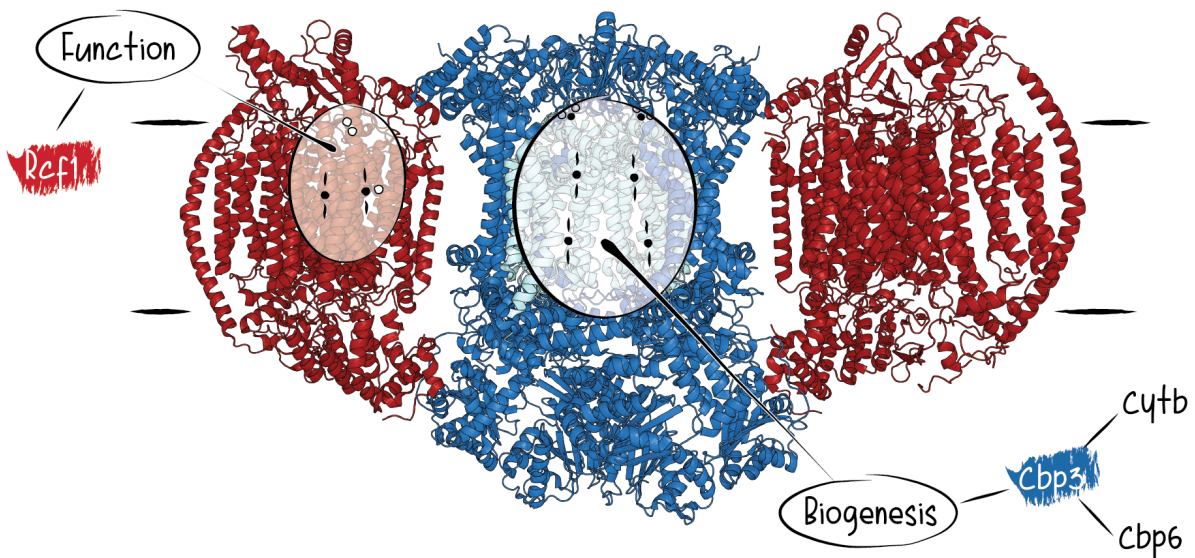


Mechanistic Insights in the Biogenesis and Function of the Respiratory Chain

Hannah Dawitz



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Academic dissertation for the Degree of Doctor of Philosophy in Biochemistry at Stockholm University to be publicly defended on Friday 6 December 2019 at 13.00 in Magnélisalen, Kemiska övningslaboratoriet, Svante Arrhenius väg 16 B.

Abstract

Mitochondria fulfill a plethora of functions, including harboring metabolic pathways and converting energy stored in metabolites into ATP, the common energy source of the cell. This last function is performed by the oxidative phosphorylation system, consisting of the respiratory chain and the ATP synthase. Electrons are channeled through the complexes of the respiratory chain, while protons are translocated across the inner mitochondrial membrane. This process establishes an electrochemical gradient, which is used by the ATP synthase to generate ATP. The subunits of two of the respiratory chain complexes, the bc_1 complex and the cytochrome c oxidase, are encoded by two genetic origins, the nuclear and the mitochondrial genome. Therefore, the assembly of these complexes needs to be coordinated and highly regulated.

Several proteins are involved in the biogenesis of the bc_1 complex. Amongst these proteins, the Cbp3-Cbp6 complex was shown to regulate translation and assembly of the bc_1 complex subunit cytochrome b . In this work, we established a homology model of yeast Cbp3. Using a site-specific crosslink approach, we identified binding sites of Cbp3 to its obligate binding partner Cbp6 and its client, cytochrome b , enabling a deeper insight in the molecular mechanisms of bc_1 complex biogenesis.

The bc_1 complex and the cytochrome c oxidase form macromolecular structures, called supercomplexes. The detailed assembly mechanisms and functions of these structures remain to be solved. Two proteins, Rcf1 and Rcf2, were identified associating with supercomplexes in the yeast *Saccharomyces cerevisiae*. Our studies demonstrate that, while Rcf1 has a minor effect on supercomplex assembly, its main function is to modulate cytochrome c oxidase activity. We show that cytochrome c oxidase is present in three structurally different populations. Rcf1 is needed to maintain the dominant population in a functionally active state. In absence of Rcf1, the abundance of a population with an altered active site is increased. We propose that Rcf1 is needed, especially under a high work load of the respiratory chain, to maintain the function of cytochrome c oxidase.

This thesis aims to unravel molecular mechanisms of proteins involved in biogenesis and functionality of respiratory chain complexes to enable a deeper understanding. Dysfunctional respiratory chain complexes lead to severe disease, emphasizing the importance of this work.

Keywords: *respiratory chain, bc1 complex, cytochrome c oxidase, Cbp3, Rcf1, Rcf2, respiratory supercomplexes, biogenesis, mitochondria, Saccharomyces cerevisiae.*

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OF THE RESPIRATORY CHAIN

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You got to have
a dream,
If you don't have
a dream,
How you gonna have
a dream come true?

– Ella Fitzgerald

List of publications

- I. Ndi M*, Masuyer G*, Dawitz H*, Carlström A, Michel M, Elofsson A, Rapp M, Stenmark P, Ott M (2019) **Structural basis for the interaction of the chaperone Cbp3 with newly synthesized cytochrome *b* during mitochondrial respiratory chain assembly.** *J. Biol. Chem.* (in press)
- II. Dawitz H, Schäfer J, Schaart JM, Magits W, Brzezinski P, Ott M **Rcf1 modulates cytochrome *c* oxidase activity especially under energy-demanding conditions.** (submitted)
- III. Schäfer J, Dawitz H, Ott M, Ädelroth P, Brzezinski P (2018) **Structural and functional heterogeneity of cytochrome *c* oxidase in *S. cerevisiae*.** *Biochim. Biophys. Acta – Bioenergetics* 1859, 699-704
- IV. Schäfer J, Dawitz H, Ott M, Ädelroth P, Brzezinski P (2018) **Regulation of cytochrome *c* oxidase activity by modulation of the catalytic site.** *Sci. Rep.* 8, 11397

Additional publications

- V. Suhm T, Kaimal JM, Dawitz H, Peselj C, Masser AE, Hanzén, S, Ambrožič M, Smialowska A, Björck ML, Brzezinski P, Nyström T, Büttner S, Andréasson C, Ott M. (2018) **Mitochondrial translation efficiency controls cytoplasmic protein homeostasis.** *Cell Metab.* 27, 1309-1322
- VI. Toth A, Aufschneider A, Fedotovskaya O, Dawitz H, Ädelroth P, Büttner S, Ott M **Absence of cytochrome *c* release from mitochondria accelerates apoptotic cell death.** (in revision)

*authors contributed equally to the work

Abbreviations

ATP	adenosine triphosphate
CI – CIV	complex I – IV of the respiratory chain
CL	cardiolipin
FAD/FADH ₂	flavin adenine dinucleotide
FeS	iron-sulfur cluster
IMM	inner mitochondrial membrane
IMS	intermembrane space
MICOS	mitochondrial contact site and cristae organizing system
mitoribosome	mitochondrial ribosome
mtDNA	mitochondrial DNA
NAD ⁺ /NADH	nicotinamide adenine dinucleotide
Q/QH ₂	ubiquinone/ubiquinol
ROS	reactive oxygen species
SC	supercomplex
TM	transmembrane

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Introduction

Mitochondria

Mitochondria are amongst the best studied organelles of the cell. Under respiratory conditions, one cell of the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) contains 20-30 mitochondria¹, while in mammalian cells up to 10^4 mitochondria can be detected, depending on the tissue². These organelles are highly dynamic and able to form a network within the cell. Mitochondria have an outer membrane, permeable for small molecules, and an almost impermeable inner membrane. The space between the membranes, the inter membrane space (IMS), is a highly oxidative environment³. The inner mitochondrial membrane (IMM) encloses the matrix, which is a hub for metabolic processes⁴. The IMM folds in invaginations called cristae, which enlarges the IMM surface significantly. In addition to protein translocation machineries, molecule channels/transporters and biosynthesis proteins, the IMM harbors the oxidative phosphorylation (OXPHOS) system. The OXPHOS system, comprised of the respiratory chain and the ATP synthase, converts chemical energy from metabolic products into a membrane potential and to ATP, the common energy currency of the cell. ATP can be used within the matrix or transported to the IMS, the cytosol and other organelles to fuel chemical reaction.

Mitochondria harbor a plethora of functions from metabolic pathways, over signaling to involvement in cell death (apoptosis)⁴. Metabolic pathways include fatty acid oxidation, tricarboxylic acid (TCA) cycle and amino acid degradation, providing metabolic precursors for the generation of macromolecules such as lipids, proteins, DNA and RNA. Additionally, metabolic pathways provide energy in the form of NADH and FADH₂ to feed electrons into the respiratory chain. The respiratory chain, together with the ATP synthase, uses this energy to generate ATP. Furthermore, mitochondria are the main production site of reactive oxygen species (ROS), highly reactive molecules, which can lead to severe damages of proteins, lipids and DNA. However, low levels of ROS are involved in signaling. In case mitochondria become dysfunctional they are able to induce organelle degradation (mitophagy) or even cell death (apoptosis).

Two genetic origins

According to the endosymbiotic theory, mitochondria are the descendants of α -proteobacteria, which were engulfed by α -archaeal cells to utilize oxygen⁵. Most of the genes were transferred to the nucleus of the host cell, but a few genes remained in the present-day mitochondria⁶. Therefore, mitochondria contain their own mitochondrial DNA (mtDNA) and an own replication, transcription and translation machinery. In yeast, mtDNA encodes for eight mitochondrial proteins, 24 tRNAs and two rRNAs⁷ (human mtDNA encodes for 13 proteins, 22 tRNAs and two rRNAs⁸). Among these proteins are one catalytic subunit of the bc_1 complex (Cytb) and the three core-subunits of cytochrome *c* oxidase (Cox1, Cox2, Cox3), while all other subunits of the respiratory chain are encoded in the nucleus. Expressing subunits from two different genetic origins leads to a complicated assembly process in which the expression and membrane insertion of mitochondrial encoded subunits needs to be coordinated with the translation, expression, transport and insertion of nuclear encoded subunits. A variety of regulatory processes are in place to ensure proper assembly. This high regulatory level might be employed to adjust complex assembly to cellular demands.

Yeast as a model organism

The yeast *S. cerevisiae* is a well-studied and widely used model organism⁹. These studies provided a tool box of biochemical approaches, ranging from simple read-outs (growth test on different carbon sources) to large scale screens (genetic and pharmacological). Additionally, *S. cerevisiae* was the first eukaryotic organism of which the entire genome was sequenced¹⁰. Due to the unique homologous recombination system of yeast¹¹ and the available biochemical tool box, pathways and genetic variations can be studied in detail. In addition, many proteins and pathways are conserved up to the mammalian system^{9,12,13}. Thus, studies in yeast can help to identify pathways and protein functions in mammals, including humans. Furthermore, yeast can be used as a simple model for human diseases. Especially when studying mitochondrial diseases, yeast is a commonly used model organism^{14,15}, mostly due to its ability to survive without functional mitochondria as long as the

cells are kept on fermentable carbon sources. Additionally, biolistic transformation gives the unique opportunity to manipulate the mitochondrial genome in yeast¹⁶, a tool that does not exist for mammalian cells.

Diseases linked to the respiratory chain

As described above, mitochondria serve multiple functions essential for cell survival. Therefore, disturbance of mitochondrial functions can lead to diseases¹⁷. One of the essential functions is conversion of metabolic products into ATP using the respiratory chain and ATP synthase. Mutations in the subunits of respiratory chain complexes as well as their assembly factors can have severe consequences^{18–24}. Mutations can lead to lower expression levels or degradation of certain subunits, leading to lower amounts of a specific complex. On the other hand, the activity of the individual complex can be affected. Dysfunctional mitochondria derived from a disturbed respiratory chain can lead to a wide range of diseases affecting muscles (myopathy) including the heart muscle (cardiomyopathy), the nervous system including the brain (encephalomyopathy), specific organs (kidney: neonatal tubulopathy; liver: hepatopathy; multisystem failure) and metabolism (ketoacidosis, lactic acidosis)¹⁴.

Due to the prominent role of the respiratory chain in mitochondrial diseases, it is important to understand the underlying mechanisms of the individual complexes and how the complexes act together.

Respiratory chain

The mammalian respiratory chain consists of four complexes: NADH:ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), bc_1 complex (complex III) and cytochrome c oxidase (complex IV).

NADH:ubiquinone oxidoreductase and succinate dehydrogenase take up electrons from the reducing equivalents NADH and $FADH_2$ on the matrix side of the IMM and channel the electrons to the electron carrier ubiquinone, which diffuses freely within the IMM (Figure 1). Reduced ubiquinol transfers the electrons to the bc_1 complex, which channels the electrons to the soluble, IMS-located electron carrier cytochrome c . Cytochrome c in turn transfers the electrons to cytochrome c oxidase, where molecular oxygen is reduced to water. The electron flow follows the electrochemical potential of the co-factors of the complexes, thereby releasing energy.

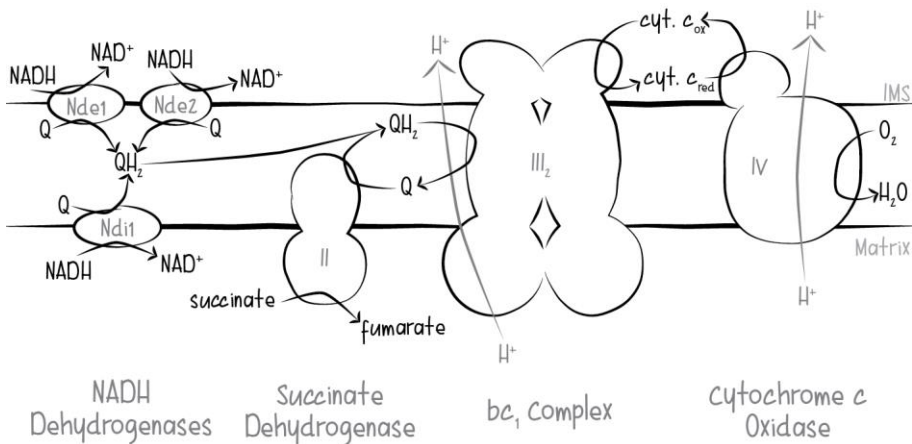


Figure 1: The respiratory chain of *S. cerevisiae*. Electrons are transferred from reducing equivalents like NADH and succinate to ubiquinol (QH_2) via NADH dehydrogenases (Nde1, Nde2 and Ndi1) or succinate dehydrogenase. Ubiquinol is oxidized to ubiquinone (Q) by the bc_1 complex, which in turn reduces cytochrome c (cyt. c). Cytochrome c oxidase transfers electrons from cytochrome c to molecular oxygen (O_2) to form water (H_2O). The bc_1 complex and the cytochrome c oxidase translocate protons across the IMM, establishing an electrochemical gradient.

The released energy is used by complex I, III and IV to translocate protons across the membrane, establishing an electrochemical gradient. This gradient is used by the ATP synthase to generate ATP. The gradient, also called

proton motive force (PMF), is composed of two parts: a pH gradient and a charge gradient. Due to tight regulation of the processes, the respiratory chain is crucial in adapting to environmental changes. Therefore, a dysfunctional respiratory chain can lead to various diseases as described on page 9.

While the composition of the respiratory chain can vary between organisms, the core subunits of the respiratory chain complexes are highly conserved. In the yeast *S. cerevisiae*, NADH:ubiquinone oxidoreductase is replaced by the NADH dehydrogenases Ndi1, Nde1 and Nde2, while the other complexes are highly conserved.

NADH dehydrogenases

In contrast to higher eukaryotes, the respiratory chain of the yeast *S. cerevisiae* does not contain NADH:ubiquinone oxidoreductase (Complex I), but three NADH dehydrogenases (also called type II NADH:ubiquinone oxidoreductase; Ndi1, Nde1 and Nde2; Figure 2). The NADH dehydrogenases are proteins located in the IMM with a size around 60 kDa, containing a FAD cofactor as the active site²⁵. These enzymes oxidize NADH and transfer the electrons to the electron carrier ubiquinone without translocating protons across the IMM. The three NADH dehydrogenases are the only proteins known in *S. cerevisiae* mitochondria to oxidize NADH^{26,27}.

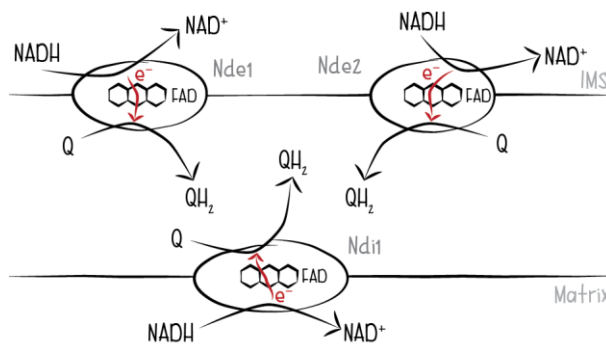


Figure 2: The NADH dehydrogenases. NADH dehydrogenases oxidize NADH and transfer electrons via the cofactor FAD to ubiquinol (QH₂). NADH is reduced in the cytosol as well as the matrix. Therefore, the yeast *S. cerevisiae* contains two “external” enzymes (Nde1 and Nde2) facing the IMS and one “internal” enzyme (Ndi1) facing the matrix.

Reduced NADH is produced in the cytosol (glycolysis) as well as in the matrix (TCA cycle). The IMM is impermeable to NADH/NAD⁺, so either NADH needs to be re-oxidized in the equivalent compartment or it needs to be shuttled between the compartments. While yeast mitochondria contain the important enzymes of the glycerol-3-phosphate dehydrogenase (G-3-PDH) system and the ethanol-acetaldehyde shuttle, a functional malate-aspartate shuttle is missing²⁸. Therefore, NADH needs to be reoxidized in both compartments to maintain the redox balance. The active site of one NADH dehydrogenase, called internal NADH dehydrogenase (Ndi1), is facing the matrix²⁹, while the other two NADH dehydrogenases, called external NADH dehydrogenase (Nde1 and Nde2) face the IMS²⁸.

Combined with complex II, these NADH dehydrogenases are the entrance of electrons to the respiratory chain.

NADH:ubiquinone oxidoreductase

In higher eukaryotes, NADH oxidation and subsequent ubiquinol reduction is coupled to proton translocation via the NADH:ubiquinone oxidoreductase, also called complex I. In human cells, this large complex consists of 45 subunits. Structural analysis showed that the complex has an L-shaped form with a hydrophobic arm embedded in the membrane, while a hydrophilic arm is extended into the matrix. The hydrophobic arm contains no cofactors. It is involved in proton translocation, but the exact pumping mechanism is not yet solved. The hydrophilic arm contains one non-covalently bound FMN and eight iron-sulfur (FeS) clusters. Electrons are transferred from NADH to FMN and then channeled through the FeS clusters (except one), following the redox potentials, to the ubiquinone binding site buried in the hydrophobic arm close to the membrane (for extensive reviews see ³⁰⁻³²).

Succinate dehydrogenase

Succinate dehydrogenase, also known as complex II of the respiratory chain, is the only respiratory chain complex with all four subunits encoded in the nuclear genome. Therefore, all subunits are synthesized in the cytosol and transported via the TOM/TIM machinery into mitochondria, where they assemble into the complex in the IMM. This complex contains several cofactors, namely three FeS clusters and one heme *b*³³ (Figure 3). Presence of this

complex is essential for respiration, as shown by a loss of respiration upon mutations in the individual subunits³⁴.

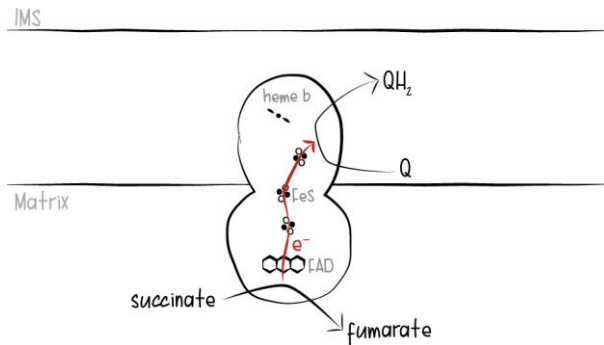


Figure 3: The succinate dehydrogenase of *S. cerevisiae*. Succinate dehydrogenase oxidizes succinate to fumarate. Electrons are transferred through the complex via the FAD and three FeS clusters to reduce ubiquinone. The function of the cofactor heme *b* is not understood yet. Succinate dehydrogenase is part of the respiratory chain as well as the tricarboxylic acid cycle.

Succinate dehydrogenase has four subunits, Sdh1-4, thereof two are integral membrane proteins (Sdh3 and Sdh4) and two are peripheral membrane proteins residing in the matrix (Sdh1 and Sdh2).

The two soluble subunits contain the catalytic core, harboring a covalently bound FAD as active site and three FeS clusters. Electrons are transferred from succinate to the FAD and further through the FeS clusters to the ubiquinone binding sites which lie in the two integral membrane proteins Sdh3 and Sdh4^{33,34}. There are two ubiquinone binding sites, one proximal (Q_p) and one distal (Q_d)³⁴. Until now, there is still a debate as to why succinate dehydrogenase has two ubiquinone binding sites. Additionally, the functional significance of the highly conserved heme *b*, located in Sdh3 and Sdh4, is not resolved yet^{33,35}. Since heme *b* is located between the two ubiquinone binding sites, it was speculated that it might transfer electrons between these two sites³³. Another proposal is that heme *b* oxidizes ubisemiquinone to avoid ROS formation³⁶.

Succinate dehydrogenase does not translocate protons across the membrane. The two protons released to the matrix from succinate during reduction of FAD are taken up from the matrix site during reduction of ubiquinone by succinate dehydrogenase.

Succinate dehydrogenase is the only complex of the respiratory chain which is also part of the TCA cycle, thereby coupling metabolism to energy production.

*bc*₁ complex

The *bc*₁ complex in *S. cerevisiae* consists of ten subunits and forms an obligate homodimer of 670 kDa³⁷. The catalytic subunits include cytochrome *b* (Cyt*b*), the Rieske iron-sulfur protein 1 (Rip1) and cytochrome *c*₁ (Cyt1) and are surrounded by seven supernumerary subunits. The catalytic subunits harbor four cofactors, heme *b*_H and *b*_L (Cyt*b*), a FeS cluster (Rip1) and heme *c*₁ (Cyt1). These cofactors are all involved in transferring electrons from ubiquinol to cytochrome *c* while translocating protons across the IMM^{38,39}. Several crystal structures^{40–43} and, in recent years, cryo-electron microscopy (cryo-EM)^{44–47} structures of the *bc*₁ complex from various species were resolved, giving mechanistic insights.

Structure and function of the subunits

The *bc*₁ complex consist of ten subunits, thereof three (Cyt*b*, Rip1 and Cyt1) are catalytically active (Figure 4).

Cyt*b* is a highly hydrophobic protein with eight transmembrane (TM) helices. It comprises the core of the dimeric complex, harboring two non-covalently bound cofactors, the high potential heme *b*_H and the low potential heme *b*_L⁴⁸. Heme *b*_L is located at the IMS side of the IMM to accept electrons from ubiquinol at the Q_o site, while heme *b*_H is located at the matrix side to reduce ubiquinone at the Q_i site. Cyt*b* is highly conserved and the only *bc*₁ complex subunit encoded in the mitochondrial DNA in all eukaryotes. Translation of Cyt*b* and assembly into the *bc*₁ complex is a highly regulated process (p. 17).

The next catalytic subunit is Rip1, which consist of a globular domain that is anchored in the IMM with one TM helix. This nuclear encoded subunit is imported into the mitochondrial matrix, where it is processed, receiving its cofactor, before its globular domain is translocated to the IMS^{49–51}. Rip1 contains an iron-sulfur cluster (2Fe-2S), which accepts electrons from ubiquinol bound at the Q_o site and transfers them to Cyt1. Interestingly, the globular domain of Rip1 resides in one monomer, while the TM helix reaches into the other monomer⁴⁰.

The last catalytic subunit is Cyt1, which also consists of a globular domain anchored in the IMM with one TM helix. The precursor polypeptide is processed twice, once when entering the matrix and once when inserted into the IMM⁵¹⁻⁵³. Before the second processing step, the cofactor heme *c* is covalently inserted into Cyt1. Cyt1 employs its cofactor to accept electrons from Rip1 and to transfer them further to the soluble electron carrier cytochrome *c*.

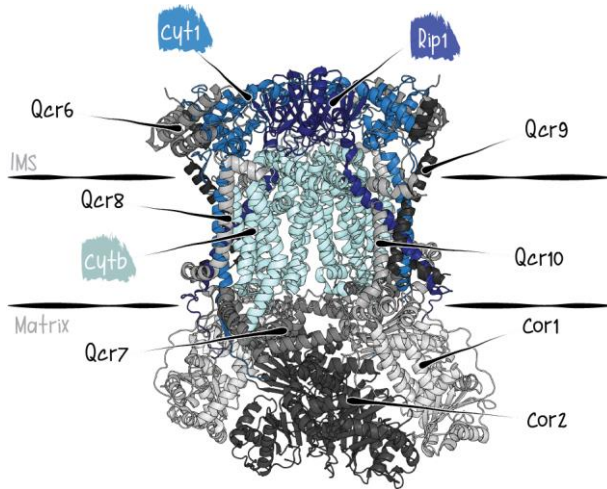


Figure 4: Structure of the bc_1 complex homodimer from *S. cerevisiae*. The catalytic subunits Cytb, Rip1 and Cyt1 are colored in blue, while the supernumerary subunits are colored in grey.

The nuclear encoded supernumerary subunits are not required for catalytic function, they neither take part in electron transfer nor in proton translocation⁵⁴. Some of the supernumerary subunits are involved in stabilizing the complex, especially its assembly intermediates (p. 17).

The supernumerary subunits Cor1 and Cor2 are soluble proteins located in the matrix, forming a tetramer in the dimeric complex. Additionally, Cor1 is part of the interaction surface with cytochrome *c* oxidase (p. 28). Cor2 forms the interface of the bc_1 complex dimer in the matrix⁵⁵ and is only attached to the complex by Cor1 and Qcr7^{46,47,56}. Cor1 and Cor2 are forming a tetrameric assembly intermediate, which is stable even in absence of Cytb⁵².

Qcr6 is also a soluble protein, but it is located next to Cyt1 in the IMS. It is the only acidic protein of the complex. It does not seem to be essential for bc_1 complex assembly or function^{57,58}. Nevertheless, it is interacting with

Cyt1, possibly preserving the heme *c* environment and supporting the interaction with cytochrome *c*^{59,60}. Loss of Qcr6 leads to lower *bc*₁ complex activity⁵⁹, while the *bc*₁ complex can still assemble^{37,61}.

Qcr7 is a membrane protein located at the matrix site. It is involved in stabilizing assembly intermediate II^{57,62,63} (p. 17).

Qcr8 and Qcr9 have one TM helix and are located next to Cyt*b*. While Qcr8 is involved in stabilizing Cyt*b* in assembly intermediate II^{57,62,64}, Qcr9 stabilizes Rip1 within the complex^{37,65}.

Qcr10 has also one TM helix, but it is sensitive to certain detergent conditions, indicating a weak binding to the complex^{47,56}. Cryo-EM structures and experiments indicate that Qcr10 interacts with Rip1 to hold this catalytic subunit in place^{40,46,47,66}.

Assembly

The subunits of *bc*₁ complex have two different genetic origins, with the catalytic subunit Cyt*b* encoded in the mitochondrial genome, while all other subunits are encoded in the nuclear genome. Therefore, the assembly of the complex must be tightly regulated. Our group and other studies described the *bc*₁ complex assembly line, showing that several proteins are needed to ensure proper assembly^{51,52,57,62,63}. In the early steps, three assembly factors, namely Cbp3, Cbp6 and Cbp4, are essential, while in the later steps Bca1, Bcs1 and Mzm1 come into play. The first and last assembly steps are resolved in detail, while the intermediate steps are still discussed (Figure 5).

Cbp3 forms an obligate complex with Cbp6 (Cbp3/6; p. 19). This complex is needed to bind to the mitochondrial ribosome (mitoribosome) at the tunnel exit and act as a translational activator for Cyt*b* translation⁶⁷. Once Cyt*b* is translated and inserted in the IMM, Cbp3/6 can bind to it, stabilizing the subunit and forming intermediate O⁶⁸. Heme *b*_L insertion follows, stabilized by subsequent binding of Cbp4 (intermediate I)⁶⁸. This intermediate is stable and accumulates in wild type cells, providing a reservoir of Cyt*b* for *bc*₁ complex assembly⁶³. Insertion of heme *b*_H leads to the release of Cbp3/6⁶⁸. After heme *b*_H insertion, Qcr7 and Qcr8 bind to Cyt*b* to stabilize the fully hemylated intermediate II³⁷. In the following, the other subunits assemble, first Cor1 and Cor2 (intermediate III), then Cyt1, Qcr6 and Qcr9, forming an intermediate of 500 kDa (intermediate IV). Finally, Qcr10 and Rip1 join to form the active complex^{37,57,62,63}. In this way, the *bc*₁ complex becomes fully assembled and functional.

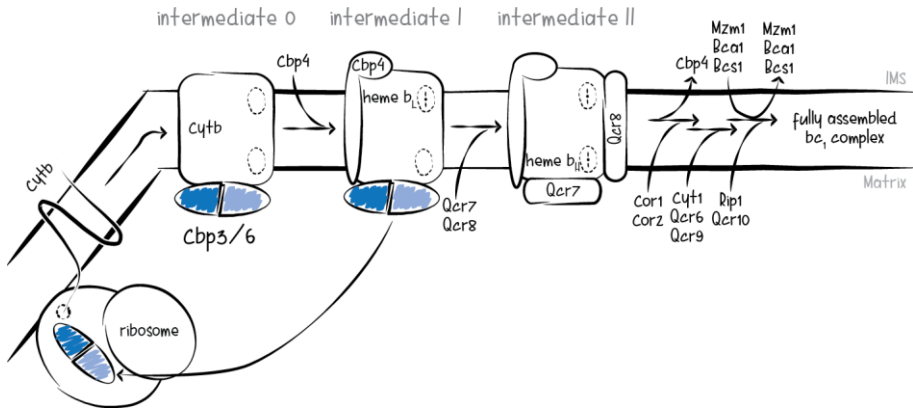


Figure 5: The Cbp3/6 complex has a dual function as a translational activator and assembly factor. Binding of Cbp3/6 to the ribosome initiates translation of *COB* mRNA. Newly synthesized, unhemylated Cytb is inserted into the IMM and stabilized by binding of Cbp3/6 (intermediate 0). First, the heme b_L site is hemylated, stabilized by the binding of the assembly factor Cbp4 (intermediate I). The second hemylation step (heme b_H) triggers the release of Cbp3/6 and binding of the subunits Qcr7 and Qcr8 (intermediate II). The assembly of the other subunits lacks detailed understanding, except of the Rip1 assembly. By employing Cbp3/6 as translational initiator and assembly factor, a direct communication between assembly and translation is established.

Bca1 is a fungi-specific assembly factor involved in a late assembly step of bc_1 complex prior to Rip1 insertion⁶⁹. Its exact function is not known.

In the last assembly step, two chaperones are involved, Mzm1 and Bcs1^{37,70,71}. These two chaperones support Rip1 maturation, translocation and subsequent assembly.

Mzm1, a chaperone conserved in higher eukaryotes⁷¹, is stabilizing Rip1 in the matrix during maturation before its globular domain is translocated to the IMS⁴⁹. The chaperone might interact with Rip1 and Qcr9 in a complex⁵¹.

The chaperone Bcs1 is involved in the translocation of Rip1 across the IMM for its maturation to occur⁴⁹⁻⁵¹. Therefore, it is essential for insertion of Rip1^{72,73}. Bcs1 is conserved in eukaryotes but absent in prokaryotes⁵¹.

Until now, it is not known at which step the bc_1 complex dimerizes, but dimerization seems to occur before the monomers are fully assembled. Conte *et al.* demonstrated for the first time that the late core intermediate (intermediate IV) is already dimerized⁷⁴. They propose that Rip1 is not essential for dimerization in contrast to previous findings.

Cbp3-Cbp6 complex

Cbp3/6 plays an important role in bc_1 complex assembly due to its dual role as translational activator and assembly factor (Figure 5).

Cbp3 is a 39 kDa protein, encoded in the nucleus⁷⁵ and imported into the mitochondria by the TOM/TIM machinery via its mitochondrial targeting sequence. It is a soluble protein located peripheral to the IMM⁷⁶. In 2001, a study investigated the importance of different regions within Cbp3⁷⁶. They showed that mutations in various regions of Cbp3 had negative effects on the assembly and functionality of bc_1 complex. This indicates that correct folding of Cbp3 is crucial to ensure stability of the protein and allow interactions with partner proteins.

Recently, a study showed that in one yeast strain (D273-10b) Cbp3 is only essential for *Cytb* hemylation and, thus, bc_1 complex assembly, but not for translational activation of *COB* mRNA⁷⁷. Interestingly, Cbp3 still interacts with the mitoribosome and *COB* mRNA in this strain. Furthermore, in this strain, in absence of Cbp3, bc_1 complex can still assemble, even into supercomplexes, however, in a non-functional state. Therefore, this study suggests that additional proteins are involved in *Cytb* synthesis in some yeast strains. The observations are supported by the fact that in the yeast *Schizosaccharomyces pombe* Cbp3/6 homologs also only support *Cytb* assembly and not translation⁷⁸.

Cbp6 is a smaller protein of 19 kDa. Cbp3 and Cbp6 can be only stably expressed in the presence of the respective other protein⁶⁷. In contrast to Cbp3, the sequence of Cbp6 is not highly conserved, but a homolog in humans was identified¹⁸.

Both proteins are necessary for respiration. Furthermore, both proteins exert different functions since overexpression of one protein cannot compensate for the absence of the other⁶⁷.

Currently, the knowledge of bc_1 complex assembly in human cells is limited. It is proposed to be similar to assembly in yeast based on identified homologs of assembly factors^{18,20,21}. Furthermore, it was shown that disturbed bc_1 complex assembly leads to diseases (p. 20). Therefore, it is essential to understand the assembly mechanisms in yeast in detail to enable knowledge for mammalian systems.

Diseases linked to Cbp3/6 homologs

Until now, two mutations were reported in the human homolog of Cbp6, UQCC2. The mutations induce *bc*₁ complex deficiency accompanied by metabolic problems (neonatal lactic acidosis), kidney dysfunction (renal tubular dysfunction) and intrauterine growth retardation¹⁸.

UQCC2 has a diverged sequence, but seems to have the same function as Cbp6. It is dependent on the presence of the Cbp3 homolog UQCC1. These two proteins interact with each other and are necessary for *Cytb* accumulation for its assembly into *bc*₁ complex.

As described above, Cbp3/6 are not only essential for hemylation and assembly of *Cytb* into *bc*₁ complex, but also for translational activation of *COB* mRNA. Whether UQCC1 and UQCC2 are also involved in translational activation is not known, since in contrast to yeast mRNA, human mRNA does not have a 5'-untranslated region (UTR) where translational activators could bind¹⁸.

Up to now, no mutations in UQCC1 were discovered. Identification of disease-inducing mutations in UQCC1 could help to understand the exact function of this protein in human mitochondria.

Catalytic cycle

The *bc*₁ complex is responsible for transferring electrons from ubiquinol, a two-electron carrier, to cytochrome *c*, a one-electron carrier (Figure 6). This process, called the Q-cycle, is a sophisticated mechanism employing a bifurcated pathway, which was first described by Mitchell in 1975³⁸.

Ubiquinol binds at the Q_o site, transferring one electron to Rip1, leaving an ubisemiquinone at the Q_o site. The electron is subsequently transferred to Cyt1 and finally to soluble cytochrome *c*. During the electron transfer, the globular domain of Rip1 undergoes a conformational change^{46,47,79}. The globular domain of Rip1 needs to move towards the Q_o site when ubiquinol is binding, building part of the actual binding site. Then, the domain rotates to Cyt1 to transfer the electron further on. The electron transfer step from ubiquinol to Rip1 or from Rip1 to Cyt1 must be the rate-limiting step according to kinetic studies⁸⁰.

The other electron reduces the low potential heme *b*_L, which transfers the electron further to the high potential heme *b*_H and to the Q_i site, where a ubiquinone accepts the electron to form ubisemiquinone. This cycle is repeated to reduce the ubisemiquinone at the Q_i site to ubiquinol. In the two

cycles necessary to fully reduce ubiquinol at the Q_i site, four protons are released to the IMS at the Q_o site, while two protons are taken up from the matrix at the Q_i site. Additionally, two cytochrome *c* molecules are reduced in the IMS.

The cryo-EM structure of the yeast supercomplexes showed a bound ubiquinone molecule in the Q_i site, but not the Q_o site, which can be explained by a higher affinity of the Q_i site for ubiquinone ^{46,47}.

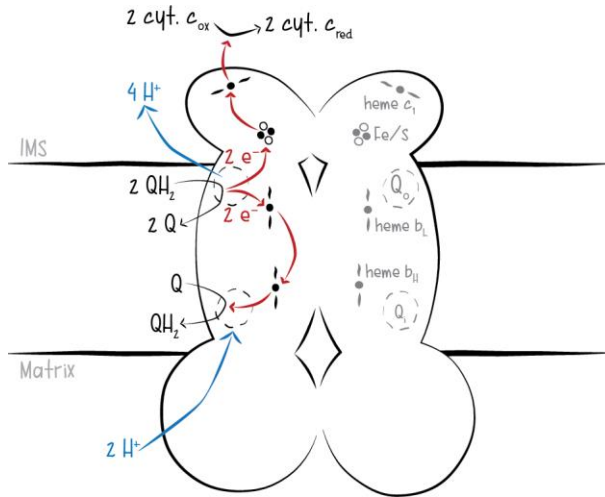


Figure 6: The Q-cycle of the bc_1 complex. Ubiquinol (QH_2) is oxidized at the Q_o site and the electrons follow a bifurcated pathway. One electron is transferred to cytochrome *c* via Rip1 and Cyt1, while a second electron is transferred to a ubiquinone (Q) at the Q_i site via heme b_L and heme b_H . Electrons from a second ubiquinol are directed the same way, reducing a second molecule of cytochrome *c* and the bisemiquinone at the Q_i site to an ubiquinol. By taking up protons (H^+) in the matrix and releasing protons to the IMS, protons are translocated across the membrane, establishing an electrochemical gradient.

Cytochrome *c* oxidase

Cytochrome *c* oxidase is the final complex of the respiratory chain. It accepts electrons from soluble cytochrome *c* and transfers them through its cofactors to molecular oxygen, reducing it to water. Already in the 1950s the metal content of the protein was determined⁸¹, while the first isolation of a functional complex was performed in the beginning of the 1960s⁸². The crystal structure from bovine cytochrome *c* oxidase was finally solved in the late

1990⁸³⁻⁸⁵. The structure of yeast cytochrome *c* oxidase in a supercomplex with the *bc*₁ complex was solved recently by cryo-EM^{46,47}.

Structure and function of the subunits

Yeast cytochrome *c* oxidase consists of twelve subunits (Figure 7). The core subunits, Cox1, Cox2 and Cox3, are mitochondrially encoded and conserved from α -proteobacteria to humans.

Cox1 is a highly hydrophobic protein consisting of twelve TM helices. This subunit harbors three out of the four metal centers of the entire complex: heme *a*, heme *a*₃ and Cu_B. Heme *a*₃ and Cu_B form the active site. Electrons are transferred through the cofactors to the active site, where oxygen binds and is reduced to water (p. 26). Cox1 harbors a conserved ring structure of five amino acids (HPEVY), of which H and Y are covalently linked^{46,85}. Since this structure ensures that the hydroxyl-group of the tyrosine is closely located to the incoming oxygen, it is thought to play an important role in catalysis². Additionally, Cox1 contains the three described proton channels, K, D and H^{46,86,87}.

Cox2 consists of two TM helices and a globular domain located in the IMS. In this globular domain the bimetallic Cu_A center is located. Thus, the metal center is accessible from the IMS and accepts electrons from soluble cytochrome *c* to transfer these electrons further to heme *a*, located in Cox1.

The last mitochondrial encoded subunit is Cox3, also a highly hydrophobic protein consisting of seven TM helices. This subunit was not found to be directly involved in electron or proton transfer, but rather stabilizing Cox1 and Cox2. Stabilizing Cox1, especially the ligand binding of Cu_B, seems to prevent suicide inactivation of cytochrome *c* oxidase⁸⁸. Furthermore, Cox3 regulates the proton pumping to ensure short lifetimes for the highly reactive oxoferryl intermediate of O₂ reduction⁸⁹. A structural feature of Cox3 is a lipid cleft containing phosphatidylethanolamine (PE) or phosphatidylglycerol (PG), depending on the organism⁸⁹. The lipids have additional contact with the Cox1 subunit, supporting the stabilizing effect of the Cox3 subunit⁸⁹. In addition, these contacts might influence the structure of the catalytic site⁸⁹. Furthermore, it was proposed that molecular oxygen diffuses through the cleft to the active site^{86,90,91}.

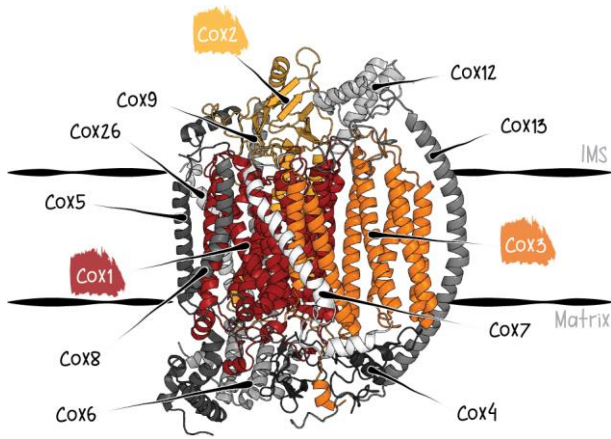


Figure 7: Structure of the cytochrome *c* oxidase of *S. cerevisiae*. The catalytic subunits Cox1, Cox2 and Cox3 are depicted in orange/red colors, while the supernumerary subunits are colored in grey.

The functions of the supernumerary subunits are only partly described. Cox4 is a globular subunit on the matrix side of the IMM interacting with Cox1, Cox3 and Cox7. It contains zinc as a cofactor, making it the only subunit with a cofactor not involved in catalytic activity. The zinc ion is needed for stability of Cox4, while Cox4 in turn is needed for the stability of cytochrome *c* oxidase⁹². It has a proposed proton collection function to support proton pumping⁹³.

Cox5 is an integral membrane protein with one TM helix. It exists in two isoforms, Cox5a and Cox5b. During normoxic conditions, the cell expresses predominantly Cox5a, while under hypoxia Cox5b is the dominating isoform. The two isoforms change the environment around the catalytic site, influencing the electron transfer in cytochrome *c* oxidase^{94,95}. Additionally, Cox5 has a proposed function in allosteric regulation of cytochrome *c* oxidase by ATP^{96,97}. As discussed on page 28, Cox5 forms the interface to the *bc*₁ complex in supercomplexes.

The subunits Cox6 (globular protein on matrix site), Cox7 and Cox9 (both integral membrane proteins) seem to be involved in assembly or stability of cytochrome *c* oxidase, since null mutants lead to respiratory deficient cells^{98–101}.

Cox12 is, like Cox4 and Cox6, a globular protein, but resides on the IMS site of the IMM. It interacts with the globular domain of Cox2 and with Cox3. The interaction of Cox12 with Cox2 might enable the binding of cytochrome *c*⁸⁵. Loss of Cox12 leads to strongly reduced cytochrome *c* oxidase

activity and assembly, but small amounts of the complex can still assemble¹⁰². Furthermore, Cox12 has a proposed function in copper insertion in Cox2¹⁰³.

Cox13 is an integral membrane protein embracing Cox3, while contacting Cox12 on the IMS site and Cox4 on the matrix site. It is proposed to protect mitochondria from ROS¹⁰⁴. Additionally, an ATP binding motive was proposed in the N-terminus, which resides in the matrix^{105,106}.

Loss of a nuclear encoded subunit (with the exception of Cox8 and Cox13) leads to a severe or complete loss of assembled cytochrome *c* oxidase or cytochrome *c* oxidase activity. Thus, Cox8 and Cox13 might not be essential for cytochrome *c* oxidase function, but might regulate its activity⁸⁷.

Recently, Cox26, was proposed to be a new stoichiometric subunit of cytochrome *c* oxidase^{107,108}. The new cryo-EM structure of supercomplexes also revealed that Cox26 is bound to CIV in stoichiometric amounts⁴⁶, supporting its claim as subunit. Based on biochemical evidence and structure analysis Cox26 interacts with Cox1, Cox9 and Cox2^{46,107}. While upon loss of Cox26 Levchenko *et al.* detected lower cytochrome *c* oxidase activity and diminished supercomplex formation already under normal conditions¹⁰⁸, Strecker *et al.* detected the same phenotypes only under stress conditions¹⁰⁷.

Assembly

Cytochrome *c* oxidase is a genetic hybrid. To ensure proper assembly of the mitochondrial and nuclear encoded subunits, there are several regulatory mechanisms in place. Part of these mechanisms is a set of yeast-specific proteins, the translational activators, interacting with the 5'-UTR of mitochondrial mRNA^{109,110}. Furthermore, the assembly of this complex is partly modular, meaning that Cox1, Cox2 and Cox3 form individual subassemblies with a specific subset of nuclear encoded subunits, before joining each other.

Cox1 has two essential translational activators, Mss51 and Pet309^{101,111}. Cox1 is cotranslationally inserted in the IMM with the help of the Oxa1 machinery¹¹². Mss51 is not only acting as translational activator, but is also part of a feedback loop that ensures proper amounts of mature Cox1 protein, as described in the following. In addition to binding to the 5'-UTR, Mss51 binds to newly synthesized Cox1 in various assembly intermediates containing the subunits Cox5a, Cox6 and Cox8 and the chaperones Shy1, Cox14, Coa3, Coa1^{113,114}. When Cox1 enters the next assembly step, Mss51 is released, enabling it to bind to and initiate a new round of translation of *COX1* mRNA¹⁰¹.

In case of disturbed assembly, Cox1 cannot enter the next assembly step and, therefore, Mss51 stays sequestered with the subassembly, unable to act as translational activator, thus, serving in an auto-regulatory mechanism.

Cox2 has one essential translational activator, Pet111¹¹⁵. While Cox1 and Cox3 are synthesized as mature proteins, Cox2 is synthesized as a precursor protein (pCox2). pCox2 is also cotranslationally inserted into the membrane via the Oxa1 machinery¹¹² and the Cox2-specific chaperone Cox18¹¹⁶. Cox18 together with Cox20, facilitates the cleavage of the precursor protein to form the mature Cox2 protein¹¹⁷. It is not clear yet if Cox2 interacts with other subunits prior to joining Cox1 and Cox3. Interactions based on assembled cytochrome *c* oxidase suggests that Cox9 and Cox12 could interact with Cox2 prior to complex assembly, while experimental data point against this proposal¹¹⁸.

Cox3 has three specific translational activators, Pet54, Pet122 and Pet494, which form a complex^{119,120}. Cox3 proceeds through several subassemblies before joining with Cox1 and Cox2. The last defined subassembly contains the subunits Cox4, Cox7, Cox13 and the auxiliary factor Rcf1¹²¹.

While Cox1 and Cox2 can still accumulate in subassemblies in case of disturbed complex assembly, Cox3 is either not translated or rapidly degraded in the absence of Cox1 or Cox2, indicating another layer of regulation¹²¹. Furthermore, the Cox3-specific translational activator Pet54 has a second role in splicing *COX1* mRNA¹²², adding another regulatory mechanism. Therefore, there seem to be several regulatory mechanisms for Cox1 and Cox3 levels, while Cox2 regulation seems to be based on the maturation of the protein.

Upon accumulation of unassembled core subunits these subunits get degraded by the ATP-dependent AAA-proteases of the IMM and matrix^{123,124}.

Nuclear encoded subunits are synthesized in the cytosol and sequestered to mitochondria via their mitochondrial targeting sequence. The proteins are imported via the TOM/TIM machinery and assemble into the respective modules.

How the individual modules assemble into the entire complex is not yet fully understood.

Metallation of the core subunits

Cox1 and Cox2 contain a total of four cofactors, which assemble into the respective subunit with help of specific chaperones.

Cox1 contains two heme *a* groups. Heme *a* is a highly reactive molecule, indicating that this cofactor should always be bound to a chaperone prior to assembly into Cox1⁸¹. The two chaperones involved in heme *a* synthesis are Cox10 and Cox15^{125,126}, two integral membrane proteins of the IMM. Therefore, heme *a* synthesis is located close to posttranslational insertion of heme *a* into Cox1⁸¹. Additionally, heme *a* synthesis is coupled to cytochrome *c* oxidase assembly steps by involving the Cox1 assembly chaperone Shy1 in heme *a* insertion¹²⁷.

Most likely simultaneous to heme *a* insertion at the Shy-containing assembly intermediate, Cu_B is inserted into Cox1¹²⁷. Copper is highly reactive and toxic, therefore, it is proposed to always bind to chaperones or transporters^{81,128}. The mitochondrial matrix contains a copper pool. The transfer of copper from the matrix to the IMS is not yet understood. In the IMS, Cox11 accepts copper from Cox17 and transfers it via an unknown mechanism to the Cu_B site^{129,130}. Since cofactors are inserted into fully translated Cox1, the protein must be flexible in the intermediate states.

The bimetallic Cu_A site is comprised of two copper ions, Cu(I) and Cu(II). Cu_A insertion into Cox2 is facilitated by the Sco proteins, Sco1 and Sco2^{131,132}. Both proteins can bind Cu(I) and Cu(II) as needed for the bimetallic center. The Cu_A binding site is quite exposed, residing in the soluble Cox2 domain explaining the post-translational Cu_A insertion. Studies showed that the flexibility of the soluble domain of Cox2 becomes restricted as soon as Cu_A is inserted¹³³.

Catalytic cycle

As described above, cytochrome *c* oxidase contains four cofactors responsible for the electron transfer from reduced cytochrome *c* through the complex to molecular oxygen, which is reduced to water at the catalytic site (Figure 8).

Electrons are transferred from cytochrome *c* to the bimetallic Cu_A site, further to heme *a* and finally to the active site consisting of the binuclear center heme *a*₃ and Cu_B. At the active site, molecular oxygen accepts the electrons and is reduced to water. During the transfer of four electrons through the complex, four protons are pumped from the matrix (also negative or N-side) to the IMS (also positive or P-side), while four more protons (chemical protons) are taken up by oxygen to form water. There are three proton channels in cytochrome *c* oxidase, the D-, K- and H-channel. All four pumped protons

are taken up by the D-channel on the matrix. Additionally, one to two chemical protons are transferred to the active site through the D-channel. The K-channel also takes up one or two chemical protons for reduction of oxygen. The function of the H-channel is still highly debated⁸⁶. The gating mechanism to avoid backflow of the protons is not yet fully understood.

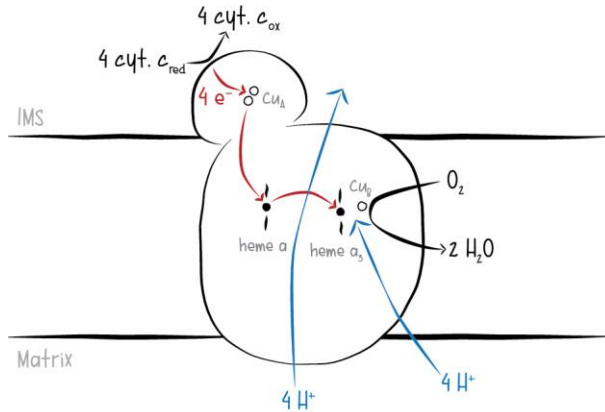


Figure 8: Catalytic cycle of the cytochrome c oxidase. Electrons are accepted from reduced cytochrome *c* (cyt. *c*) at the Cu_A site, transferred further to heme *a* and finally to the catalytic site, consisting of heme a_3 and Cu_B . At the catalytic site, molecular oxygen (O_2) is reduced to water (H_2O). For the reduction of one molecule of O_2 to two molecules of H_2O , four protons are taken up from the matrix, while four additional protons are pumped across the membrane to the IMS, thus establishing an electrochemical gradient.

Supercomplexes

For a long time the complexes of the respiratory chain were proposed to assemble in rigid entities, described in the solid-state model^{134,135}. In this model, ubiquinone and cytochrome *c* are bound to the complexes in a way that electron transfer is independent of diffusion. Later on, this model was challenged by the random-collision model, according to which all proteins move freely in the IMM and electron transfer is completely dependent on diffusion¹³⁶.

In 2000, this model was also challenged on the basis of experiments demonstrating that the respiratory chain complexes can be purified both as individual complexes and in macromolecular structures, called supercomplexes or respirasomes¹³⁷. Therefore, a new model, the plasticity model, was proposed¹³⁸. This model is a combination and extension of the two previous

models: complexes can either associate with other complexes to form active entities or the complexes can diffuse freely in an active state in the membrane. The model proposes a dynamic equilibrium between the individual complexes and the supercomplexes, which depends on physiological conditions^{138–141}. Whether ubiquinone and cytochrome *c* diffuse freely or are associated to the complexes is still under debate (p. 30). The plasticity model incorporates structural evidence as well as kinetic evidence^{138,142,143}.

Supercomplexes can be found in many organisms in multiple compositions under various conditions. Supercomplexes were detected *in vivo* by live cell imaging¹⁴⁴ and tomography. Recent advances in cryo-EM led to several high-resolution structures of supercomplexes from different organisms, enabling researchers to verify previous theories and investigate the individual complexes and supercomplexes on a structural basis.

Structure

Structure of yeast supercomplexes

In yeast, low resolution structures of supercomplexes showed that the dimer of *bc*₁ complex is flanked by one or two copies of cytochrome *c* oxidase^{145,146}. This was verified by high resolution structures resolving the III₂IV and III₂IV₂ supercomplexes (at up to 3.5Å; Figure 9)^{46,47}. While the structure of the *bc*₁ complex was already resolved by X-ray crystallography, the structure of yeast cytochrome *c* oxidase had only been published as a homology model⁸⁷. The structural features of the individual complexes are described on pages 15 and 22.

The interface between the complexes is formed by one cytochrome *c* oxidase subunit (Cox5) and five *bc*₁ complex subunits (Cor1, Qcr6, Cyt1, Rip1 and Qcr8). Several protein-protein interactions are observed in the matrix (Cor1-Cox5) and the IMS (Qcr6-Cox5, Cyt1-Cox5), while the interface in the IMM is mediated by lipids (cardiolipin (CL) and phosphocholine (PC) connecting Cox5 with Rip1 and Qcr8). Several additional lipids were identified within the core of the complexes.

Interestingly, the only subunit of cytochrome *c* oxidase which is part of the interface, Cox5, has two isoforms: Cox5a and Cox5b. However, cryo-EM structures showed that interacting residues of Cox5 are conserved within the isoforms in a way that the interaction with Cor1 is independent of the Cox5 isoform⁴⁷.

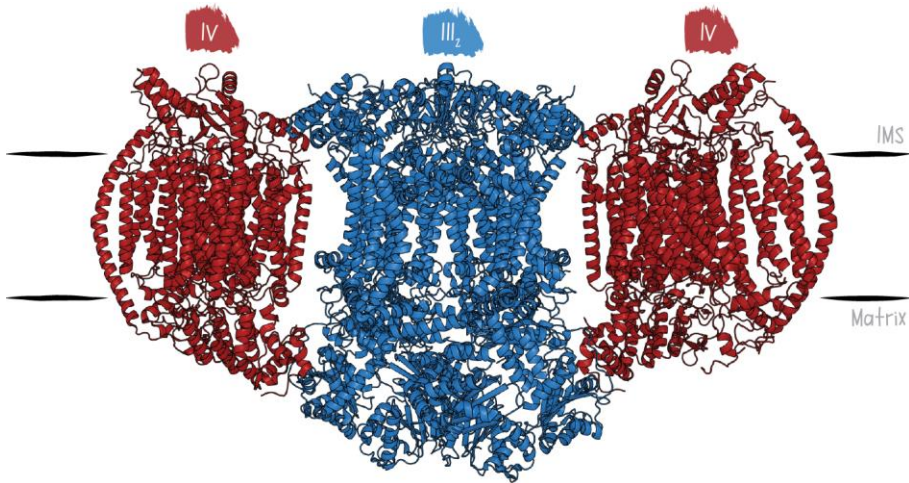


Figure 9: Cryo-EM structure of III₂IV₂ supercomplex of *S. cerevisiae* (PDB ID: 6HU9)⁴⁶. In yeast *bc*₁ complex (complex III) and cytochrome *c* oxidase (complex IV) can form supercomplexes in two different stoichiometries: III₂IV and III₂IV₂.

Structure of mammalian supercomplexes

The mammalian respiratory chain contains an additional complex, complex I (NADH:ubiquinone oxidoreductase), which is absent in *S. cerevisiae*. Therefore, several higher order structures can form in mammals: I₁III₂IV₁₋₄, I₁III₂ and III₂IV₁₋₂ (Figure 10). Structures containing all three complexes are called respirasomes, while structures with only two different complexes are called supercomplexes.

While the structure of the individual complexes is well conserved between mammals and yeast, the interaction between the complexes seems to vary due to the additional participation of complex I (CI). The *bc*₁ complex engages mostly the same subunits in forming an interface, independent on the interacting complex⁴⁶. In contrast, interaction of cytochrome *c* oxidase with *bc*₁ complex varies depending on the architecture of the macromolecular structure.

In the mammalian respirasome, the *bc*₁ complex subunits UQCRC1 (yeast homolog: Cor1; location: matrix) and UQCR11 (Qcr10; IMS) interact with the cytochrome *c* oxidase subunit COX7A (Cox7). Furthermore, cytochrome *c* oxidase is positioned towards CI and *bc*₁ complex in a way that COX7A (Cox7), COX6A (Cox13) and COX3 (Cox3) form the interface (most likely without protein-protein interactions). Interestingly, in this way the lipid cleft of Cox3 is close to the other complexes. Since the lipids of this cleft have contacts to the active site⁸⁹, the question remains if these contacts can influence the

structure of the active site. Furthermore, the interaction sites of bc_1 complex and cytochrome *c* oxidase in the mammalian respirasome compared to the yeast supercomplex differ tremendously, even if the structures of the individual complexes are well conserved. Until now, there are no structures for the mammalian III_2IV_{1-2} supercomplexes available. It will be interesting to see which subunits form the interaction sites in such a complex.

Additionally, the bc_1 complex monomers seem to be not symmetrically active in a supercomplex indicated by different occupation of ubiquinone/ubiquinol binding sites⁴⁴.

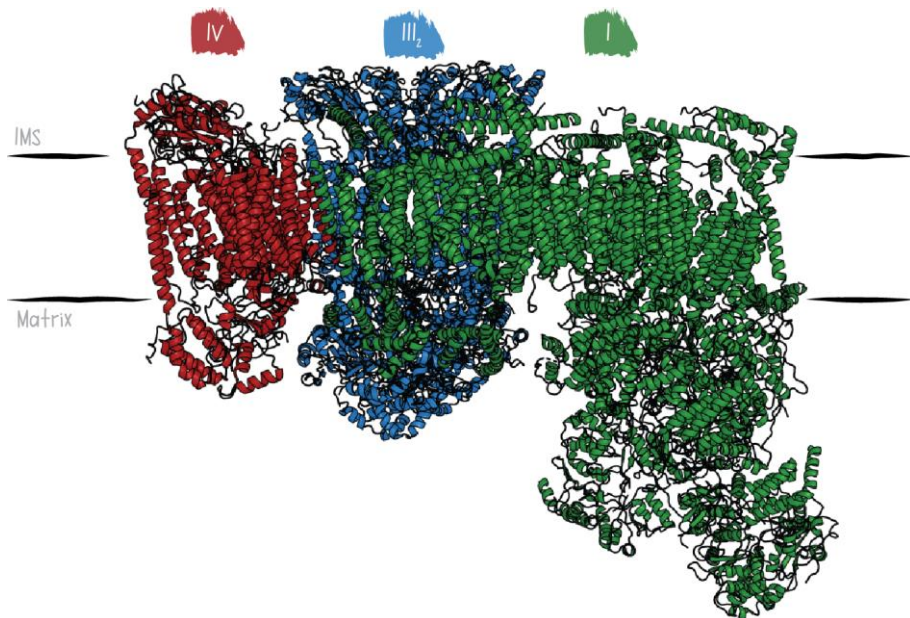


Figure 10: Cryo-EM structure of mammalian $I_1III_2IV_1$ respirasome (PDB ID: 5J4Z)¹⁴⁷. In mammals NADH:ubiquinone oxidoreductase (complex I), bc_1 complex (complex III) and cytochrome *c* oxidase (complex IV) can form higher order structures: $I_1III_2IV_{1-4}$, I_1III_2 and III_2IV_{1-2} .

Function of supercomplexes in yeast and mammals

Despite the recent cryo-EM structures and decades of research on the respiratory chain, the exact function of supercomplexes is still highly debated^{138,148–150}. Several proposals were made, ranging from increasing electron flux to avoiding aggregation in crowded membranes (Figure 11).

Increased electron flux could be achieved by several different mechanisms. First, substrate channeling between the complexes could enhance

electron flux. While initial studies supported this idea^{137,140,143}, strong evidence against substrate channeling was presented employing biophysical, biochemical and structural analysis, as described in the following. Substrate channeling is dependent on either a physical (tunnel or linker) or an electrostatic component¹⁵¹. The physical component was excluded since the recent cryo-EM structures of respirasomes revealed no protein channels restricting either ubiquinol or cytochrome *c* from diffusing into a pool^{45–47,148,152}. The electrostatic component was excluded based on a series of biochemical and biophysical experiments. Using bovine heart sub-mitochondrial particles (SMPs) and introducing an alternative quinol oxidase (AOX), Hirst and colleagues showed that ubiquinol reduced by complex I (assembled in respirasomes) can be reoxidized by AOX. Therefore, ubiquinol is in free exchange with the pool¹⁵³. Additionally, Letts *et al.* showed that trapping of ubiquinone in the I₁III₂ supercomplex limits complex I turnover⁴⁴. Moreover, individual complexes can be purified in their active state¹³⁶. Blaza *et al.* demonstrated that cytochrome *c* can be reduced by feeding electrons into the respiratory chain by either NADH or succinate¹⁵⁴. Since succinate is oxidized by complex II, which is not part of higher order structures, but *bc*₁ complex is part of supercomplexes and respirasomes, cytochrome *c* must exchange with the pool.

Another way of enhancing electron flow is to reduce the diffusion time of the substrates by keeping the individual complexes close to each other. The respirasome and supercomplex structures show a close proximity of the respective active sites, e.g. in *S. cerevisiae* the cytochrome *c* binding sites of the *bc*₁ complex and the cytochrome *c* oxidase are 70 Å apart ensuring no direct electron transfer but also minimizing the diffusion distance⁴⁷.

Furthermore, increased electron flow can be achieved by enhanced catalysis due to structural changes. While there is no direct evidence, Letts and colleagues showed that structural changes within the mammalian complex I lead to conformational changes in the *bc*₁ complex (in the core subunit MT-CYB), suggesting crosstalk between the complexes⁴⁴.

Based on the assumption that supercomplexes enable more efficient catalysis, reduced ROS production was proposed. The *bc*₁ complex and, in mammals, complex I are the main production sites of ROS in mitochondria¹⁵⁵. Assuming an increased efficiency of electron transfer, lower unspecific loss of electrons would lead to reduced production of ROS. Contradicting views exist about ROS production due to supercomplex formation^{148,156–158}. It is important to keep in mind that in all experiments the assembly of the respiratory chain was changed. Therefore, detected ROS production could be a by-

product of instable individual complexes or disturbed electron flow and is not necessarily a direct effect of the absence of supercomplexes. To investigate if increased ROS production is a direct effect of disturbed supercomplex assembly, experiments have to be designed in a way that supercomplex assembly is disturbed without affecting assembly and functionality of the individual complexes.

Another purpose of supercomplexes could be to regulate the activity of the respiratory chain. In mammals, it was demonstrated that the I_1III_2 supercomplex displays a lower activity than the respirasome $I_1III_2IV^{159}$, supporting this idea. Other studies showed that under stress conditions, such as ER/nutrient stress¹⁶⁰, exercise¹⁶¹ and adaption to carbon sources^{138,140}, more supercomplexes form. Furthermore, in mammals, two subunits involved in complex interactions (NDUFB7, UQCRH) contain disulfide bonds, indicating a possible redox regulation¹⁴⁷. Another subunit involved in inter-complex interaction is Cox5, which exhibits allosteric inhibition of cytochrome *c* oxidase by ATP^{162,163}. The question remains whether supercomplex formation influences this function.

Furthermore, supercomplex formation might be a way to avoid unintended aggregation in the highly crowded IMM¹⁵⁴, which contains only 20% lipids¹⁶⁴. By favoring weak but specific interactions, strong random interactions are avoided. Unintended strong interactions could affect activity or lead to aggregation.

Finally, evidence accumulates that the stability of supercomplexes and the individual complexes is interdependent. Experiments showed that supercomplex/respirasome assembly is disturbed in the absence of complex I, bc_1 complex, cytochrome *c* oxidase and cytochrome $c^{165-168}$, while further investigations indicated that complex I is unstable in the absence of bc_1 complex¹⁶⁶ or cytochrome *c* oxidase¹⁶⁷. On the other hand, experiments demonstrated that this interdependence only occurs under non-physiological oxygen concentrations, therefore, the physiological relevance is not clear yet¹³⁸. Additionally, the individual complexes can be purified in their active state^{82,136,169}, showing that at least the fully assembled complexes are stable individually.

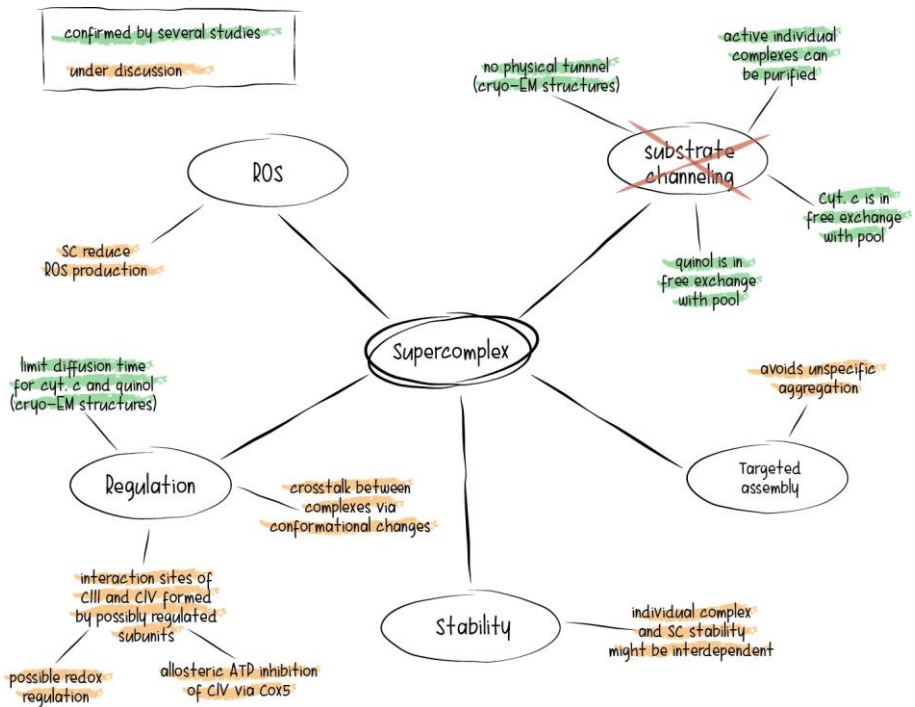


Figure 11: Summary of discussed functions of the supercomplexes. While most evidence speaks against substrate channeling, other functions like reduced ROS production or regulation of the function of the respiratory chain are still highly debated.

Assembly of supercomplexes

Until now, not much is known about the assembly of supercomplexes. There is evidence that the individual complexes assemble first before the complexes assemble into supercomplexes/respirasomes^{170,171}. This is supported by the fact that complexes can be purified individually in a catalytically active state^{82,136,169}. On the other hand, there are experiments suggesting that under certain conditions, assembly intermediates of different complexes can interact in a supercomplex intermediate before the rest of the subunits assemble^{165,172}. Furthermore, Trumpower and colleagues proposed a complex between subassemblies of bc_1 complex (intermediate II) and cytochrome *c* oxidase⁵⁷.

The importance of individual subunits for supercomplex assembly is still under debate. Zara *et al.* showed that Qcr9 is essential for supercomplex assembly, while Qcr6 is not³⁷. This is in line with findings from Cruciat *et al.*⁶¹ but in contrast to findings from Pfeiffer and colleagues¹⁷³. Additionally, the

newly identified subunit of cytochrome *c* oxidase, Cox26, might be involved in stabilizing the interaction surface of cytochrome *c* oxidase with *bc*₁ complex¹⁰⁷.

As mentioned before the IMM forms cristae. This morphology is important for respiratory complex stability and supercomplex formation¹⁷⁴. Furthermore, supercomplex stability depends on the presence of specific lipids. Cardiolipin (CL), the signature lipid of mitochondria, is important for supercomplex stability. Strains deficient in CL show decreased supercomplex assembly^{173,175} and individually purified complexes need CL to be able to assemble into supercomplexes when reconstituted into liposomes^{176,177}. In contrast, phosphatidylethanolamine (PE) destabilizes supercomplexes¹⁷⁸. Nevertheless, both lipids are necessary to maintain the membrane potential¹⁷⁸. This demonstrates that lipids need to exist in a perfect balance in mitochondria to ensure assembly and function of the individual proteins and organelle morphology.

Assembly of the individual complexes and supercomplexes can not only be affected by mutations/deletions in genes encoding subunits or assembly factors, but also in genes encoding proteins involved in lipid synthesis. The most prominent example is found to be part of the genetic reason for Barth syndrome. Mutations in the taffazin gene, a lipid synthase essential for CL modulation, strongly reduce CL levels in mitochondria. Loss of CL in turn leads to loss of supercomplexes^{179,180}.

Proteins associated with supercomplexes

Analyzing supercomplexes and respirasomes in yeast and mammals led to the discovery of several proteins associated with these structures without being subunits of the individual complexes including the Rcf proteins, SCAFI, Coi1 and Aac2.

Rcf1 and Rcf2

Rcf1 (Aim31) and Rcf2 (Aim38) were discovered in 2012 by three independent groups^{104,181,182}, while later also Rcf3 was described¹⁸³. The proteins were proposed to influence supercomplex assembly and therefore called respiratory supercomplex factors. All three proteins are integral membrane proteins and belong to the conserved Hig1 protein family. Members of this family were identified due to their structural similarity defined by the Hig-domain

(PF04588)¹⁸⁴. Most of the already characterized proteins of this family are induced under various stress conditions like low glucose, hypoxia, oxidative stress and light^{184–188}.

While Rcf1 is highly conserved, Rcf2 and Rcf3 are fungi specific.

Structure

Based on biochemical data and computational analysis Rcf1 was proposed to consist of two TM helices and a long soluble fungi specific C-terminal tail. The N- as well as C-termini are proposed to reside in the IMS^{104,181,182}.

While there is no structure of the proteins associated with cytochrome c oxidase or supercomplexes, a structure of Rcf1 in micelles was solved by NMR¹⁸⁹. To resolve this structure, Zhou *et al.* purified Rcf1 overexpressed in *E. coli*, denatured the protein and refolded it in the presence of dodecylphosphocholine (DPC). In contrast to the proposed structure, in this environment Rcf1 consists of five TM helices, with TM4 and TM5 forming the charged interface of a homodimer. Therefore, the N- and C- termini are on opposite sides of the membrane. The study also proposed a model in which Rcf1 is in an equilibrium between monomer and dimer. It was proposed that TM4 and TM5 could flip out of the membrane, splitting the dimer and interact with other proteins.

A specific structural feature, the QRRQ motif [(Q/I)₃(R/H)XRX₃Q] within the Hig domain, was investigated in detail¹⁹⁰. Mutations of the QRRQ motif led to the proposal that this motif is on the one hand needed to stabilize Rcf1 within the membrane. On the other hand, the QRRQ motif is involved in the transient association of Rcf1 with a late-stage assembly intermediate of cytochrome c oxidase, thereby modifying its activity.

Function and interaction partners

The exact function of Rcf1 and Rcf2 are still under debate (Figure 12). Growth assays showed that loss of Rcf1 leads to a slightly diminished respiratory ability, while loss of Rcf2 has no impact on overall respiration. Surprisingly, loss of both proteins leads to a strong defect in respiration^{104,181,182}.

As mentioned above, Rcf1 and Rcf2 were discovered as proteins associating with supercomplexes. Interestingly, Rcf1 as well as Rcf2 also comigrate with lower molecular weight complexes and in a free form¹⁸¹. This observation is supported by the fact that Rcf1 protein levels are not affected in the absence of assembled cytochrome c oxidase¹⁸¹. While Rcf1 and Rcf2 display

a stronger association with cytochrome *c* oxidase, in its absence both proteins can interact with the *bc*₁ complex^{181,182}. In line with this observation, loss of Rcf1 affects cytochrome *c* oxidase on different levels, while *bc*₁ complex is mostly unaffected^{104,181,182}.

In the absence of Rcf1 reduced levels of cytochrome *c* oxidase subunits are detected^{104,181}. Additionally, it was reported that Rcf1 is needed for the correct assembly of Rcf2, Cox12 and Cox13 into cytochrome *c* oxidase^{104,181}. This led to the observation of two distinct cytochrome *c* oxidase forms, differing in the content of Cox13¹⁰⁴. The Cox13-containing form seems to protect mitochondria from ROS, since in its absence ROS production increases. *rcf1Δ* mitochondria show increased oxidative damage supposedly partly because of loss of Cox13 from the cytochrome *c* oxidase^{104,182}. Therefore, it seems that several forms of assembled cytochrome *c* oxidase, and likely also several forms of supercomplexes, exist, depending on the presence of Rcf1, Rcf2 and Cox13.

Furthermore, it was shown that Rcf1 can interact with newly synthesized Cox3, one of the cytochrome *c* oxidase core subunit^{181,182,191}. While it is expected that this interaction is also true for assembled cytochrome *c* oxidase, it was not shown yet. Rcf1, binding to Cox3 (and possibly Cox12 and Cox13), could influence the binding site of cytochrome *c* and, thus, influence cytochrome *c* oxidase activity.

Analysis of the activity of the individual complexes showed that loss of Rcf1 leads to reduced cytochrome *c* oxidase activity. The reduction compared to WT activity varied between 30% to 70% in different studies^{104,181,182,191,192}. Cytochrome *c* oxidase activity is not affected in the absence of Rcf2^{104,181}. In the absence of both proteins, Strogolova *et al.* detected only 25% of wild-type cytochrome *c* activity¹⁸¹.

Due to the effect of Rcf1 on cytochrome *c* oxidase activity, the catalytic site of cytochrome *c* oxidase was analyzed in the absence and presence of Rcf1 and Rcf2 by monitoring CO-recombination in whole mitochondria¹⁹². The study proposes two populations of cytochrome *c* oxidase with different activities, dependent on presence of Rcf1 and Rcf2. In the absence of Rcf1 or Rcf2, a change in the active site of cytochrome *c* oxidase was proposed, with Rcf1 showing the more pronounced phenotype. Furthermore, following cytochrome *c* oxidation, it was proposed that cytochrome *c* is closely bound between *bc*₁ complex and cytochrome *c* oxidase with the help of Rcf1. In absence of Rcf1, electron transfer between the *bc*₁ complex and cytochrome *c*

oxidase depends solely on diffusion, which would be slower than electron transfer via bound cytochrome *c* mediated by Rcf1.

Two studies proposed that Rcf1 and Rcf2 are involved in regulating proton pumping and proton leakage via Cox3 and its integral lipids and, thus, regulating the activity of cytochrome *c* oxidase^{191,193}. The studies showed that the PMF declines upon loss of Rcf1, Rcf2 and both proteins together. Therefore, they proposed a role of Rcf1 and Rcf2 in folding and lipid association of Cox3 either with newly synthesized Cox3 or Cox3 damaged during the catalytic cycle.

Interestingly, Rcf1 also interacts with the ADP/ATP carrier Aac2 (Pet9)¹⁸¹. This protein is highly abundant in the IMM¹⁹⁴, therefore, the interaction could be random. Nevertheless, inhibitors of Aac2 influencing its conformation have been shown to display different effects on Rcf1 crosslink abilities to Aac2, indicating that Rcf1 might sense different conformation of the protein and, thus, different metabolic states in mitochondria¹⁸¹.

Upon loss of Rcf1, supercomplex assembly is affected. The higher molecular weight supercomplex, III₂IV₂, is assembled in lower amounts, therefore, more III₂IV and individual complexes accumulate^{104,181,182}. It was suggested that Rcf1 and Rcf2 localize at the interface of the supercomplexes due to their ability to independently interact with cytochrome *c* oxidase as well as *bc*₁ complex^{181,182}. They were proposed to act as a bridge or glue between the complexes. In light of the recent yeast supercomplex structures and indications of Rcf1 binding to Cox3, a bridging function of Rcf1 seems unlikely. Nevertheless, it was not shown yet if Rcf1 and Rcf2 can interact with multiple subunits of the individual complexes under different conditions.

Mass spectrometric analysis revealed that Rcf1 and Rcf2 were present in the final preparation of supercomplexes used for cryo-EM studies⁴⁶. The fact that Rcf1 and Rcf2 were not identified in the structure supports the idea that these proteins only bind transiently to some supercomplexes or cytochrome *c* oxidase.

Rcf1 belongs to the Hig1 protein family, a family of which most of the members are expressed under stress conditions. Two research groups investigated if Rcf1 expression is important under hypoxic conditions^{104,182}. They came to opposite conclusions, leaving the question unanswered if Rcf1 is induced under stress conditions.

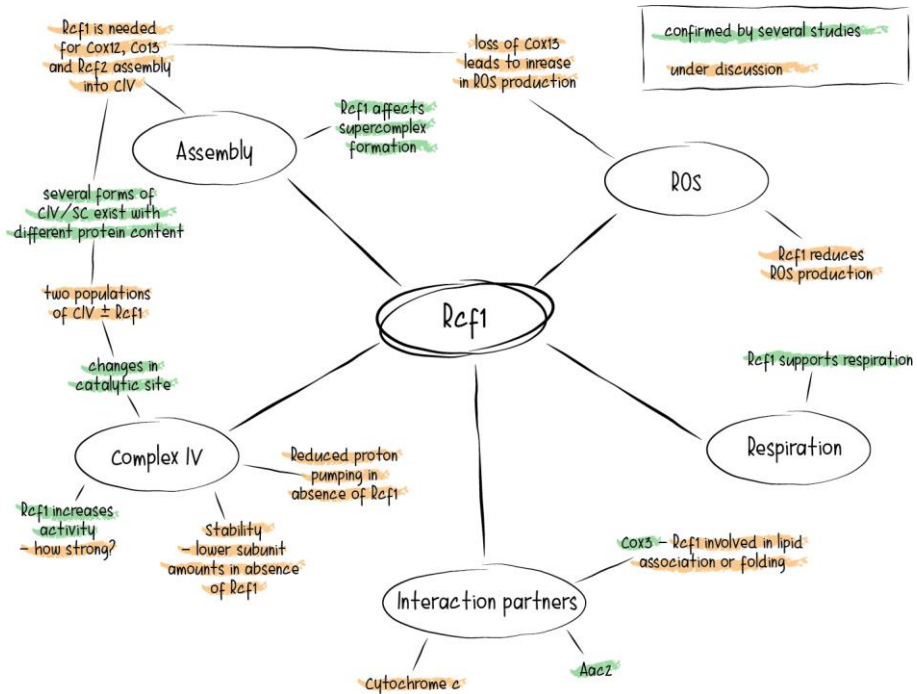


Figure 12: Summary of possible functions of Rcf1. Rcf1 was discovered as a protein associated with supercomplexes (SC). While it affects supercomplex assembly, recent studies propose a more direct role of Rcf1 in regulation of cytochrome *c* oxidase (CIV) activity.

In contrast to the clear phenotypes in absence of Rcf1, absence of Rcf2 seems to not influence either cytochrome *c* oxidase activity or assembly, including supercomplex assembly. Interestingly, loss of Rcf2 still leads to increased ROS production, similar to loss of Rcf1¹⁰⁴.

Rcf1 homologs

While Rcf2 and Rcf3 are fungi specific, Rcf1 has several homologs in mammals, with HIGD1A and HIGD2A (also RCF1A and RCF1B) best studied. Both proteins are expressed under several stress conditions like hypoxia, low glucose, light and in different developmental stages^{184–187,195,196}. Interestingly, both proteins localize to mitochondria, but can relocalize to the nucleus^{195,197}. This nuclear localization can be found under physiological conditions as well as under stress conditions during disease processes^{195,197}.

HIGD1A is the more intensely studied homolog. It was discovered already in 2000¹⁹⁶ and was later found to be expressed in different tissues under multiple conditions^{185,186,188,195,198,199}. Several functions are proposed for HIGD1A,

including repression of oxygen consumption, enhancing AMPK activity, lowering cellular ROS and involvement in cancer^{196,200}. In addition, HIGD1A is involved in anti-apoptotic responses²⁰⁰ by counteracting cytochrome *c* release¹⁹⁹. Moreover, it was demonstrated that HIGD1A is required for the morphology of mitochondria by interacting with OPA1²⁰¹. Furthermore, Hayashi *et al.* showed that bovine HIGD1A can associate with fully assembled cytochrome *c* oxidase and regulate its activity in a positive manner²⁰². Based on simulations, this group proposed that HIGD1A binds to COX1 (*ScCox1*), COX2 (*ScCox2*), COX6c (*ScCox9*) and COX4 (*ScCox5*) and changes the structural environment of heme *a*. This in turn should lead to an increase in proton pumping.

HIGD2A is implicated in changes of the mitochondrial morphology via OPA1¹⁹⁵. It also enhances cell survival under hypoxic conditions by counteracting apoptosis¹⁹⁹. Two research groups showed that homologs of Rcf1 (human HIGD2A and *C. elegans* M05D6.5) can rescue the growth phenotype in *rcf1Δ* yeast strains, suggesting that the conserved Hig-domain has a conserved function^{181,182}.

Rcf3

In 2016 one research group¹⁸³ discovered that Rcf2 is proteolytically processed into a stable C-terminal fragment and a labile N-terminal fragment. Interestingly, the C-terminus contains not only the Hig-domain, but is also encoded by another open reading frame, YBR255c-A, now called Rcf3. Rcf3 is also an integral membrane protein and can associate with cytochrome *c* oxidase as well as supercomplexes. Only upon loss of both proteins, Rcf2 and Rcf3, lower respiration is detected, indicating an overlapping function of these two proteins. Interestingly, the assembly state of the complexes is not affected upon absence of Rcf2 and Rcf3. This emphasizes the differences between Rcf1 and the Rcf2/Rcf3 protein pair: while Rcf1 affects not only cytochrome *c* oxidase activity but also its assembly, including supercomplex assembly, the Rcf2/Rcf3 protein pair can influence cytochrome *c* oxidase activity without changing the assembly states. Based on copurifications another study proposed that Rcf3 might mediate contacts between supercomplexes and the mitochondrial contact site and cristae organizing system (MICOS) complex¹⁹⁴.

SCAFI

In mammals, SCAFI, supercomplex assembly factor 1 (also COX7A2L or COX7RP), was discovered in association with supercomplexes and respirasomes containing cytochrome *c* oxidase. While this protein is a homolog of the cytochrome *c* oxidase subunit COX7A, its exact function is controversially debated. There are two forms of SCAFI, a short (SCAFI₁₁₁) and a long form (SCAFI₁₁₃)¹⁴⁰. The function of the long form is quite agreed upon: most studies showed that SCAFI₁₁₃ is involved in assembly of the III₂IV supercomplex as well as in the assembly of respirasomes^{140,203–206}.

The function of the short form is still strongly discussed. Some studies do not detect a difference in respirasome formation in mice strains expressing the different isoforms^{141,205–208}, while other studies proposed that the short form of SCAFI cannot support supercomplex assembly in specific tissues^{140,203}. To explain this contradiction, tissue specificity, strain differences and experimental variations must be taken into consideration. Nevertheless, it seems that SCAFI is affecting assembly of supercomplexes and respirasomes without affecting respiratory function.

In the analysis of respirasomes, SCAFI was not identified in the recent cryo-EM structures¹⁴⁷. Since SCAFI is a homolog of COX7A it was proposed that these two polypeptides are interchangeable in the structure^{147,203}. This proposal is supported by the sequence similarity of these two polypeptides: all the major interaction residues are conserved¹⁴⁷. Unfortunately, the resolution of the cryo-EM structure is not high enough to identify the highly flexible N-terminus of SCAFI, distinguishing it from COX7A¹⁴⁷. Nevertheless, the idea of the interchanging isoforms is supported by the detection of two respirasome populations, one containing SCAFI and the other one containing COX7A^{203,204}.

Since a study demonstrated that SCAFI interacts predominantly with *bc*₁ complex it was speculated that cytochrome *c* oxidase can assemble lacking COX7A and subsequently assemble with *bc*₁ complex containing SCAFI²⁰⁴. By binding to *bc*₁ complex, SCAFI, in contrast to COX7A, can introduce a bridge with its extra domain to interact with cytochrome *c* oxidase (tested by simulations²⁰³).

Coi1

Cytochrome *c* oxidase interacting protein, Coi1, is a fungi specific integral membrane protein suggested to be involved in supercomplex formation. The only extensive study was performed by Singhal *et al.*²⁰⁹. The group showed that Coi1 interacts with both *bc*₁ complex and cytochrome *c* oxidase subunits, Rcf1 and with MICOS components. In the absence of Coi1, respiratory function is decreased and assembly of *bc*₁ complex as well as cytochrome *c* oxidase and subsequently assembly of supercomplexes is disturbed. In contrast to these strong phenotypes, mitochondrial lipid composition and morphology seem to be not affected.

ADP/ATP carrier

The ADP/ATP carriers (AAC) are located in the IMM. These integral membrane proteins exchange cytosolic ADP with matrix-generated ATP. Three isoforms exist, Aac1-3, thereof Aac2 (Pet9) is the most abundant. Several studies demonstrated that Aac2 associates with supercomplexes^{181,210}, while one study showed a direct interaction with Rcf1¹⁸¹. The interaction with supercomplexes was proposed to be beneficial for Aac2, so the higher local membrane potential can be employed to drive the ADP/ATP exchange²¹¹.

Paper summary and future perspectives

One of the main functions of mitochondria is the transformation of energy stored in metabolites into ATP, the common energy currency of the cell. This process is performed by the respiratory chain and the ATP synthase. To ensure proper function, several layers of regulation on protein level and assembly of complexes are employed. Despite decades of research on the respiratory chain, the assembly and regulation of these structures are still not fully understood.

This thesis aims to understand molecular mechanisms in the assembly of respiratory chain complexes as well as supercomplexes and in the regulation of respiratory chain complex activity. These new insights will help to understand the function of individual complexes and supercomplexes better and might help to identify therapeutic targets to tackle mitochondria-derived diseases.

In the following I will summarize the papers included in this thesis. I will describe open questions and possible approaches to answer these questions. The first paper describes our findings about an assembly factor of bc_1 complex, Cbp3, while the three other papers address Rcf1 and Rcf2, two proteins involved in the regulation of cytochrome *c* oxidase activity.

In **paper I**, we solved the structure of a Cbp3 homolog from *Brucella abortus*, *BaCbp3*. The structure was used to construct a homology model of the highly conserved C-terminal ubiquinol-cytochrome *c* chaperone domain of Cbp3 of *S. cerevisiae* (*ScCbp3*). Based on this model, residues residing on the surface of the globular domain were identified for modification. An unnatural amino acid was introduced at the identified positions to enable photo-induced crosslinking. With this approach we were able to identify binding surfaces on Cbp3 with its obligate partner, Cbp6, and with its client, the newly synthesized *Cytb*. The binding sites are located on opposite sides of the glob-

ular domain of Cbp3, indicating that both interactions can occur simultaneously, in line with the current assembly model⁶³. Since only the structure of the conserved domain was resolved, there is no structural information about the yeast-specific N-terminus. Previous studies showed an interaction between Cbp3 and the mitoribosome. Since we could not identify an interaction of the C-terminal domain of Cbp3 with the mitoribosome, we speculate that the N-terminus is involved in the interaction with the mitoribosome to facilitate translational activation of *COB* mRNA.

In the future, there are several possibilities to investigate the interactions of Cbp3 with its partners to gain deeper insight into the molecular details of its function.

First, the interaction site with the mitoribosome has to be identified. Since there is no structural information about the N-terminus available to identify suitable residues, the photocrosslink approach becomes more complicated. The unnatural amino acid used in this study, p-benzoyl-L-phenylalanine (pBpa), resembles an aromatic amino acid. Therefore, aromatic residues in the N-terminus could be used as sites to incorporate pBpa to ensure proper folding of the N-terminus and identify interaction partners. To enhance the probability of detecting interactions, several residues could be targeted at once. Additionally, the charges of the surface around the ribosomal tunnel exit can be determined to modify appropriately charged residues in the N-terminus of Cbp3. Another approach would include employing chemical crosslink reagents. This approach was used previously to identify proteins interacting with Cbp3⁶⁷. Since only the N-terminus of Cbp3 contains cysteines, cysteine-specific crosslink reagents can be used to identify interactions specifically between the N-terminus and other proteins. With this approach not only proteins at the tunnel exit (which contain cysteines on the surface of the mitoribosome), but also possible additional interactors could be identified.

Second, the translational activation function of Cbp3/6 is not fully understood. Identifying interaction partners might help to unravel this mechanism. As described before and shown in paper I, Cbp3 has a variety of interaction partners, which even interact with the same residues, indicating a quite dynamic behavior of Cbp3. Identifying possible additional interaction partners by mass spectrometry might elucidate the molecular mechanism of translational activation in yeast. This insight might help to determine whether the human homolog, UQCC1, functions in a similar way.

Third, it would be interesting to determine the molecular changes leading to the release of Cbp3/6 from bc_1 assembly intermediate I. Upon full hemylation, *Cytb* could undergo a conformational change, disrupting the interaction with Cbp3/6. On the other hand, interaction of Qcr7 with *Cytb* or Cbp3 could lead to the release of Cbp3/6.

Last, it would be of high interest to identify the interaction sites of *Cytb* or Cbp6 with Cbp3. For those two proteins pathogenic mutations are described. Determining if these mutations disrupt the interaction with Cbp3 and, thus, disrupt bc_1 complex assembly, might help to get a deeper insight into the cause of their pathogenicity.

Until today, there are no mutations identified in the human Cbp3-homolog UQCC1. As soon as a mutation is identified, the homology model of ScCbp3 and interaction sites presented in paper I can help to identify the underlying mechanism of the disease-causing mutation.

Papers II, III and IV address Rcf1 and Rcf2, proteins involved in cytochrome *c* oxidase activity as well as supercomplex assembly. There are several studies published with partly contradicting results discussing the function of Rcf1 and Rcf2.

In **paper II**, we show that, while Rcf1 only has a small effect on supercomplex assembly, it strongly decreases cytochrome *c* oxidase activity. Interestingly, Rcf2 shows the opposite effect on cytochrome *c* oxidase activity, but with a weaker phenotype compared to Rcf1. Therefore, we speculate that these two proteins are involved in regulating cytochrome *c* oxidase activity.

This hypothesis is supported by our earlier studies, **papers III and IV**, in which we investigated the catalytic site of cytochrome *c* oxidase in detail. Yeast cytochrome *c* oxidase was purified from strains expressing or lacking Rcf1. The purified oxidase was analyzed by measuring light absorption spectra and ligand-binding kinetics. Comparing wild type and *rcf1* Δ oxidase, changes in the light absorption spectra and ligand-binding were evident. These observations indicate alterations around the catalytic site upon loss of Rcf1. We demonstrated that cytochrome *c* oxidase exists in three different populations: 1) fully active, 2) altered catalytic site, but overall intact structure, 3) altered catalytic site as well as overall structure. All three populations exist in wild type strains, thereof the first population is dominant, while in absence of Rcf1, there is a significant shift to the other two, structurally altered, populations. Nevertheless, in absence of Rcf1, fully active cytochrome *c* oxidase can assemble. Due to the alterations around the catalytic

site, the midpoint potential in the cytochrome *c* oxidase is lowered. We speculate that this structural change influences O₂ binding and, thus, regulate energy conservation of the enzyme.

Furthermore, in **paper II** we show that interaction of Rcf1 and Rcf2 with the supercomplexes is dependent on the carbon source. Under respiratory conditions, more cytochrome *c* oxidase assembles into supercomplexes compared to fermentable conditions. However, relatively more Rcf1 and Rcf2 are assembled into supercomplexes under respiratory conditions. This indicates a stronger need for regulation of the processes during a high work load of the respiratory chain. Additionally, we show that the capacity of Rcf1 to promote respiration is conserved in the Hig-domain, while interactions with other proteins are enabled by both domains. The interaction with cytochrome *c* oxidase subunit Cox3 could facilitate the observed alterations in the catalytic site. We show that these interactions persist in cytochrome *c* oxidase as well as in supercomplexes.

While our findings help to better describe the function of Rcf1 on a molecular level as well a physiological level, many open questions still remain. How does Rcf1 influence cytochrome *c* oxidase activity? Rcf1 can affect cytochrome *c* oxidase activity in three principle ways: 1) By binding to Cox3, Rcf1 could simultaneously interact with Cox1, directly altering the structure around the catalytic site. 2) Rcf1 might structurally modulate the lipid-binding cleft of Cox3, changing the lipid interaction with the catalytic site in Cox1 (most likely with Cu_B⁸⁹), altering the structure of the catalytic site. 3) The Rcf1 binding site could be normally occupied by lipids. Upon binding of Rcf1, the lipids could be released and the active site structurally altered.

Does Rcf1 regulate cytochrome *c* oxidase activity or does it rather have a maintaining function? While the regulating function of Rcf1 cannot be excluded, the maintaining function is more in line with our experimental evidence. A fully active, structurally unaltered cytochrome *c* oxidase population is present in the absence of Rcf1. Additionally, during high turnover of cytochrome *c* oxidase, likely relatively more Rcf1 is needed in the supercomplexes to maintain its function. To identify the exact molecular mechanism of Rcf1 function, a structure of cytochrome *c* oxidase-Rcf1 complex or supercomplex-Rcf1 complex is essential.

Why does Rcf1 and Rcf2 assemble preferentially in the supercomplexes under respiratory conditions? Our data demonstrate that, under respiratory

conditions, more Rcf1 and Rcf2 assemble into supercomplexes. Several possible explanations exist for this observation: 1) The combination of Rcf1 and Rcf2 might enhance respiratory efficiency under respiratory conditions; 2) The turn-over of cytochrome *c* oxidase might be higher under respiratory conditions. Therefore, Rcf1 might be needed to maintain cytochrome *c* oxidase function. Furthermore, the question remains how Rcf1 and Rcf2 assemble into supercomplexes under different conditions. This question might be answered with the help of radio-labeling in assembly studies.

Populärvetenskaplig sammanfattning

Mobiltelefon, kaffebryggare, skrivbordslampa – alla maskiner vi använder behöver ström ur eluttaget. Denna el skapas i kraftverk från olika resurser, såsom vind, vatten eller kärnkraft. För att fungera som en människa behöver vi inte el från uttaget. Men hur får vi vår energi? Vilka är våra kraftverk?

Vår kropp består av många enskilda celler, som i sin tur är uppdelade i olika fack, så kallade organeller. Varje organell är specialiserad på vissa funktioner. En typ av organell, mitokondrierna, är känd som "cellens kraftverk." Varje cell har flera mitokondrier. Dessa överför energi från en mängd resurser till den universella energibäraren: adenosintrifosfat, även kallat ATP. ATP är den viktigaste energibäraren, cellens "el", som kan användas i hela cellen för att driva olika kemiska processer.

Mitokondrier är i sin tur indelade i distinkta områden separerade av ett membran. I detta membran inbäddas flera biologiska maskiner, så kallade enzymer. Dessa antar den ovannämnda uppgiften att konvertera energi från råmaterial till den universella energibäraren ATP. Fyra av dessa enzymer, komplex I till IV, är anslutna i serie och bildar andningskedjan, som således ansvarar för att tillgodose majoriteten av cellens energibehov. Hos människor har många genetiska förändringar identifierats som orsaker till en felaktig andningskedja, och kan orsaka sjukdomar. Dessa sjukdomar kan försvaga musklerna, bland annat skada hjärtmuskeln, men också nervsystemet och därmed påverka till exempel hjärnan.

Forskning om mänskliga celler medför olika begränsningar. Därför används jästceller som en modellorganism i denna doktorsavhandling. Till skillnad från mänskliga celler kan jästceller överleva utan funktionella mitokondrier, vilket möjliggör karaktäriseringen av allvarliga genetiska förändringar som hotar funktionen hos mitokondrier.

Enzymer, liksom de fyra komplexen i andningskedjan, tillhör molekylklassen proteiner. Denna klass av molekyler utför en mängd olika uppgifter i cellen. Förutom enzymer kan proteiner fungera som transportörer eller ta emot och överföra signaler. Dessutom kan proteiner hjälpa andra proteiner att anta

rätt form vid rätt tidpunkt och interagera med andra proteiner. Dessa sistnämnda proteiner kallas också chaperoner.

Denna doktorsavhandling behandlar bland annat chaperonet Cbp3, vilket är ansvarig för sammansättningen av komplex III i andningskedjan. Cbp3 har en annan chaperon som sin obligatoriska partner, Cbp6. Tillsammans med Cbp6 stöder Cbp3 integrationen av en del av komplex III (cytokrom *b*) i membranet. Vi identifierade de exakta interaktionsytorna på Cbp3 med dess två interaktionspartners, Cbp6 och cytokrom *b*. Genetiska förändringar kan påverka chaperonen Cbp3. Så snart sådana genetiska förändringar har identifierats, kan effekten på komplex III bestämmas med hjälp av denna avhandling.

Den andra delen av denna avhandling behandlar proteinerna Rcf1 och Rcf2. I synnerhet Rcf1 hjälper till att säkerställa att komplex IV i andningskedjan fungerar optimalt. Vi beskriver hur Rcf1 håller det aktiva centrumet av komplex IV i rätt position för att säkerställa optimal aktivitet av komplex IV.

Många mitokondriesjukdomar kan för närvarande bara behandlas symptomatiskt. Detta arbete bidrar till en djupare förståelse av enskilda processer i den mitokondriella andningskedjan. Denna kunskap kan i framtiden användas för att optimera behandlingar av dessa sjukdomar.

Populärwissenschaftliche Zusammenfassung

Handy, Kaffeemaschine, Schreibtischlampe – alle Geräte, die wir benutzen, brauchen Strom, der aus der Steckdose kommt. Dieser wird in Kraftwerken aus unterschiedlichsten Ressourcen, wie Wind, Wasser oder fossilen Brennstoffen, gewonnen. Um als Mensch zu funktionieren, brauchen wir keinen Strom aus der Steckdose. Aber wie bekommen wir dann unsere Energie? Was sind die Kraftwerke unseres Körpers?

Unser Körper besteht aus vielen einzelnen Zellen, die wiederum in verschiedene Abteile, sogenannte Organellen, gegliedert sind. Jedes Organell ist auf bestimmte Funktionen spezialisiert. Eine Art von Organellen, die Mitochondrien, sind als „Kraftwerke der Zelle“ bekannt. Jede Zelle hat eine Vielzahl an Mitochondrien. Diese übertragen Energie aus verschiedensten Ressourcen auf einen universellen Energieträger: Adenosintriphosphat, kurz ATP. ATP ist der Hauptenergieträger, der „Strom“, der in der gesamten Zelle genutzt werden kann, um verschiedenste Prozesse anzutreiben.

Mitochondrien sind wiederum in verschiedene Bereiche unterteilt, die durch eine Membran getrennt sind. In dieser Membran sind mehrere biologische Maschinen, sogenannte Enzyme, eingebettet. Diese übernehmen die oben genannte Aufgabe, Energie aus Rohstoffen in den universellen Energieträger ATP umzuwandeln. Vier dieser Enzyme, Komplex I bis IV, sind in Reihe geschaltet und bilden die Atmungskette, welche somit mitverantwortlich ist, den Energiebedarf der gesamten Zelle zu decken. Bei Menschen wurden viele genetische Veränderungen identifiziert, die eine fehlerhafte Atmungskette hervorrufen und damit Krankheiten auslösen. Diese Krankheiten können Muskeln schwächen, unter anderem den Herzmuskel, aber auch das Nervensystem schädigen und damit zum Beispiel auch das Gehirn beeinträchtigen.

Forschung an menschlichen Zellen bringt verschiedene Einschränkungen mit sich. Deshalb werden in dieser Doktorarbeit Hefezellen als Modellorganismus genutzt. Hefezellen können, im Gegensatz zu menschlichen Zellen, ohne funktionale Mitochondrien überleben und

ermöglichen somit die Charakterisierung schwerwiegender genetischer Veränderungen, die die Funktionalität der Mitochondrien beeinträchtigen.

Enzyme, wie die vier Komplexe der Atmungskette, gehören zu der Molekülklasse der Proteine. Diese Molekülklasse übernimmt eine Vielzahl von Aufgaben in der Zelle. Proteine können neben Enzymen auch als Transporter fungieren oder Signale empfangen und weitergeben. Des Weiteren können Proteine andere Proteine dabei unterstützen, sich zum richtigen Zeitpunkt in die richtige Form zu bringen und mit anderen Proteinen zu interagieren. Diese letzte Art nennt man auch Chaperone.

Diese Doktorarbeit beschäftigt sich unter anderem mit dem Chaperon Cbp3. Dieser Chaperon ist für die Zusammensetzung von Komplex III der Atmungskette mitverantwortlich. Cbp3 hat als obligatorischen Partner einen weiteren Chaperon, Cbp6. Zusammen mit Cbp6 unterstützt Cbp3 die Integration eines Einzelteils von Komplex III (Cytochrom *b*) in die Membran. Wir haben die genauen Interaktionsflächen von Cbp3 mit seinen zwei Interaktionspartnern, Cbp6 und Cytochrom *b*, identifiziert. Genetische Veränderungen können den Chaperon Cbp3 verändern. Sobald in Zukunft solche genetischen Veränderungen identifiziert werden, kann die Auswirkung auf die Zusammensetzung von Komplex III mit Hilfe dieser Doktorarbeit bestimmt werden.

Der zweite Teil dieser Arbeit befasst sich mit den Proteinen Rcf1 und Rcf2. Insbesondere Rcf1 hilft dabei, sicherzustellen, dass Komplex IV der Atmungskette optimal funktioniert. Wir beschreiben, wie Rcf1 das aktive Zentrum von Komplex IV in der richtigen Position hält um optimale Aktivität von Komplex IV zu gewährleisten.

Viele mitochondriale Erkrankungen können derzeit nur symptomatisch behandelt werden. Diese Arbeit trägt dazu bei, ein tiefergehendes Verständnis einzelner Prozesse in der mitochondrialen Atmungskette zu erreichen. Dieses Wissen kann in Zukunft genutzt werden, um Therapien zu optimieren.

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