory media and is important for nucleotide hydrolysis. Other mutations may not present a clear respiratory phenotype in yeast, such as N314S and Q302E, equivalents of human N277S and Q334E, responsible for mild complex III deficiency and Bjornstad syndrome, respectively (Hinson et al., 2007; de Lonlay et al., 2001; Fig. IV-2). This may be due to differences in the severity of *BCS1L*-related syndromes, as well as divergence between the yeast and human proteins. However, a detailed biochemical characterization of these mutants might also reveal defects in the activity of Bcs1. Both types of

studies would help to dissect the interactions and functions of residues and domains within Bcs1.

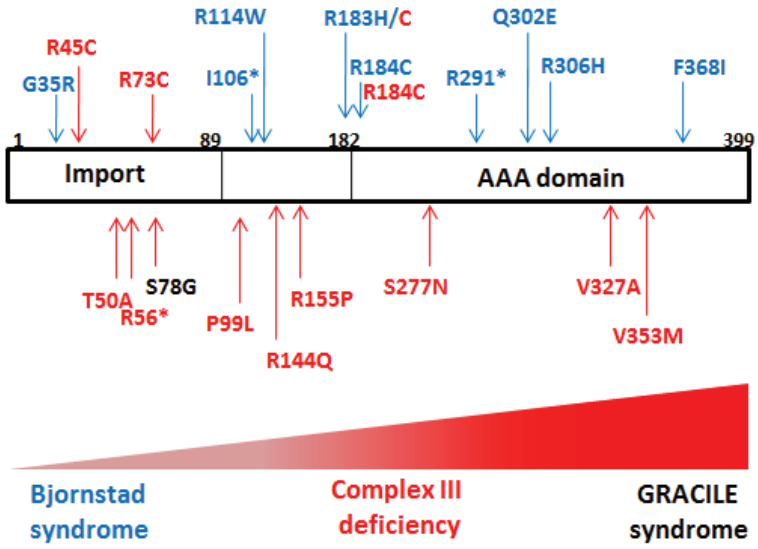


Figure IV-1 Schematic representation of BCS1L primary structure.
Positions of disease-related mutations are indicated. Color code is blue for Bjornstad syndrome , red for complex III deficiencies and black for GRACIE syndrome. Severity of the diseases is depicted in the bottom drawing.

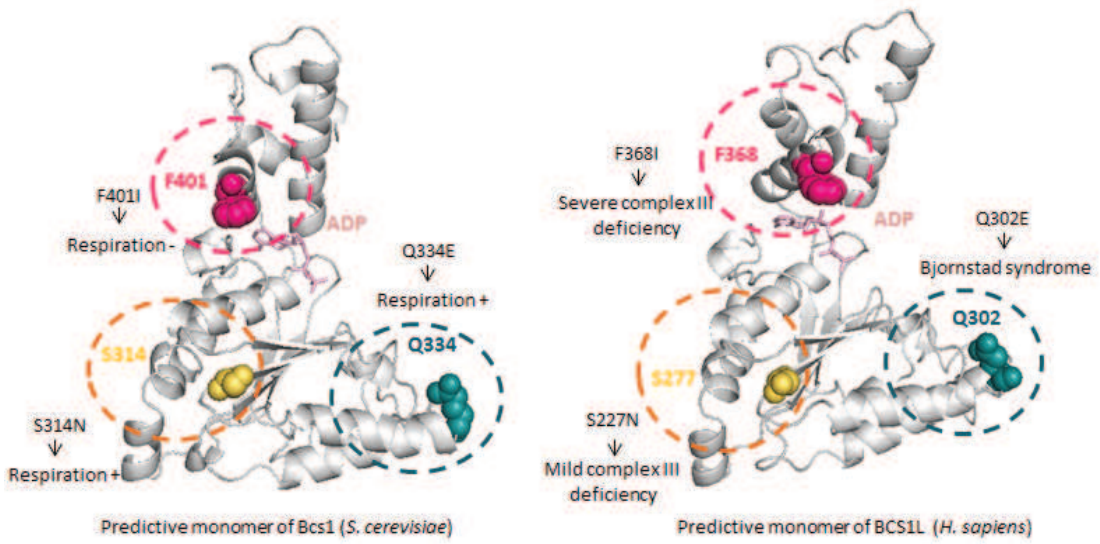


Figure IV-2 Predictive models of AAA domains in monomeric Bcs1 and BCS1L.
Equivalent residues from *S.cerevisiae* Bcs1 and *H. sapiens* BCS1L are shown as colored spheres. Consequences of mutations of indicated residues are shown for both yeast and human proteins. The image was generated by Pymol v1.3 software.

Disease mutations modeled in yeast may also be the starting point in the search for genetic or chemical compensation, as presented in this manuscript for genetic compensation. Mutations in human *ATP6*, responsible for NARP (Neuropathy, Ataxia and Retinitis Pigmentosa) and MILS (Maternally Inherited Leigh Syndrome) have been modeled in yeast (Kucharczyk et al., 2009a, 2009b, 2010). The yeast strains carrying modeled human *atp6* mutations were used in a rapid high-throughput yeast-based assay to screen chemical libraries for drugs able to compensate the respiratory deficiency (Couplan et al., 2011). In the long term, this assay may be instrumental for development of treatments for mitochondrial diseases; large number of mutations can be rapidly constructed in yeast and compensation tested with numerous chemical libraries, which would point to potentially useful molecules and also provide hints for drug design. Recently, such an approach has been successfully undertaken for the *bcs1*-F401I mutation, which can be genetically compensated by modifying the activity of the ATP synthase. The possibility of chemical compensation for this mutant suggests that the intramitochondrial adenine nucleotide pool may be a potential target for the treatment of *Bcs1*-related disorders.

3. Defects in mitochondrial translation lead to pathologies in humans

An important number of pathogenic mutations affecting the mitochondrial translational apparatus have been described, including those in tRNA, rRNA, ribosomal proteins, elongation and termination factors and translational activators (for review Rötig, 2011). Although several mutations have been found for the elongation factor $EFG1_{mt}$ (Coenen et al., 2004; Valente et al., 2007), no disease-causing mutation has been reported until now for the ribosome recycling factors $EFG2_{mt}$ or mtRRF. As new techniques for gene identification emerge, it is possible that the two ribosome recycling factors may be added to the list of proteins that can be mutated in defective mitochondrial synthesis. If this happens, it will be interesting to see if the resulting deficiency of the OXPHOS complexes could correlate with the main defects in complex IV and ATP synthase, seen in our characterization of *Δrrf1* mutant in *S. cerevisiae*. Since we have shown that the specific effect on mitochondrial translationin the *Δrrf1* strain could be complemented by the human mtRRF, the conservation of at least some aspects of Rrf1 function

could be expected. Following this line of thinking, mutations in a certain number of genes involved in mitochondrial protein synthesis might have been missed due to unexpected effects on the OXPHOS complexes, thus obscuring the origin of the defect. Given the general conservation of the steps of translation, the approach used in the presented study of Rrf1 might be re-used to characterize the effects of other mutated translation factors in *S. cerevisiae*, which may, in some cases, orient the search for the causative mutations in combined OXPHOS deficiencies. In the laboratory, we plan to develop the study of the Rrf1 partner, Mef2, the homolog of the human $EFG2_{m.}$

References

Abrahams, J.P., Leslie, A.G., Lutter, R., and Walker, J.E. (1994). Structure at 2.8 A resolution of F1-ATPase from bovine heart mitochondria. Nature *370*, 621–628.

Acehan, D., Malhotra, A., Xu, Y., Ren, M., Stokes, D.L., and Schlame, M. (2011). Cardiolipin Affects the Supramolecular Organization of ATP Synthase in Mitochondria. Biophysical Journal *100*, 2184–2192.

Acín-Pérez, R., Fernández-Silva, P., Peleato, M.L., Pérez-Martos, A., and Enriquez, J.A. (2008). Respiratory active mitochondrial supercomplexes. Mol. Cell *32*, 529–539.

Ackerman, S.H., and Tzagoloff, A. (1990). Identification of two nuclear genes (ATP11, ATP12) required for assembly of the yeast F1-ATPase. Proceedings of the National Academy of Sciences *87*, 4986–4990.

Agrawal, R., Sharma, M., Yassin, A., Lahiri, I., and Spremulli, indaL. (2011). Structure and function of organellar ribosomes as revealed by cryo-EM. In Ribosomes, M. Rodnina, W. Wintermeyer, and R. Green, eds. (Springer Vienna), pp. 83–96.

Aldridge, C., Spence, E., Kirkilionis, M.A., Frigerio, L., and Robinson, C. (2008). Tat-dependent targeting of Rieske iron-sulphur proteins to both the plasma and thylakoid membranes in the cyanobacterium Synechocystis PCC6803. Mol. Microbiol. *70*, 140–150.

Allen, J.F. (1996). Separate sexes and the mitochondrial theory of ageing. Journal of Theoretical Biology *180*, 135–140.

Allue, I., Gandelman, O., Dementieva, E., Ugarova, N., and Cobbold, P. (1996). Evidence for rapid consumption of millimolar concentrations of cytoplasmic ATP during rigor-contracture of metabolically compromised single cardiomyocytes. Biochemical Journal *319*, 463.

Althoff, T., Mills, D.J., Popot, J.-L., and Kühlbrandt, W. (2011). Arrangement of electron transport chain components in bovine mitochondrial supercomplex I1III2IV1. EMBO J. *30*, 4652–4664.

Amiott, E.A., and Jaehning, J.A. (2006). Mitochondrial Transcription Is Regulated via an ATP "Sensing" Mechanism that Couples RNA Abundance to Respiration. Molecular Cell *22*, 329–338.

Anand, R., Langer, T., and Baker, M.J. (2013). Proteolytic control of mitochondrial function and morphogenesis. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1833*, 195–204.

Antonicka, H., Leary, S.C., Guercin, G.-H., Agar, J.N., Horvath, R., Kennaway, N.G., Harding, C.O., Jaksch, M., and Shoubridge, E.A. (2003a). Mutations in COX10 result in a defect in mitochondrial heme A biosynthesis and account for multiple, early-onset clinical phenotypes associated with isolated COX deficiency. Human Molecular Genetics *12*, 2693–2702.

Antonicka, H., Mattman, A., Carlson, C.G., Glerum, D.M., Hoffbuhr, K.C., Leary, S.C., Kennaway, N.G., and Shoubridge, E.A. (2003b). Mutations in COX15 Produce a Defect in the Mitochondrial Heme Biosynthetic Pathway, Causing Early-Onset Fatal Hypertrophic Cardiomyopathy. The American Journal of Human Genetics *72*, 101–114.

Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R.A., and Schägger, H. (1998). Yeast mitochondrial F1F0-ATP synthase exists as a dimer: identification of three dimer-specific subunits. The EMBO Journal *17*, 7170–7178.

Atkinson, A., Smith, P., Fox, J.L., Cui, T.-Z., Khalimonchuk, O., and Winge, D.R. (2011). The LYR Protein Mzm1 Functions in the Insertion of the Rieske Fe/S Protein in Yeast Mitochondria. Molecular and Cellular Biology *31*, 3988–3996.

Augustin, S., Gerdes, F., Lee, S., Tsai, F.T.F., Langer, T., and Tatsuta, T. (2009). An intersubunit signaling network coordinates ATP hydrolysis by m-AAA proteases. Mol. Cell *35*, 574–585.

Baker, L.A., Watt, I.N., Runswick, M.J., Walker, J.E., and Rubinstein, J.L. (2012). Arrangement of subunits in intact mammalian mitochondrial ATP synthase determined by cryo-EM. Proceedings of the National Academy of Sciences *109*, 11675–11680.

Barat, C., Datta, P.P., Raj, V.S., Sharma, M.R., Kaji, H., Kaji, A., and Agrawal, R.K. (2007). Progression of the ribosome recycling factor through the ribosome dissociates the two ribosomal subunits. Mol. Cell *27*, 250–261.

Barel, O., Shorer, Z., Flusser, H., Ofir, R., Narkis, G., Finer, G., Shalev, H., Nasasra, A., Saada, A., and Birk, O.S. (2008). Mitochondrial Complex III Deficiency Associated with a Homozygous Mutation in UQCRQ. The American Journal of Human Genetics *82*, 1211–1216.

Barrientos, A., Barros, M.H., Valnot, I., Rötig, A., Rustin, P., and Tzagoloff, A. (2002a). Cytochrome oxidase in health and disease. Gene *286*, 53–63.

Barrientos, A., Korr, D., and Tzagoloff, A. (2002b). Shy1p is necessary for full expression of mitochondrial COX1 in the yeast model of Leigh's syndrome. EMBO J. *21*, 43–52.

Barrientos, A., Zambrano, A., and Tzagoloff, A. (2004). Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in Saccharomyces cerevisiae. The EMBO Journal *23*, 3472–3482.

Barros, M.H., Carlson, C.G., Glerum, M.D., and Tzagoloff, A. (2001). Involvement of mitochondrial ferredoxin and Cox15p in hydroxylation of heme O.

Beauvoit, B., and Rigoulet, M. (2001). Regulation of Cytochrome c Oxidase by Adenylic Nucleotides. Is Oxidative Phosphorylation Feedback Regulated by its End-Products? IUBMB Life *52*, 143–152.

Beis, I., and Newsholme, E.A. (1975). The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. Biochem. J. *152*, 23–32.

Bénit, P., Goncalves, S., Philippe Dassa, E., Brière, J.-J., Martin, G., and Rustin, P. (2006). Three spectrophotometric assays for the measurement of the five respiratory chain complexes in minuscule biological samples. Clinica Chimica Acta *374*, 81–86.

Bernard, D.G. (2003). Overlapping Specificities of the Mitochondrial Cytochrome c and c1 Heme Lyases. Journal of Biological Chemistry *278*, 49732–49742.

Bestwick, M., Khalimonchuk, O., Pierrel, F., and Winge, D.R. (2010). The Role of Coa2 in Hemylation of Yeast Cox1 Revealed by Its Genetic Interaction with Cox10. Molecular and Cellular Biology *30*, 172–185.

Biagini, G.A., Fisher, N., Shone, A.E., Mubaraki, M.A., Srivastava, A., Hill, A., Antoine, T., Warman, A.J., Davies, J., Pidathala, C., et al. (2012). Generation of quinolone antimalarials targeting the Plasmodium falciparum mitochondrial respiratory chain for the treatment and prophylaxis of malaria. Proceedings of the National Academy of Sciences *109*, 8298–8303.

Blázquez, A., Gil-Borlado, M.C., Morán, M., Verdú, A., Cazorla-Calleja, M.R., Martín, M.A., Arenas, J., and Ugalde, C. (2009). Infantile mitochondrial encephalomyopathy with unusual phenotype caused by a novel BCS1L mutation in an isolated complex III-deficient patient. Neuromuscular Disorders *19*, 143–146.

Boekema, E.J., and Braun, H.-P. (2007). Supramolecular structure of the mitochondrial oxidative phosphorylation system. J. Biol. Chem. *282*, 1–4.

Bonnefoy, N., Kermorgant, Mich., Groudinsky, O., Minet, Mich., Slonimski, P.P., and Dujardin, Genevi. (1994). Cloning of a human gene involved in cytochrome oxidase assembly by functional complementation of an oxa1-mutation in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences *91*, 11978–11982.

Bonnefoy, N., Fiumera, H., Dujardin, G., and Fox, T. (2009). Roles of Oxa1-related inner-membrane translocases in assembly of respiratory chain complexes. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1793*, 60–70.

Boyer, P.D. (1997). The ATP synthase-a splendid molecular machine. Annual Review of Biochemistry *66*, 717–749.

Brandt, U. (2006). Energy converting NADH: quinone oxidoreductase (complex I). Annu. Rev. Biochem. *75*, 69–92.

Brandt, U., Yu, L., Yu, C.A., and Trumpower, B.L. (1993). The mitochondrial targeting presequence of the Rieske iron-sulfur protein is processed in a single step after insertion into the cytochrome bc1 complex in mammals and retained as a subunit in the complex. Journal of Biological Chemistry *268*, 8387–8390.

Braun, H.-P., Emmermann, M., Kruft, V., and Schmitz, U.K. (1992). The general mitochondrial processing peptidase from potato is an integral part of cytochrome c reductase of the respiratory chain. The EMBO Journal *11*, 3219.

Briggs, L.C., Baldwin, G.S., Miyata, N., Kondo, H., Zhang, X., and Freemont, P.S. (2008). Analysis of nucleotide binding to P97 reveals the properties of a tandem AAA hexameric ATPase. J. Biol. Chem. *283*, 13745–13752.

Brindefalk, B., Ettema, T.J.G., Viklund, J., Thollesson, M., and Andersson, S.G.E. (2011). A Phylometagenomic Exploration of Oceanic Alphaproteobacteria Reveals Mitochondrial Relatives Unrelated to the SAR11 Clade. PLoS ONE *6*, e24457.

Brown, N.G., Costanzo, M.C., and Fox, T.D. (1994). Interactions among three proteins that specifically activate translation of the mitochondrial COX3 mRNA in Saccharomyces cerevisiae. Molecular and Cellular Biology *14*, 1045–1053.

Brunori, M., ANTONINI, G., MALATESTA, F., SARTI, P., and WILSON, M.T. (1987). Cytochrome-c oxidase. European Journal of Biochemistry *169*, 1–8.

Bryan, A.C., Rodeheffer, M.S., Wearn, C.M., and Shadel, G.S. (2002). Sls1p is a membrane-bound regulator of transcription-coupled processes involved in Saccharomyces cerevisiae mitochondrial gene expression. Genetics *160*, 75–82.

Burgess, S.M., Delannoy, M., and Jensen, R.E. (1994). MMM1 encodes a mitochondrial outer membrane protein essential for establishing and maintaining the structure of yeast mitochondria. The Journal of Cell Biology *126*, 1375–1391.

Burke, P.V., Raitt, D.C., Allen, L.A., Kellogg, E.A., and Poyton, R.O. (1997). Effects of oxygen concentration on the expression of cytochrome c and cytochrome c oxidase genes in yeast. J. Biol. Chem. *272*, 14705–14712.

Callegari, S., McKinnon, R.A., Andrews, S., and de Barros Lopes, M.A. (2011). The MEF2 gene is essential for yeast longevity, with a dual role in cell respiration and maintenance of mitochondrial membrane potential. FEBS Lett. *585*, 1140–1146.

Carlson, M. (1999). Glucose repression in yeast. Current Opinion in Microbiology *2*, 202–207.

Carr, H.S., and Winge, D.R. (2003). Assembly of cytochrome c oxidase within the mitochondrion. Accounts of Chemical Research *36*, 309–316.

Chen, W., and Dieckmann, C.L. (1997). Genetic evidence for interaction between Cbp1 and specific nucleotides in the 5'untranslated region of mitochondrial cytochrome b mRNA in Saccharomyces cerevisiae. Molecular and Cellular Biology *17*, 6203–6211.

Chen, Y.-C., Taylor, E.B., Dephoure, N., Heo, J.-M., Tonhato, A., Papandreou, I., Nath, N., Denko, N.C., Gygi, S.P., and Rutter, J. (2012). Identification of a protein mediating respiratory supercomplex stability. Cell Metab. *15*, 348–360.

Chernoff, Y.O., Lindquist, S.L., Ono, B., Inge-Vechtomov, S.G., and Liebman, S.W. (1995). Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. Science *268*, 880–884.

Chi, A., Huttenhower, C., Geer, L.Y., Coon, J.J., Syka, J.E.P., Bai, D.L., Shabanowitz, J., Burke, D.J., Troyanskaya, O.G., and Hunt, D.F. (2007). Analysis of phosphorylation sites on proteins from

Saccharomyces cerevisiae by electron transfer dissociation (ETD) mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. *104*, 2193–2198.

Choquet, Y., Wostrikoff, K., Rimbault, B., Zito, F., Girard-Bascou, J., Drapier, D., and F-A Wollman (2000). Assembly-controlled regulation of chloroplast gene translation. Ind. J. Biochem. Biophys *37*, 441–446.

Christian, B.E., and Spremulli, L.L. (2009). Evidence for an active role of IF3mt in the initiation of translation in mammalian mitochondria. Biochemistry *48*, 3269–3278.

Ciccone, S., Maiani, E., Bellusci, G., Diederich, M., and Gonfloni, S. (2013). Parkinson's disease: a complex interplay of mitochondrial DNA alterations and oxidative stress. Int J Mol Sci *14*, 2388–2409.

Clark-Walker, G.D., and Linnane, A.W. (1966). In vivo differentiation of yeast cytoplasmic and mitochondrial protein synthesis with antibiotics. Biochem. Biophys. Res. Commun. *25*, 8–13.

Cobine, P.A., Ojeda, L.D., Rigby, K.M., and Winge, D.R. (2004). Yeast Contain a Non-proteinaceous Pool of Copper in the Mitochondrial Matrix. Journal of Biological Chemistry *279*, 14447–14455.

Coenen, M.J.H., Antonicka, H., Ugalde, C., Sasarman, F., Rossi, R., Heister, J.G.A.M.A., Newbold, R.F., Trijbels, F.J.M.F., van den Heuvel, L.P., Shoubridge, E.A., et al. (2004). Mutant mitochondrial elongation factor G1 and combined oxidative phosphorylation deficiency. N. Engl. J. Med. *351*, 2080–2086.

Contamine, V., and Picard, M. (2000). Maintenance and Integrity of the Mitochondrial Genome: a Plethora of Nuclear Genes in the Budding Yeast. Microbiology and Molecular Biology Reviews *64*, 281–315.

Couplan, E., Aiyar, R.S., Kucharczyk, R., Kabala, A., Ezkurdia, N., Gagneur, J., St Onge, R.P., Salin, B., Soubigou, F., Le Cann, M., et al. (2011). A yeast-based assay identifies drugs active against human mitochondrial disorders. Proc. Natl. Acad. Sci. U.S.A. *108*, 11989–11994.

Crofts, A.R., Hong, S., Ugulava, N., Barquera, B., Gennis, R., Guergova-Kuras, M., and Berry, E.A. (1999). Pathways for proton release during ubihydroquinone oxidation by the bc1 complex. Proceedings of the National Academy of Sciences *96*, 10021–10026.

Cross, R.L., and Müller, V. (2004). The evolution of A-, F-, and V-type ATP synthases and ATPases: reversals in function and changes in the H+/ATP coupling ratio. FEBS Lett. *576*, 1–4.

Cui, T.-Z., Smith, P.M., Fox, J.L., Khalimonchuk, O., and Winge, D.R. (2012). Late-stage maturation of the Rieske Fe/S protein: Mzm1 stabilizes Rip1 but does not facilitate its translocation by the AAA ATPase Bcs1. Mol. Cell. Biol. *32*, 4400–4409.

Cuperus, R., Leen, R., Tytgat, G.A.M., Caron, H.N., and Kuilenburg, A.B.P. (2009). Fenretinide induces mitochondrial ROS and inhibits the mitochondrial respiratory chain in neuroblastoma. Cellular and Molecular Life Sciences *67*, 807–816.

Dautant, A., Velours, J., and Giraud, M.-F. (2010). Crystal structure of the Mg·ADP-inhibited state of the yeast F1c10-ATP synthase. J. Biol. Chem. *285*, 29502–29510.

Davies, K.M., Anselmi, C., Wittig, I., Faraldo-Gómez, J.D., and Kühlbrandt, W. (2012). Structure of the yeast F1Fo-ATP synthase dimer and its role in shaping the mitochondrial cristae. Proceedings of the National Academy of Sciences *109*, 13602–13607.

Dawson, T.M. (2003). Molecular Pathways of Neurodegeneration in Parkinson's Disease. Science *302*, 819–822.

Decoster, E., Simon, M., Hatat, D., and Faye, G. (1990). The MSS51 gene product is required for the translation of the COX1 mRNA in yeast mitochondria. Mol. Gen. Genet. *224*, 111–118.

Von der Malsburg, K., Müller, J.M., Bohnert, M., Oeljeklaus, S., Kwiatkowska, P., Becker, T., Loniewska-Lwowska, A., Wiese, S., Rao, S., Milenkovic, D., et al. (2011). Dual Role of Mitofilin in Mitochondrial Membrane Organization and Protein Biogenesis. Developmental Cell *21*, 694–707.

Devaux, F., Lelandais, G., Garcia, M., Goussard, S., and Jacq, C. (2010). Posttranscriptional control of mitochondrial biogenesis: spatio-temporal regulation of the protein import process. FEBS Lett. *584*, 4273–4279.

Dienhart, M.K., and Stuart, R.A. (2008). The yeast Aac2 protein exists in physical association with the cytochrome bc1-COX supercomplex and the TIM23 machinery. Molecular Biology of the Cell *19*, 3934–3943.

Ding, M.G., Butler, C.A., Saracco, S.A., Fox, T.D., Godard, F., di Rago, J.-P., and Trumpower, B.L. (2008). Introduction of cytochrome b mutations in Saccharomyces cerevisiae by a method that allows selection for both functional and non-functional cytochrome b proteins. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1777*, 1147–1156.

Drobinskaya, I.Y., Kozlov, I.A., Murataliev, M.B., and Vulfson, E.N. (1985). Tightly bound adenosine diphosphate, which inhibits the activity of mitochondrial F1-ATPase, is located at the catalytic site of the enzyme. FEBS Lett. *182*, 419–424.

Dudek, J., Rehling, P., and van der Laan, M. (2013). Mitochondrial protein import: common principles and physiological networks. Biochim. Biophys. Acta *1833*, 274–285.

Dudkina, N.V., Kouril, R., Peters, K., Braun, H.-P., and Boekema, E.J. (2010). Structure and function of mitochondrial supercomplexes. Biochim. Biophys. Acta *1797*, 664–670.

Dumont, M.E., Cardillo, T.S., Hayes, M.K., and Sherman, F. (1991). Role of cytochrome c heme lyase in mitochondrial import and accumulation of cytochrome c in Saccharomyces cerevisiae. Molecular and Cellular Biology *11*, 5487–5496.

Feng, Y., Li, W., Li, J., Wang, J., Ge, J., Xu, D., Liu, Y., Wu, K., Zeng, Q., Wu, J.-W., et al. (2012). Structural insight into the type-II mitochondrial NADH dehydrogenases. Nature *491*, 478–482.

Ferguson, S.J. (2010). ATP synthase: From sequence to ring size to the P/O ratio. Proceedings of the National Academy of Sciences *107*, 16755–16756.

Fernandez Vizarra, E., Tiranti, V., and Zeviani, M. (2009). Assembly of the oxidative phosphorylation system in humans: What we have learned by studying its defects. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1793*, 200–211.

Fernandez-Vizarra, E., Bugiani, M., Goffrini, P., Carrara, F., Farina, L., Procopio, E., Donati, A., Uziel, G., Ferrero, I., and Zeviani, M. (2007). Impaired complex III assembly associated with BCS1L gene mutations in isolated mitochondrial encephalopathy. Human Molecular Genetics *16*, 1241–1252.

Fiori, A., Perez-Martinez, X., and Fox, T.D. (2005). Overexpression of the COX2 translational activator, Pet111p, prevents translation of COX1 mRNA and cytochrome c oxidase assembly in mitochondria of Saccharomyces cerevisiae. Molecular Microbiology *56*, 1689–1704.

Fiumera, H.L., Dunham, M.J., Saracco, S.A., Butler, C.A., Kelly, J.A., and Fox, T.D. (2009). Translocation and Assembly of Mitochondrially Coded Saccharomyces cerevisiae Cytochrome c Oxidase Subunit Cox2 by Oxa1 and Yme1 in the Absence of Cox18. Genetics *182*, 519–528.

Fontanesi, F., Soto, I.C., Horn, D., and Barrientos, A. (2009). Mss51 and Ssc1 Facilitate Translational Regulation of Cytochrome c Oxidase Biogenesis. Molecular and Cellular Biology *30*, 245–259.

Forsburg, S.L. (2001). The art and design of genetic screens: yeast.

Fox, T.D. (2012). Mitochondrial protein synthesis, import, and assembly. Genetics *192*, 1203–1234.

Fu, W., Japa, S., and Beattie, D.S. (1990). Import of the iron-sulfur protein of the cytochrome b. c1 complex into yeast mitochondria. Journal of Biological Chemistry *265*, 16541–16547.

Gadir, N., Haim-Vilmovsky, L., Kraut-Cohen, J., and Gerst, J.E. (2011). Localization of mRNAs coding for mitochondrial proteins in the yeast Saccharomyces cerevisiae. RNA *17*, 1551–1565.

Gao, N., Zavialov, A.V., Li, W., Sengupta, J., Valle, M., Gursky, R.P., Ehrenberg, M., and Frank, J. (2005). Mechanism for the disassembly of the posttermination complex inferred from cryo-EM studies. Mol. Cell *18*, 663–674.

Gebert, N., Chacinska, A., Wagner, K., Guiard, B., Koehler, C.M., Rehling, P., Pfanner, N., and Wiedemann, N. (2008). Assembly of the three small Tim proteins precedes docking to the mitochondrial carrier translocase. EMBO Rep. *9*, 548–554.

Gebert, N., Ryan, M.T., Pfanner, N., Wiedemann, N., and Stojanovski, D. (2011a). Mitochondrial protein import machineries and lipids: A functional connection. Biochimica et Biophysica Acta (BBA) - Biomembranes *1808*, 1002–1011.

Gebert, N., Gebert, M., Oeljeklaus, S., von der Malsburg, K., Stroud, D.A., Kulawiak, B., Wirth, C., Zahedi, R.P., Dolezal, P., Wiese, S., et al. (2011b). Dual function of Sdh3 in the respiratory chain and TIM22 protein translocase of the mitochondrial inner membrane. Mol. Cell *44*, 811–818.

Georgiades, K., Madoui, M.-A., Le, P., Robert, C., and Raoult, D. (2011). Phylogenomic Analysis of Odyssella thessalonicensis Fortifies the Common Origin of Rickettsiales, Pelagibacter ubique and Reclimonas americana Mitochondrion. PLoS ONE *6*, e24857.

Ghezzi, D., Arzuffi, P., Zordan, M., Da Re, C., Lamperti, C., Benna, C., D'Adamo, P., Diodato, D., Costa, R., Mariotti, C., et al. (2011). Mutations in TTC19 cause mitochondrial complex III deficiency and neurological impairment in humans and flies. Nature Genetics *43*, 259–263.

Gil-Borlado, M.C., González-Hoyuela, M., Blázquez, A., García-Silva, M.T., Gabaldón, T., Manzanares, J., Vara, J., Martín, M.A., Seneca, S., Arenas, J., et al. (2009). Pathogenic mutations in the 5' untranslated region of BCS1L mRNA in mitochondrial complex III deficiency. Mitochondrion *9*, 299–305.

Girzalsky, W., Platta, H.W., and Erdmann, R. (2009). Protein transport across the peroxisomal membrane. Biol. Chem. *390*, 745–751.

Glerum, D.M., and Tzagoloff, A. (1994). Isolation of a human cDNA for heme A: farnesyltransferase by functional complementation of a yeast cox10 mutant. Proceedings of the National Academy of Sciences *91*, 8452–8456.

Glerum, D.M., Shtanko, A., and Tzagoloff, A. (1996). Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. Journal of Biological Chemistry *271*, 14504–14509.

Graack, H.R., and Wittmann-Liebold, B. (1998). Mitochondrial ribosomal proteins (MRPs) of yeast. Biochem. J. *329 (Pt 3)*, 433–448.

Graham, L.A., and Trumpower, B.L. (1991). Mutational analysis of the mitochondrial Rieske iron-sulfur protein of Saccharomyces cerevisiae. III. Import, protease processing, and assembly into the cytochrome bc1 complex of iron-sulfur protein lacking the iron-sulfur cluster. J. Biol. Chem. *266*, 22485–22492.

Gray, M.W. (1999). Mitochondrial Evolution. Science *283*, 1476–1481.

Gray, M.W. (2012). Mitochondrial Evolution. Cold Spring Harbor Perspectives in Biology *4*, a011403–a011403.

Gray, R.E., Grasso, D.G., Maxwell, R.J., Finnegan, P.M., Nagley, P., and Devenish, R.J. (1990). Identification of a 66 KDa protein associated with yeast mitochondrial ATP synthase as heat shock protein hsp60. FEBS Lett. *268*, 265–268.

Gresser, M.J., Myers, J.A., and Boyer, P.D. (1982). Catalytic site cooperativity of beef heart mitochondrial F1 adenosine triphosphatase. Correlations of initial velocity, bound intermediate, and oxygen exchange measurements with an alternating three-site model. J. Biol. Chem. *257*, 12030–12038.

Gribble, F.M., Loussouarn, G., Tucker, S.J., Zhao, C., Nichols, C.G., and Ashcroft, F.M. (2000). A novel method for measurement of submembrane ATP concentration. J. Biol. Chem. *275*, 30046–30049.

Gruschke, S., Grone, K., Heublein, M., Holz, S., Israel, L., Imhof, A., Herrmann, J.M., and Ott, M. (2010). Proteins at the Polypeptide Tunnel Exit of the Yeast Mitochondrial Ribosome. Journal of Biological Chemistry *285*, 19022–19028.

Gruschke, S., Kehrein, K., Rompler, K., Grone, K., Israel, L., Imhof, A., Herrmann, J.M., and Ott, M. (2011). Cbp3-Cbp6 interacts with the yeast mitochondrial ribosomal tunnel exit and promotes cytochrome b synthesis and assembly. The Journal of Cell Biology *193*, 1101–1114.

Gyllensten, U., Wharton, D., Josefsson, A., and Wilson, A.C. (1991). Paternal inheritance of mitochondrial DNA in mice. Nature *352*, 255–257.

Hanson, P.I., and Whiteheart, S.W. (2005). AAA+ proteins: have engine, will work. Nat. Rev. Mol. Cell Biol. *6*, 519–529.

Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H., and Neupert, W. (1986). Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. Cell *47*, 939–951.

Haut, S., Brivet, M., Touati, G., Rustin, P., Lebon, S., Garcia-Cazorla, A., Saudubray, J.M., Boutron, A., Legrand, A., and Slama, A. (2003). A deletion in the human QP-C gene causes a complex III deficiency resulting in hypoglycaemia and lactic acidosis. Hum. Genet. *113*, 118–122.

Hell, K., Tzagoloff, A., Neupert, W., and Stuart, R.A. (2000). Identification of Cox20p, a novel protein involved in the maturation and assembly of cytochrome oxidase subunit 2. Journal of Biological Chemistry *275*, 4571–4578.

Hell, K., Neupert, W., and Stuart, R.A. (2001). Oxa1p acts as a general membrane insertion machinery for proteins encoded by mitochondrial DNA. The EMBO Journal *20*, 1281–1288.

Helling, S., Hüttemann, M., Ramzan, R., Kim, S.H., Lee, I., Müller, T., Langenfeld, E., Meyer, H.E., Kadenbach, B., Vogt, S., et al. (2012). Multiple phosphorylations of cytochrome c oxidase and their functions. Proteomics *12*, 950–959.

Helsen, C.W., and Glover, J.R. (2012). A new perspective on Hsp104-mediated propagation and curing of the yeast prion [PSI (+)]. Prion *6*, 234–239.

Herrin, D.L., and Schmidt, G.W. (1988). Rapid, reversible staining of northern blots prior to hybridization. BioTechniques *6*, 196–197, 199–200.

Herrmann, J.M., and Riemer, J. (2010). The intermembrane space of mitochondria. Antioxidants & Redox Signaling *13*, 1341–1358.

Herrmann, J.M., Woellhaf, M.W., and Bonnefoy, N. (2013). Control of protein synthesis in yeast mitochondria: The concept of translational activators. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1833*, 286–294.

Hersch, G.L., Burton, R.E., Bolon, D.N., Baker, T.A., and Sauer, R.T. (2005). Asymmetric interactions of ATP with the AAA+ ClpX6 unfoldase: allosteric control of a protein machine. Cell *121*, 1017–1027.

Hinson, J.T., Fantin, V.R., Schönberger, J., Breivik, N., Siem, G., McDonough, B., Sharma, P., Keogh, I., Godinho, R., and Santos, F. (2007). Missense mutations in the BCS1L gene as a cause of the Björnstad syndrome. New England Journal of Medicine *356*, 809–819.

Hoeh, W.R., Blakley, K.H., and Brown, W.M. (1991). Heteroplasmy suggests limited biparental inheritance of Mytilus mitochondrial DNA. Science *251*, 1488–1490.

Hofmann, E., and Kopperschla¨ger, G. (1982). Phosphofructokinase from yeast. In Methods in Enzymology, Willis A. Wood, ed. (Academic Press), pp. 49–60.

Hoppins, S., Lackner, L., and Nunnari, J. (2007). The Machines that Divide and Fuse Mitochondria. Annual Review of Biochemistry *76*, 751–780.

Horn, D., and Barrientos, A. (2008). Mitochondrial copper metabolism and delivery to cytochrome c oxidase. IUBMB Life *60*, 421–429.

Horng, Y.-C., Cobine, P.A., Maxfield, A.B., Carr, H.S., and Winge, D.R. (2004). Specific Copper Transfer from the Cox17 Metallochaperone to Both Sco1 and Cox11 in the Assembly of Yeast Cytochrome c Oxidase. Journal of Biological Chemistry *279*, 35334–35340.

Horvat, S., Beyer, C., and Arnold, S. (2006). Effect of hypoxia on the transcription pattern of subunit isoforms and the kinetics of cytochrome c oxidase in cortical astrocytes and cerebellar neurons. Journal of Neurochemistry *99*, 937–951.

Hosler, J.P., Ferguson-Miller, S., and Mills, D.A. (2006). Energy transduction: proton transfer through the respiratory complexes. Annual Review of Biochemistry *75*, 165.

Houštěk, J., Pícková, A., Vojtíšková, A., Mráček, T., Pecina, P., and Ješina, P. (2006). Mitochondrial diseases and genetic defects of ATP synthase. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1757*, 1400–1405.

Huigsloot, M., Nijtmans, L.G., Szklarczyk, R., Baars, M.J.H., van den Brand, M.A.M., HendriksFranssen, M.G.M., van den Heuvel, L.P., Smeitink, J.A.M., Huynen, M.A., and Rodenburg, R.J.T. (2011). A Mutation in C2orf64 Causes Impaired Cytochrome c Oxidase Assembly and Mitochondrial Cardiomyopathy. The American Journal of Human Genetics *88*, 488–493.

Hunte, C., Koepke, J., Lange, C., Roßmanith, T., and Michel, H. (2000). Structure at 2.3 A resolution of the cytochrome bc 1 complex from the yeast Saccharomyces cerevisiae co-crystallized with an antibody Fv fragment. Structure *8*, 669–684.

Hüttemann, M., Kadenbach, B., and Grossman, L.I. (2001). Mammalian subunit IV isoforms of cytochrome c oxidase. Gene *267*, 111–123.

Ito, K., Fujiwara, T., Toyoda, T., and Nakamura, Y. (2002). Elongation factor G participates in ribosome disassembly by interacting with ribosome recycling factor at their tRNA-mimicry domains. Mol. Cell *9*, 1263–1272.

Iwata, S. (1998). Complete Structure of the 11-Subunit Bovine Mitochondrial Cytochrome bc1 Complex. Science *281*, 64–71.

Jacobs, H.T. (2001). Making mitochondrial mutants. Trends in Genetics *17*, 653–660.

Janosi, L., Shimizu, I., and Kaji, A. (1994). Ribosome recycling factor (ribosome releasing factor) is essential for bacterial growth. Proc. Natl. Acad. Sci. U.S.A. *91*, 4249–4253.

Janosi, L., Mottagui-Tabar, S., Isaksson, L.A., Sekine, Y., Ohtsubo, E., Zhang, S., Goon, S., Nelken, S., Shuda, M., and Kaji, A. (1998). Evidence for in vivo ribosome recycling, the fourth step in protein biosynthesis. EMBO J. *17*, 1141–1151.

Jiang, P., and Ninfa, A.J. (2007). Escherichia coli PII Signal Transduction Protein Controlling Nitrogen Assimilation Acts As a Sensor of Adenylate Energy Charge in Vitro. Biochemistry *46*, 12979–12996.

Jouaville, L.S., Pinton, P., Bastianutto, C., Rutter, G.A., and Rizzuto, R. (1998). Regulation of mitochondrial ATP synthesis by calcium: Evidence for a long-term metabolic priming.

Kabaleeswaran, V., Puri, N., Walker, J.E., Leslie, A.G.W., and Mueller, D.M. (2006). Novel features of the rotary catalytic mechanism revealed in the structure of yeast F1 ATPase. EMBO J. *25*, 5433–5442.

Kane, L.A., Youngman, M.J., Jensen, R.E., and Van Eyk, J.E. (2009). Phosphorylation of the F1Fo ATP Synthase Subunit: Functional and Structural Consequences Assessed in a Model System. Circulation Research *106*, 504–513.

Kara, B., Arıkan, M., Maraş, H., Abacı, N., Cakıris, A., and Ustek, D. (2012). Whole mitochondrial genome analysis of a family with NARP/MILS caused by m.8993T>C mutation in the MT-ATP6 gene. Mol. Genet. Metab. *107*, 389–393.

Kelley, L.A., and Sternberg, M.J.E. (2009). Protein structure prediction on the Web: a case study using the Phyre server. Nature Protocols *4*, 363–371.

Khalimonchuk, O., and Rödel, G. (2005). Biogenesis of cytochrome c oxidase. Mitochondrion *5*, 363–388.

Khalimonchuk, O., Bestwick, M., Meunier, B., Watts, T.C., and Winge, D.R. (2009). Formation of the Redox Cofactor Centers during Cox1 Maturation in Yeast Cytochrome Oxidase. Molecular and Cellular Biology *30*, 1004–1017.

Kim, H.J., Khalimonchuk, O., Smith, P.M., and Winge, D.R. (2012). Structure, function, and assembly of heme centers in mitochondrial respiratory complexes. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1823*, 1604–1616.

Kim, K.K., Min, K., and Suh, S.W. (2000). Crystal structure of the ribosome recycling factor from Escherichia coli. EMBO J. *19*, 2362–2370.

Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999). The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. The EMBO Journal *18*, 3981–3989.

Koonin, E.V. (2010). The origin and early evolution of eukaryotes in the light of phylogenomics. Genome Biol *11*, 209.

Kornmann, B., Currie, E., Collins, S.R., Schuldiner, M., Nunnari, J., Weissman, J.S., and Walter, P. (2009). An ER-Mitochondria Tethering Complex Revealed by a Synthetic Biology Screen. Science *325*, 477–481.

Kotarsky, H., Karikoski, R., Mörgelin, M., Marjavaara, S., Bergman, P., Zhang, D.-L., Smet, J., van Coster, R., and Fellman, V. (2010). Characterization of complex III deficiency and liver dysfunction in GRACILE syndrome caused by a BCS1L mutation. Mitochondrion *10*, 497–509.

Krause, K., de Souza, R.L., Roberts, D.G., and Dieckmann, C.L. (2004). The mitochondrial message-specific mRNA protectors Cbp1 and Pet309 are associated in a high-molecular weight complex. Molecular Biology of the Cell *15*, 2674–2683.

Krayl, M., Lim, J.H., Martin, F., Guiard, B., and Voos, W. (2006). A Cooperative Action of the ATP-Dependent Import Motor Complex and the Inner Membrane Potential Drives Mitochondrial Preprotein Import. Molecular and Cellular Biology *27*, 411–425.

Kucharczyk, R., Rak, M., and di Rago, J.-P. (2009a). Biochemical consequences in yeast of the human mitochondrial DNA 8993T>C mutation in the ATPase6 gene found in NARP/MILS patients. Biochim. Biophys. Acta *1793*, 817–824.

Kucharczyk, R., Salin, B., and di Rago, J.-P. (2009b). Introducing the human Leigh syndrome mutation T9176G into Saccharomyces cerevisiae mitochondrial DNA leads to severe defects in the incorporation of Atp6p into the ATP synthase and in the mitochondrial morphology. Hum. Mol. Genet. *18*, 2889–2898.

Kucharczyk, R., Ezkurdia, N., Couplan, E., Procaccio, V., Ackerman, S.H., Blondel, M., and di Rago, J.-P. (2010). Consequences of the pathogenic T9176C mutation of human mitochondrial DNA on yeast mitochondrial ATP synthase. Biochim. Biophys. Acta *1797*, 1105–1112.

Van der Laan, M., Bohnert, M., Wiedemann, N., and Pfanner, N. (2012). Role of MINOS in mitochondrial membrane architecture and biogenesis. Trends in Cell Biology *22*, 185–192.

Lange, C., and Hunte, C. (2002). Crystal structure of the yeast cytochrome bc1 complex with its bound substrate cytochrome c. Proceedings of the National Academy of Sciences *99*, 2800–2805.

Lapuente-Brun, E., Moreno-Loshuertos, R., Acín-Pérez, R., Latorre-Pellicer, A., Colás, C., Balsa, E., Perales-Clemente, E., Quirós, P.M., Calvo, E., Rodríguez-Hernández, M.A., et al. (2013). Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. Science *340*, 1567–1570.

Lau, W.C.Y., Baker, L.A., and Rubinstein, J.L. (2008). Cryo-EM structure of the yeast ATP synthase. J. Mol. Biol. *382*, 1256–1264.

Lefebvre-Legendre, L. (2000). Identification of a Nuclear Gene (FMC1) Required for the Assembly/Stability of Yeast Mitochondrial F1-ATPase in Heat Stress Conditions. Journal of Biological Chemistry *276*, 6789–6796.

Lemaire, C., and Dujardin, G. (2008). Preparation of respiratory chain complexes from Saccharomyces cerevisiae wild-type and mutant mitochondria : activity measurement and subunit composition analysis. Methods Mol. Biol. *432*, 65–81.

Leonhard, K., Herrmann, J.M., Stuart, R.A., Mannhaupt, G., Neupert, W., and Langer, T. (1996). AAA proteases with catalytic sites on opposite membrane surfaces comprise a proteolytic system for the ATP-dependent degradation of inner membrane proteins in mitochondria. The EMBO Journal *15*, 4218.

Leonhard, K., Stiealer, A., Neupert, W., and Langer, T. (1999). Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease.

Levéen, P., Kotarsky, H., Mörgelin, M., Karikoski, R., Elmér, E., and Fellman, V. (2011). The GRACILE mutation introduced into Bcs1l causes postnatal complex III deficiency: a viable mouse model for mitochondrial hepatopathy. Hepatology *53*, 437–447.

Lill, R. (2009). Function and biogenesis of iron–sulphur proteins. Nature *460*, 831–838.

Lill, R., and Mühlenhoff, U. (2005). Iron–sulfur-protein biogenesis in eukaryotes. Trends in Biochemical Sciences *30*, 133–141.

Liu, C.-M., Fann, C.S.-J., Chen, C.-Y., Liu, Y.-L., Oyang, Y.-J., Yang, W.-C., Chang, C.-C., Wen, C.-C., Chen, W.J., Hwang, T.-J., et al. (2011). ANXA7, PPP3CB, DNAJC9, and ZMYND17 genes at chromosome 10q22 associated with the subgroup of schizophrenia with deficits in attention and executive function. Biol. Psychiatry *70*, 51–58.

Lode, A., Kuschel, M., Paret, C., and Rödel, G. (2000). Mitochondrial copper metabolism in yeast: interaction between Sco1p and Cox2p. FEBS Letters *485*, 19–24.

Logan, D.C. (2006). The mitochondrial compartment. Journal of Experimental Botany *57*, 1225–1243.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953–961.

De Lonlay, P., Valnot, I., Barrientos, A., Gorbatyuk, M., Tzagoloff, A., Taanman, J.-W., Benayoun, E., Chrétien, D., Kadhom, N., Lombès, A., et al. (2001). A mutant mitochondrial respiratory chain assembly protein causes complex III deficiency in patients with tubulopathy, encephalopathy and liver failure.

Lucas, M.F., Rousseau, D.L., and Guallar, V. (2011). Electron transfer pathways in cytochrome c oxidase. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1807*, 1305–1313.

Lupo, D., Vollmer, C., Deckers, M., Mick, D.U., Tews, I., Sinning, I., and Rehling, P. (2011). Mdm38 is a 14-3-3-Like Receptor and Associates with the Protein Synthesis Machinery at the Inner Mitochondrial Membrane. Traffic *12*, 1457–1466.

Luttik, M.A., Overkamp, K.M., Kötter, P., de Vries, S., van Dijken, J.P., and Pronk, J.T. (1998). The Saccharomyces cerevisiae NDE1 andNDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. Journal of Biological Chemistry *273*, 24529–24534.

Maheshwari, K.K., and Marzuki, S. (1984). The formation of a defective small subunit of the mitochondrial ribosomes in petite mutants of Saccharomyces cerevisiae. Biochim. Biophys. Acta *781*, 153–164.

Mai, Z., Ghosh, S., Frisardi, M., Rosenthal, B., Rogers, R., and Samuelson, J. (1999). Hsp60 is targeted to a cryptic mitochondrion-derived organelle ("crypton") in the microaerophilic protozoan parasite Entamoeba histolytica. Molecular and Cellular Biology *19*, 2198–2205.

Mannella, C.A. (2006). The relevance of mitochondrial membrane topology to mitochondrial function. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease *1762*, 140–147.

Manthey, G.M., and McEwen, J.E. (1995). The product of the nuclear gene PET309 is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial COX1 locus of Saccharomyces cerevisiae.

Maréchal, A., Meunier, B., Lee, D., Orengo, C., and Rich, P.R. (2012). Yeast cytochrome c oxidase: A model system to study mitochondrial forms of the haem–copper oxidase superfamily. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1817*, 620–628.

Marella, M., Seo, B.B., Yagi, T., and Matsuno-Yagi, A. (2009). Parkinson's disease and mitochondrial complex I: a perspective on the Ndi1 therapy. Journal of Bioenergetics and Biomembranes *41*, 493–497.

Marella, M., Seo, B.B., Thomas, B.B., Matsuno-Yagi, A., and Yagi, T. (2010). Successful Amelioration of Mitochondrial Optic Neuropathy Using the Yeast NDI1 Gene in a Rat Animal Model. PLoS ONE *5*, e11472.

Margineantu, D.H., Brown, R.M., Brown, G.K., Marcus, A.H., and Capaldi, R.A. (2002). Heterogeneous distribution of pyruvate dehydrogenase in the matrix of mitochondria. Mitochondrion *1*, 327–338.

Martin, W., and Müller, M. (1998). The hydrogen hypothesis for the first eukaryote. Nature *392*, 37–41.

Martin, A., Baker, T.A., and Sauer, R.T. (2005). Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines. Nature *437*, 1115–1120.

Martin, W., Hoffmeister, M., Rotte, C., and Henze, K. (2001). An overview of endosymbiotic models for the origins of eukaryotes, their ATP-producing organelles (mitochondria and hydrogenosomes), and their heterotrophic lifestyle. Biol. Chem. *382*, 1521–1539.

Mashkevich, G., Repetto, B., Glerum, D.M., Jin, C., and Tzagoloff, A. (1997). SHY1, the Yeast Homolog of the MammalianSURF-1 Gene, Encodes a Mitochondrial Protein Required for Respiration. Journal of Biological Chemistry *272*, 14356–14364.

Massa, V., Fernandez-Vizarra, E., Alshahwan, S., Bakhsh, E., Goffrini, P., Ferrero, I., Mereghetti, P., D'Adamo, P., Gasparini, P., and Zeviani, M. (2008). Severe Infantile Encephalomyopathy Caused by a Mutation in COX6B1, a Nucleus-Encoded Subunit of Cytochrome C Oxidase. The American Journal of Human Genetics *82*, 1281–1289.

Mathieu, L., Marsy, S., Saint-Georges, Y., Jacq, C., and Dujardin, G. (2011). A transcriptome screen in yeast identifies a novel assembly factor for the mitochondrial complex III. Mitochondrion *11*, 391–396.

McStay, G.P., Su, C.H., and Tzagoloff, A. (2013a). Stabilization of Cox1p intermediates by the Cox14p-Coa3p complex. FEBS Lett. *587*, 943–949.

McStay, G.P., Su, C.H., and Tzagoloff, A. (2013b). Modular assembly of yeast cytochrome oxidase. Mol. Biol. Cell *24*, 440–452.

De Meirleir, L. (2004). Respiratory chain complex V deficiency due to a mutation in the assembly gene ATP12. Journal of Medical Genetics *41*, 120–124.

De Meirleir, L., Seneca, S., Damis, E., Sepulchre, B., Hoorens, A., Gerlo, E., García Silva, M.T., Hernandez, E.M., Lissens, W., and Van Coster, R. (2003). Clinical and diagnostic characteristics of complex III deficiency due to mutations in the BCS1L gene. Am. J. Med. Genet. A *121A*, 126–131.

Meisinger, C., Rissler, M., Chacinska, A., Szklarz, L.K.S., Milenkovic, D., Kozjak, V., Schönfisch, B., Lohaus, C., Meyer, H.E., and Yaffe, M.P. (2004). The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. Developmental Cell *7*, 61–71.

Melo, A.M.P., Bandeiras, T.M., and Teixeira, M. (2004). New Insights into Type II NAD(P)H:Quinone Oxidoreductases. Microbiology and Molecular Biology Reviews *68*, 603–616.

Metelkin, E., Demin, O., Kovács, Z., and Chinopoulos, C. (2009). Modeling of ATP-ADP steady-state exchange rate mediated by the adenine nucleotide translocase in isolated mitochondria. FEBS J. *276*, 6942–6955.

Meunier, B., Fisher, N., Ransac, S., Mazat, J.-P., and Brasseur, G. (2012). Respiratory complex III dysfunction in humans and the use of yeast as a model organism to study mitochondrial myopathy and associated diseases. Biochimica et Biophysica Acta (BBA) - Bioenergetics.

Michel, A.H., and Kornmann, B. (2012). The ERMES complex and ER-mitochondria connections. Biochem. Soc. Trans. *40*, 445–450.

Mick, D.U., Wagner, K., van der Laan, M., Frazier, A.E., Perschil, I., Pawlas, M., Meyer, H.E., Warscheid, B., and Rehling, P. (2007). Shy1 couples Cox1 translational regulation to cytochrome c oxidase assembly. The EMBO Journal *26*, 4347–4358.

Mick, D.U., Vukotic, M., Piechura, H., Meyer, H.E., Warscheid, B., Deckers, M., and Rehling, P. (2010). Coa3 and Cox14 are essential for negative feedback regulation of COX1 translation in mitochondria. The Journal of Cell Biology *191*, 141–154.

Mick, D.U., Fox, T.D., and Rehling, P. (2011). Inventory control: cytochrome c oxidase assembly regulates mitochondrial translation. Nat. Rev. Mol. Cell Biol. *12*, 14–20.

Mileykovskaya, E., Penczek, P.A., Fang, J., Mallampalli, V.K.P.S., Sparagna, G.C., and Dowhan, W. (2012). Arrangement of the respiratory chain complexes in Saccharomyces cerevisiae supercomplex III2IV2 revealed by single particle cryo-electron microscopy. J. Biol. Chem. *287*, 23095–23103.

Mokranjac, D. (2005). Role of Tim21 in Mitochondrial Translocation Contact Sites. Journal of Biological Chemistry *280*, 23437–23440.

Morán, M., Moreno-Lastres, D., Marín-Buera, L., Arenas, J., Martín, M.A., and Ugalde, C. (2012). Mitochondrial respiratory chain dysfunction: implications in neurodegeneration. Free Radic. Biol. Med. *53*, 595–609.

Moreno-Lastres, D., Fontanesi, F., García-Consuegra, I., Martín, M.A., Arenas, J., Barrientos, A., and Ugalde, C. (2012). Mitochondrial Complex I Plays an Essential Role in Human Respirasome Assembly. Cell Metabolism *15*, 324–335.

Mulero, J.J., and Fox, T.D. (1993). PETl 11 Acts in the 5' Leader of the Saccharomyces cerevisiae Mitochondrial COX2 mRNA to Promote Its Translation.

Naithani, S., Saracco, S.A., Butler, C.A., and Fox, T.D. (2003). Interactions among COX1, COX2, andCOX3 mRNA-specific Translational Activator Proteins on the Inner Surface of the Mitochondrial Inner Membrane ofSaccharomyces cerevisiae. Molecular Biology of the Cell *14*, 324–333.

Nakamoto, R.K., Baylis Scanlon, J.A., and Al-Shawi, M.K. (2008). The rotary mechanism of the ATP synthase. Arch. Biochem. Biophys. *476*, 43–50.

Nakano, H., Yoshida, T., Uchiyama, S., Kawachi, M., Matsuo, H., Kato, T., Ohshima, A., Yamaichi, Y., Honda, T., Kato, H., et al. (2003). Structure and binding mode of a ribosome recycling factor (RRF) from mesophilic bacterium. J. Biol. Chem. *278*, 3427–3436.

Napiwotzki, J., and Kadenbach, B. (1998). Extramitochondrial ATP/ADP-ratios regulate cytochrome c oxidase activity via binding to the cytosolic domain of subunit IV. Biol. Chem. *379*, 335–339.

Nett, J.H., Denke, E., and Trumpower, B.L. (1997). Two-step processing is not essential for the import and assembly of functionally active iron-sulfur protein into the cytochrome bc1 complex in Saccharomyces cerevisiae. Journal of Biological Chemistry *272*, 2212–2217.

Nezich, C.L., and Youle, R.J. (2013). Make or break for mitochondria. eLife *2*, e00804–e00804.

Nijtmans, L.G., Henderson, N.S., and Holt, I.J. (2002). Blue native electrophoresis to study mitochondrial and other protein complexes. Methods *26*, 327–334.

Nouet, C., Bourens, M., Hlavacek, O., Marsy, S., Lemaire, C., and Dujardin, G. (2006). Rmd9p Controls the Processing/Stability of Mitochondrial mRNAs and Its Overexpression Compensates for a Partial Deficiency of Oxa1p in Saccharomyces cerevisiae. Genetics *175*, 1105–1115.

Nouet, C., Truan, G., Mathieu, L., and Dujardin, G. (2009). Functional Analysis of Yeast bcs1 Mutants Highlights the Role of Bcs1p-Specific Amino Acids in the AAA Domain. Journal of Molecular Biology *388*, 252–261.

Nunnari, J., Fox, T.D., and Walter, P. (1993). A mitochondrial protease with two catalytic subunits of nonoverlapping specificities. Science *262*, 1997–2004.

Osman, C., Wilmes, C., Tatsuta, T., and Langer, T. (2007). Prohibitins interact genetically with Atp23, a novel processing peptidase and chaperone for the F1Fo-ATP synthase. Molecular Biology of the Cell *18*, 627–635.

Oswald, C., Krause-Buchholz, U., and Rödel, G. (2009). Knockdown of Human COX17 Affects Assembly and Supramolecular Organization of Cytochrome c Oxidase. Journal of Molecular Biology *389*, 470–479.

Ott, M., and Herrmann, J.M. (2010). Co-translational membrane insertion of mitochondrially encoded proteins. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1803*, 767–775.

Ott, M., Prestele, M., Bauerschmitt, H., Funes, S., Bonnefoy, N., and Herrmann, J.M. (2006). Mba1, a membrane-associated ribosome receptor in mitochondria. The EMBO Journal *25*, 1603–1610.

Oudshoorn, P., Steeg, H., Swinkels, B.W., Schoppink, P., and Grivell, L.A. (1987). Subunit II of yeast QH2: cytochrome-c oxidoreductase. European Journal of Biochemistry *163*, 97–103.

Pai, R.D., Zhang, W., Schuwirth, B.S., Hirokawa, G., Kaji, H., Kaji, A., and Cate, J.H.D. (2008). Structural Insights into ribosome recycling factor interactions with the 70S ribosome. J. Mol. Biol. *376*, 1334–1347.

Palade, G.E. (1952). The fine structure of mitochondria. Anat. Rec. *114*, 427–451.

Palmer, T., and Berks, B.C. (2012). The twin-arginine translocation (Tat) protein export pathway. Nat. Rev. Microbiol. *10*, 483–496.

Palmieri, F., Agrimi, G., Blanco, E., Castegna, A., Di Noia, M.A., Iacobazzi, V., Lasorsa, F.M., Marobbio, C.M.T., Palmieri, L., Scarcia, P., et al. (2006). Identification of mitochondrial carriers in Saccharomyces cerevisiae by transport assay of reconstituted recombinant proteins. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1757*, 1249–1262.

Partikian, A., Ölveczky, B., Swaminathan, R., Li, Y., and Verkman, A.S. (1998). Rapid diffusion of green fluorescent protein in the mitochondrial matrix. The Journal of Cell Biology *140*, 821–829.

Paumard, P., Vaillier, J., Coulary, B., Schaeffer, J., Soubannier, V., Mueller, D.M., Brèthes, D., di Rago, J.-P., and Velours, J. (2002). The ATP synthase is involved in generating mitochondrial cristae morphology. The EMBO Journal *21*, 221–230.

Perez-Martinez, X., Funes, S., Tolkunova, E., Davidson, E., King, M., and González-Halphen, D. (2002). Structure of nuclear-localized cox3 genes in Chlamydomonas reinhardtii and in its colorless close relative Polytomella sp. Current Genetics *40*, 399–404.

Perez-Martinez, X., Broadley, S.A., and Fox, T.D. (2003). Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p. The EMBO Journal *22*, 5951–5961.

Perez-Martinez, X., Butler, C.A., Shingu-Vazquez, M., and Fox, T.D. (2009). Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome c oxidase in Saccharomyces cerevisiae mitochondria. Molecular Biology of the Cell *20*, 4371–4380.

Pfeiffer, K. (2003). Cardiolipin Stabilizes Respiratory Chain Supercomplexes. Journal of Biological Chemistry *278*, 52873–52880.

Pierrel, F., Bestwick, M.L., Cobine, P.A., Khalimonchuk, O., Cricco, J.A., and Winge, D.R. (2007). Coa1 links the Mss51 post-translational function to Cox1 cofactor insertion in cytochrome c oxidase assembly. EMBO J. *26*, 4335–4346.

Pietromonaco, S.F., Denslow, N.D., and O'Brien, T.W. (1991). Proteins of mammalian mitochondrial ribosomes. Biochimie *73*, 827–835.

Pizzo, P., and Pozzan, T. (2007). Mitochondria–endoplasmic reticulum choreography: structure and signaling dynamics. Trends in Cell Biology *17*, 511–517.

Porcelli, A.M., Ghelli, A., Zanna, C., Pinton, P., Rizzuto, R., and Rugolo, M. (2005). pH difference across the outer mitochondrial membrane measured with a green fluorescent protein mutant. Biochemical and Biophysical Research Communications *326*, 799–804.

Poulton, J., Luan, J., Macaulay, V., Hennings, S., Mitchell, J., and Wareham, N.J. (2002). Type 2 diabetes is associated with a common mitochondrial variant: evidence from a population-based case-control study. Hum. Mol. Genet. *11*, 1581–1583.

Prelich, G. (1999). Suppression mechanisms: themes from variations. Trends in Genetics *15*, 261–266.

Prescott, M., Higuti, T., Nagley, P., and Devenish, R.J. (1995). The functional expression of a rat cDNA encoding OSCP in the yeast Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. *207*, 943–949.

Prodromou, C., Roe, M.S., O'Brien, R., Landbury, J.E., Piper, P.W., and Pearl, L.H. (1997). Identification and Structural Characterization of the ATP/ADP-Binding Site in the Hsp90 Molecular Chaperone.

Pullman, M.E., and Monroy, G.C. (1963). A naturally occuring inhibitor of mitochondrial adenosine triphosphatase. J. Biol. Chem. *238*, 3762–3769.

Rainey, R.N., Glavin, J.D., Chen, H.-W., French, S.W., Teitell, M.A., and Koehler, C.M. (2006). A New Function in Translocation for the Mitochondrial i-AAA Protease Yme1: Import of Polynucleotide Phosphorylase into the Intermembrane Space. Molecular and Cellular Biology *26*, 8488–8497.

Rak, M., and Tzagoloff, A. (2009a). F1-dependent translation of mitochondrially encoded Atp6p and Atp8p subunits of yeast ATP synthase. Proceedings of the National Academy of Sciences *106*, 18509–18514.

Rak, M., and Tzagoloff, A. (2009b). F1-dependent translation of mitochondrially encoded Atp6p and Atp8p subunits of yeast ATP synthase. Proc. Natl. Acad. Sci. U.S.A. *106*, 18509–18514.

Rak, M., Gokova, S., and Tzagoloff, A. (2011). Modular assembly of yeast mitochondrial ATP synthase.

Rees, D.M., Leslie, A.G., and Walker, J.E. (2009). The structure of the membrane extrinsic region of bovine ATP synthase. Proceedings of the National Academy of Sciences *106*, 21597–21601.

Reinders, J., Wagner, K., Zahedi, R.P., Stojanovski, D., Eyrich, B., Van der Laan, M., Rehling, P., Sickmann, A., Pfanner, N., and Meisinger, C. (2007). Profiling phosphoproteins of yeast mitochondria reveals a role of phosphorylation in assembly of the ATP synthase. Molecular & Cellular Proteomics *6*, 1896–1906.

Robinson, C., Matos, C.F.R.O., Beck, D., Ren, C., Lawrence, J., Vasisht, N., and Mendel, S. (2011). Transport and proofreading of proteins by the twin-arginine translocation (Tat) system in bacteria. Biochim. Biophys. Acta *1808*, 876–884.

Rorbach, J., Richter, R., Wessels, H.J., Wydro, M., Pekalski, M., Farhoud, M., Kühl, I., Gaisne, M., Bonnefoy, N., Smeitink, J.A., et al. (2008). The human mitochondrial ribosome recycling factor is essential for cell viability. Nucleic Acids Res. *36*, 5787–5799.

Rötig, A. (2011). Human diseases with impaired mitochondrial protein synthesis. Biochim. Biophys. Acta *1807*, 1198–1205.

Rouillard, J.M., Dufour, M.E., Theunissen, B., Mandart, E., Dujardin, G., and Lacroute, F. (1996). SLS1, a new Saccharomyces cerevisiae gene involved in mitochondrial metabolism, isolated as a syntheticlethal in association with an SSM4 deletion. Mol. Gen. Genet. *252*, 700–708.

Saikrishnan, K., Kalapala, S.K., Varshney, U., and Vijayan, M. (2005). X-ray structural studies of Mycobacterium tuberculosis RRF and a comparative study of RRFs of known structure. Molecular plasticity and biological implications. J. Mol. Biol. *345*, 29–38.

Saint-Georges, Y., Garcia, M., Delaveau, T., Jourdren, L., Le Crom, S., Lemoine, S., Tanty, V., Devaux, F., and Jacq, C. (2008). Yeast mitochondrial biogenesis: a role for the PUF RNA-binding protein Puf3p in mRNA localization. PLoS ONE *3*, e2293.

Schaefer, A.M., Taylor, R.W., Turnbull, D.M., and Chinnery, P.F. (2004). The epidemiology of mitochondrial disorders—past, present and future. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1659*, 115–120.

Schägger, H., and von Jagow, G. (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. *199*, 223–231.

Schägger, H., and Pfeiffer, K. (2000). Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J. *19*, 1777–1783.

Schliebs, W., Girzalsky, W., and Erdmann, R. (2010). Peroxisomal protein import and ERAD: variations on a common theme. Nature Reviews Molecular Cell Biology *11*, 885–890.

Schon, E.A., and Area-Gomez, E. (2013). Mitochondria-associated ER membranes in Alzheimer disease. Molecular and Cellular Neuroscience *55*, 26–36.

Schreiner, B., Westerburg, H., Forné, I., Imhof, A., Neupert, W., and Mokranjac, D. (2012). Role of the AAA protease Yme1 in folding of proteins in the intermembrane space of mitochondria. Molecular Biology of the Cell *23*, 4335–4346.

Schwartz, M., and Vissing, J. (2002). Paternal inheritance of mitochondrial DNA. N. Engl. J. Med. *347*, 576–580.

Selmer, M., Al-Karadaghi, S., Hirokawa, G., Kaji, A., and Liljas, A. (1999). Crystal structure of Thermotoga maritima ribosome recycling factor: a tRNA mimic. Science *286*, 2349–2352.

Sesaki, H., and Jensen, R.E. (1999). Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. The Journal of Cell Biology *147*, 699–706.

Shingu-Vazquez, M., Camacho-Villasana, Y., Sandoval-Romero, L., Butler, C.A., Fox, T.D., and Perez-Martinez, X. (2010). The Carboxyl-terminal End of Cox1 Is Required for Feedback Assembly Regulation of Cox1 Synthesis in Saccharomyces cerevisiae Mitochondria. Journal of Biological Chemistry *285*, 34382–34389.

Sjostrand, F.S. (1953). Electron Microscopy of Mitochondria and Cytoplasmic Double Membranes: Ultra-Structure of Rod-shaped Mitochondria. Nature *171*, 30–31.

Sloan, D.B., Alverson, A.J., Chuckalovcak, J.P., Wu, M., McCauley, D.E., Palmer, J.D., and Taylor, D.R. (2012). Rapid Evolution of Enormous, Multichromosomal Genomes in Flowering Plant Mitochondria with Exceptionally High Mutation Rates. PLoS Biology *10*, e1001241.

Smith, D. (2004). Assembly of Cytochrome-c Oxidase in the Absence of Assembly Protein Surf1p Leads to Loss of the Active Site Heme. Journal of Biological Chemistry *280*, 17652–17656.

Smith, D.M., Fraga, H., Reis, C., Kafri, G., and Goldberg, A.L. (2011). ATP Binds to Proteasomal ATPases in Pairs with Distinct Functional Effects, Implying an Ordered Reaction Cycle. Cell *144*, 526–538.

Smith, P.M., Fox, J.L., and Winge, D.R. (2012). Biogenesis of the cytochrome bc1 complex and role of assembly factors. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1817*, 276–286.

Smits, P., Smeitink, J., and van den Heuvel, L. (2010). Mitochondrial translation and beyond: processes implicated in combined oxidative phosphorylation deficiencies. J. Biomed. Biotechnol. *2010*, 737385.

Snider, J., and Houry, W.A. (2008). AAA+ proteins: diversity in function, similarity in structure. Biochem. Soc. Trans. *36*, 72–77.

Sogo, L.F., and Yaffe, M.P. (1994). Regulation of mitochondrial morphology and inheritance by Mdm10p, a protein of the mitochondrial outer membrane. The Journal of Cell Biology *126*, 1361–1373.

Soto, I.C., Fontanesi, F., Liu, J., and Barrientos, A. (2012). Biogenesis and assembly of eukaryotic cytochrome c oxidase catalytic core. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1817*, 883–897.

Soubannier, V., Vaillier, J., Paumard, P., Coulary, B., Schaeffer, J., and Velours, J. (2002). In the absence of the first membrane-spanning segment of subunit 4(b), the yeast ATP synthase is functional but does not dimerize or oligomerize. J. Biol. Chem. *277*, 10739–10745.

Stock, D., Leslie, A.G., and Walker, J.E. (1999). Molecular architecture of the rotary motor in ATP synthase. Science *286*, 1700–1705.

Stock, D., Gibbons, C., Arechaga, I., Andew GW, L., and Walker, J.E. (2000). The rotary mechanism of ATP synthase.

Stojanovski, D., Bragoszewski, P., and Chacinska, A. (2012). The MIA pathway: A tight bond between protein transport and oxidative folding in mitochondria. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1823*, 1142–1150.

Strater, N., Marek, S., Kuettner, E.B., Kloos, M., Keim, A., Bruser, A., Kirchberger, J., and Schoneberg, T. (2010). Molecular architecture and structural basis of allosteric regulation of eukaryotic phosphofructokinases. The FASEB Journal *25*, 89–98.

Strauss, M., Hofhaus, G., Schröder, R.R., and Kühlbrandt, W. (2008). Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. The EMBO Journal *27*, 1154–1160.

Strogolova, V., Furness, A., Robb-McGrath, M., Garlich, J., and Stuart, R.A. (2012). Rcf1 and Rcf2, members of the hypoxia-induced gene 1 protein family, are critical components of the mitochondrial cytochrome bc1-cytochrome c oxidase supercomplex. Mol. Cell. Biol. *32*, 1363–1373.

Suzuki, T., Murakami, T., Iino, R., Suzuki, J., Ono, S., Shirakihara, Y., and Yoshida, M. (2003). F0F1-ATPase/Synthase Is Geared to the Synthesis Mode by Conformational Rearrangement of Subunit in Response to Proton Motive Force and ADP/ATP Balance. Journal of Biological Chemistry *278*, 46840–46846.

Szklarczyk, R., Wanschers, B.F., Cuypers, T.D., Esseling, J.J., Riemersma, M., van den Brand, M.A., Gloerich, J., Lasonder, E., van den Heuvel, L.P., Nijtmans, L.G., et al. (2012). Iterative orthology prediction uncovers new mitochondrial proteins and identifies C12orf62 as the human ortholog of COX14, a protein involved in the assembly of cytochrome c oxidase. Genome Biol. *13*, R12.

Szyrach, G., Ott, M., Bonnefoy, N., Neupert, W., and Herrmann, J.M. (2003). Ribosome binding to the Oxa1 complex facilitates co-translational protein insertion in mitochondria. The EMBO Journal *22*, 6448–6457.

Tamai, S., Iida, H., Yokota, S., Sayano, T., Kiguchiya, S., Ishihara, N., Hayashi, J.-I., Mihara, K., and Oka, T. (2008). Characterization of the mitochondrial protein LETM1, which maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L. Journal of Cell Science *121*, 2588–2600.

Tavares-Carreon, F., Camacho-Villasana, Y., Zamudio-Ochoa, A., Shingu-Vazquez, M., Torres-Larios, A., and Perez-Martinez, X. (2007). The Pentatricopeptide Repeats Present in Pet309 Are Necessary for Translation but Not for Stability of the Mitochondrial COX1 mRNA in Yeast. Journal of Biological Chemistry *283*, 1472–1479.

Teixeira, P.F., and Glaser, E. (2013). Processing peptidases in mitochondria and chloroplasts. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1833*, 360–370.

Teyssier, E., Hirokawa, G., Tretiakova, A., Jameson, B., Kaji, A., and Kaji, H. (2003). Temperature-sensitive mutation in yeast mitochondrial ribosome recycling factor (RRF). Nucleic Acids Res. *31*, 4218–4226.

Tiranti, V., Galimberti, C., Nijtmans, L., Bovolenta, S., Perini, M.P., and Zeviani, M. (1999). Characterization of SURF-1 expression and Surf-1p function in normal and disease conditions.

Torraco, A., Verrigni, D., Rizza, T., Meschini, M.C., Vazquez-Memije, M.E., Martinelli, D., Bianchi, M., Piemonte, F., Dionisi-Vici, C., Santorelli, F.M., et al. (2012). TMEM70: a mutational hot spot in nuclear ATP synthase deficiency with a pivotal role in complex V biogenesis. Neurogenetics *13*, 375–386.

Tovar, J., León-Avila, G., Sánchez, L.B., Sutak, R., Tachezy, J., van der Giezen, M., Hernández, M., Müller, M., and Lucocq, J.M. (2003). Mitochondrial remnant organelles of Giardia function in iron-sulphur protein maturation. Nature *426*, 172–176.

Towpik, J. (2005). Regulation of mitochondrial translation in yeast. Cellular and Molecular Biology Letters *10*, 571.

Toyoda, T., Tin, O.F., Ito, K., Fujiwara, T., Kumasaka, T., Yamamoto, M., Garber, M.B., and Nakamura, Y. (2000). Crystal structure combined with genetic analysis of the Thermus thermophilus ribosome recycling factor shows that a flexible hinge may act as a functional switch. RNA *6*, 1432–1444.

Trouillard, M., Meunier, B., and Rappaport, F. (2011). Questioning the functional relevance of mitochondrial supercomplexes by time-resolved analysis of the respiratory chain. Proc. Natl. Acad. Sci. U.S.A. *108*, E1027–1034.

Truscott, K.N., Lowth, B.R., Strack, P.R., and Dougan, D.A. (2010). Diverse functions of mitochondrial AAA+ proteins: protein activation, disaggregation, and degradationThis paper is one of a selection of papers published in this special issue entitled 8th International Conference on AAA Proteins and has undergone the Journal's usual peer review process. Biochemistry and Cell Biology *88*, 97–108.

Tsuboi, M., Morita, H., Nozaki, Y., Akama, K., Ueda, T., Ito, K., Nierhaus, K.H., and Takeuchi, N. (2009). EF-G2mt is an exclusive recycling factor in mammalian mitochondrial protein synthesis. Mol. Cell *35*, 502–510.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995). Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 A. Science *269*, 1069–1074.

Tzagoloff, A. (2004). Atp10p Assists Assembly of Atp6p into the F0 Unit of the Yeast Mitochondrial ATPase. Journal of Biological Chemistry *279*, 19775–19780.

Tzagoloff, A., Wu, M.A., and Crivellone, M. (1986). Assembly of the mitochondrial membrane system. Characterization of COR1, the structural gene for the 44-kilodalton core protein of yeast coenzyme QH2-cytochrome c reductase. Journal of Biological Chemistry *261*, 17163–17169.

Ulery, T.L., Jang, S.H., and Jaehning, J.A. (1994). Glucose repression of yeast mitochondrial transcription: kinetics of derepression and role of nuclear genes. Mol. Cell. Biol. *14*, 1160–1170.

Valente, L., Tiranti, V., Marsano, R.M., Malfatti, E., Fernandez-Vizarra, E., Donnini, C., Mereghetti, P., De Gioia, L., Burlina, A., Castellan, C., et al. (2007). Infantile encephalopathy and defective mitochondrial DNA translation in patients with mutations of mitochondrial elongation factors EFG1 and EFTu. Am. J. Hum. Genet. *80*, 44–58.

Velours, J., and Arselin, G. (2000). The Saccharomyces cerevisiae ATP synthase. J. Bioenerg. Biomembr. *32*, 383–390.

Velours, J., Vaillier, J., Paumard, P., Soubannier, V., Lai-Zhang, J., and Mueller, D.M. (2001). Bovine coupling factor 6, with just 14.5% shared identity, replaces subunit h in the yeast ATP synthase. J. Biol. Chem. *276*, 8602–8607.

Vijayasarathy, C., Biunno, I., Lenka, N., Yang, M., Basu, A., Hall, I.P., and Avadhani, N.G. (1998). Variations in the subunit content and catalytic activity of the cytochrome c oxidase complex from different tissues and different cardiac compartments. Biochim. Biophys. Acta *1371*, 71–82.

Visapää, I., Fellman, V., Vesa, J., Dasvarma, A., Hutton, J.L., Kumar, V., Payne, G.S., Makarow, M., Van Coster, R., and Taylor, R.W. (2002). GRACILE Syndrome, a Lethal Metabolic Disorder with Iron Overload, Is Caused by a Point Mutation in< i> BCS1L</i>. The American Journal of Human Genetics *71*, 863–876.

Vogtle, F.-N., Burkhart, J.M., Rao, S., Gerbeth, C., Hinrichs, J., Martinou, J.-C., Chacinska, A., Sickmann, A., Zahedi, R.P., and Meisinger, C. (2012). Intermembrane Space Proteome of Yeast Mitochondria. Molecular & Cellular Proteomics *11*, 1840–1852.

Voulhoux, R. (2003). Role of a Highly Conserved Bacterial Protein in Outer Membrane Protein Assembly. Science *299*, 262–265.

Vukotic, M., Oeljeklaus, S., Wiese, S., Vögtle, F.N., Meisinger, C., Meyer, H.E., Zieseniss, A., Katschinski, D.M., Jans, D.C., Jakobs, S., et al. (2012). Rcf1 mediates cytochrome oxidase assembly and

respirasome formation, revealing heterogeneity of the enzyme complex. Cell Metab. *15*, 336–347.

Wagener, N., and Neupert, W. (2012). Bcs1, a AAA protein of the mitochondria with a role in the biogenesis of the respiratory chain. J. Struct. Biol. *179*, 121–125.

Wagner, K., Perschil, I., Fichter, C.D., and van der Laan, M. (2010). Stepwise assembly of dimeric F1Fo-ATP synthase in mitochondria involves the small Fo-subunits k and i. Molecular Biology of the Cell *21*, 1494–1504.

Wallace, D.C. (2012). Mitochondria and cancer. Nat. Rev. Cancer *12*, 685–698.

Wallis, M.G., Groudinsky, O., Slonimski, P.P., and Dujardin, G. (1994). The NAM1 protein (NAM1p), which is selectively required for cox1, cytb and atp6 transcript processing/stabilisation, is located in the yeast mitochondrial matrix. European Journal of Biochemistry *222*, 27–32.

Wang, Z.G., White, P.S., and Ackerman, S.H. (2001). Atp11p and Atp12p are assembly factors for the F(1)-ATPase in human mitochondria. J. Biol. Chem. *276*, 30773–30778.

Waterland, R.A., Basu, A., Chance, B., and Poyton, R.O. (1991). The isoforms of yeast cytochrome c oxidase subunit V alter the in vivo kinetic properties of the holoenzyme. J. Biol. Chem. *266*, 4180–4186.

Weber, J., and Senior, A.E. (2000). ATP synthase: what we know about ATP hydrolysis and what we do not know about ATP synthesis.

Weber, J., and Senior, A.E. (2003). ATP synthesis driven by proton transport in F1F0-ATP synthase. FEBS Letters *545*, 61–70.

Weber, E.R., Hanekamp, T., and Thorsness, P.E. (1996). Biochemical and functional analysis of the YME1 gene product, an ATP and zinc-dependent mitochondrial protease from S. cerevisiae. Molecular Biology of the Cell *7*, 307.

Weraarpachai, W., Sasarman, F., Nishimura, T., Antonicka, H., Auré, K., Rötig, A., Lombès, A., and Shoubridge, E.A. (2012). Mutations in C12orf62, a factor that couples COX I synthesis with cytochrome c oxidase assembly, cause fatal neonatal lactic acidosis. Am. J. Hum. Genet. *90*, 142–151.

Westermann, B., Gaume, B., Herrmann, J.M., Neupert, W., and Schwarz, E. (1996). Role of the mitochondrial DnaJ homolog Mdj1p as a chaperone for mitochondrially synthesized and imported proteins. Molecular and Cellular Biology *16*, 7063–7071.

Wiedemann, N., Urzica, E., Guiard, B., Muller, H., Lohaus, C., Meyer, H.E., Ryan, M.T., Meisinger, C., and Muhlenhoff, U. (2006). Essential role of Isd11 in mitochondrial iron–sulfur cluster synthesis on Isu scaffold proteins. The EMBO Journal *25*.

Wiedemann, N., van der Laan, M., Hutu, D.P., Rehling, P., and Pfanner, N. (2007). Sorting switch of mitochondrial presequence translocase involves coupling of motor module to respiratory chain. The Journal of Cell Biology *179*, 1115–1122.

Wikström, M. (2004). Cytochrome c oxidase: 25 years of the elusive proton pump. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1655*, 241–247.

Wittig, I., Velours, J., Stuart, R., and Schägger, H. (2008). Characterization of domain interfaces in monomeric and dimeric ATP synthase. Molecular & Cellular Proteomics *7*, 995–1004.

Wostrikoff, K., and Stern, D. (2007). Rubisco large-subunit translation is autoregulated in response to its assembly state in tobacco chloroplasts. Proceedings of the National Academy of Sciences *104*, 6466–6471.

Yanamura, W., Zhang, Y.Z., Takamiya, S., and Capaldi, R.A. (1988). Tissue-specific differences between heart and liver cytochrome c oxidase. Biochemistry *27*, 4909–4914.

Yokoyama, T., Shaikh, T.R., Iwakura, N., Kaji, H., Kaji, A., and Agrawal, R.K. (2012). Structural insights into initial and intermediate steps of the ribosome-recycling process. EMBO J. *31*, 1836–1846.

Yoshida, T., Uchiyama, S., Nakano, H., Kashimori, H., Kijima, H., Ohshima, T., Saihara, Y., Ishino, T., Shimahara, H., Yoshida, T., et al. (2001). Solution structure of the ribosome recycling factor from Aquifex aeolicus. Biochemistry *40*, 2387–2396.

Young, J.C., Hoogenraad, N.J., and Hartl, F.U. (2003). Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. Cell *112*, 41–50.

Zara, V., Conte, L., and Trumpower, B.L. (2007). Identification and characterization of cytochrome bc1 subcomplexes in mitochondria from yeast with single and double deletions of genes encoding cytochrome bc1 subunits. FEBS Journal *274*, 4526–4539.

Zara, V., Conte, L., and Trumpower, B. (2009). Biogenesis of the yeast cytochrome bc1 complex. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1793*, 89–96.

Zavialov, A.V., Hauryliuk, V.V., and Ehrenberg, M. (2005). Splitting of the posttermination ribosome into subunits by the concerted action of RRF and EF-G. Mol. Cell *18*, 675–686.

Zeng, X., Neupert, W., and Tzagoloff, A. (2007a). The metalloprotease encoded by ATP23 has a dual function in processing and assembly of subunit 6 of mitochondrial ATPase. Molecular Biology of the Cell *18*, 617–626.

Zeng, X., Hourset, A., and Tzagoloff, A. (2007b). The Saccharomyces cerevisiae ATP22 gene codes for the mitochondrial ATPase subunit 6-specific translation factor. Genetics *175*, 55–63.

Zeng, X., Barros, M.H., Shulman, T., and Tzagoloff, A. (2008). ATP25, a new nuclear gene of Saccharomyces cerevisiae required for expression and assembly of the Atp9p subunit of mitochondrial ATPase. Mol. Biol. Cell *19*, 1366–1377.

Zhao, J., Lendahl, U., and Nistér, M. (2012). Regulation of mitochondrial dynamics: convergences and divergences between yeast and vertebrates. Cellular and Molecular Life Sciences *70*, 951–976.

Zheng, L., Cash, V.L., Flint, D.H., and Dean, D.R. (1998). Assembly of Iron-Sulfur Clusters IDENTIFICATION OF AN iscSUA-hscBA-fdx GENE CLUSTER FROM AZOTOBACTER VINELANDII. Journal of Biological Chemistry *273*, 13264–13272.

Zinser, E., Sperka-Gottlieb, C.D., Fasch, E.-V., Kohlwein, S.D., Paltauf, F., and Daum, G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. Journal of Bacteriology *173*, 2026–2034.