# Yeast mitochondrial copper metabolism: topology and role of Cox11p

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Zur Erlangung des akademischen Grades
Doktor der Naturwissenschaften
(Dr. rer. nat.)

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**Oleh Khalimonchuk**

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# Contents

# Abbreviations

| | |
|---|---|
| A | Absorption |
| aa | Amino acid |
| ADH | Alcohol dehydrogenase |
| AEBSF | 4-(2-aminoethyl)-benzenesulfonyl fluoride |
| APS | Ammoniumperoxisulfate |
| BN-PAGE | Blue-native polyacrylamide gel electrophoresis |
| bp | Base pairs |
| Cal | Calmodulin |
| C-terminal | Carboxy-terminal |
| Cu | Copper |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleosidetriphosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylendiamine-tetraacetic acid |
| HRP | Horseradish peroxidase |
| IMM | Inner mitochondrial membrane |
| IMS | Mitochondrial intermembrane space |
| Kan | Kanamycin |
| kDa | Kilodalton |
| MOPS | 4-Morpholinepropanesulfonic acid |
| mt | Mitochondrial |
| N-terminal | Amino-terminal |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| PEG | Polyethyleneglycol |
| PI | Protease inhibitor |
| PMSF | Phenylmethylsulfonyl fluoride |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| RNAasein | Ribonuclease inhibitor |

| | |
|---|---|
| RT | Room temperature |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| TAP | Tandem affinity purification |
| TEV protease | Tobacco etching virus protease |
| TBE | Tris Borate EDTA |
| TBS | Tris-buffered saline |
| TEMED | N,N,N',N'-Tetramethylethylenediamine |
| TM | Transmembrane |
| Tris | Tris(hydroximethyl)aminometane |
| V | Volt |
| v/v | Volume per volume |
| w/v | Weight per volume |
| wt | Wild-type |
| YNB | Yeast nitrogen base |

## Amino acid code

| | | | | | |
|---|---|---|---|---|---|
| A | Ala | Alanine | M | Met | Methionine |
| C | Cys | Cysteine | N | Asn | Asparagine |
| D | Asp | Aspartic acid | P | Pro | Proline |
| E | Glu | Glutamic acid | Q | Gln | Glutamine |
| F | Phe | Phenylalanine | R | Arg | Arginine |
| G | Gly | Glycine | S | Ser | Serine |
| H | His | Histidine | T | Thr | Threonine |
| I | Ile | Isoleucine | V | Val | Valine |
| K | Lys | Lysine | W | Trp | Tryptophan |
| L | Leu | Leucine | Y | Tyr | Tyrosine |

# Aim of the work

Cytochrome *c* oxidase (COX) is the terminal enzyme of the eukaryotic respiratory chain, reducing oxygen to water and generating a proton gradient, that drives ATP synthesis. In the yeast, *Saccharomyces (S.) cerevisiae* it is composed of 11 subunits, three of which are encoded by mitochondrial DNA. The latter form the catalytic core of COX including two hemes, a magnesium ion, a zinc ion and three copper ions. Since free copper is not available in the cells a chaperone system is necessary to deliver copper to the mitochondria and COX. It has been postulated that Cox17p transfers copper to the proteins of the Sco-protein-family which are involved in copper insertion into the binuclear $Cu_A$ site in Cox2p subunit. Recently it was demonstrated that Cox11p, a protein essential for respiratory growth, is implicated in the assembly of $Cu_B$ site of COX. However little is still known about the mechanisms of both processes. Interestingly, the genome of the fission yeast *Schizosaccharomyces (S.) pombe* contains two almost identical versions of *cox11*$^+$ gene: *cox11*$^+$and *cox11b*$^+$. Both proteins contain a large N-terminal extension that is homologous to *S. cerevisiae* Rsm22p, a protein of the small subunit of mitochondrial ribosome. The reason for existence of such a fusion remains unclear.

It is proposed to characterize the proteins involved in copper insertion into COX using methods of molecular genetics and biochemistry, with special emphasis on Cox11p. The functional complementation of Cox11p family members in the heterologous yeast systems will be analyzed. Topogenesis and topological properties of Cox11p from *S. cerevisiae* as well as from *S. pombe* will be characterized. In order to get a clue how the $Cu_B$ is formed, interactions of Cox11p with proteins involved in COX assembly will be analyzed. The question why fission yeast require two versions of *cox11*$^+$ and if both version might be functional and necessary for the *S. pombe* cells, will be addressed.

# Chapter 1

# Introduction

## 1.1 Copper and its trafficking in the cell

Copper is a transition metal that has the ability to cycle between two redox states, oxidized $Cu^{2+}$ and reduced $Cu^{+}$. It is an essential nutrient required for the activity of a number of enzymes with diverse biological roles. Virtually all organisms require copper as a catalytical cofactor for a number of biological processes including respiration, iron transport, oxidative stress protection, pigmentation, blood clotting and normal cell growth and development (for review see Harris 2000; Puig and Thiele, 2002). Copper as well as the most of the other metals are not freely available within the cells (Rae et al., 1999; Outten and O'Halloran, 2001). Meanwhile it seems clear that metal ions cannot simply diffuse to the respective compartment to be inserted into molecular structures. As free copper is chemically active, a strigent control system for Cu homeostasis must exist to protect the cell from the toxic effects caused by the redox properties of this element. To avoid the oxidative damage of proteins, lipids and nucleic acids caused by the damaging hydroxyl radicals thar are formed from oxygen in the presence of free copper (Halliwell and Gutteridge, 1992), specific pathways for Cu trafficking and delivery into the various cellular compartments have evolved (Bartnikas and Gitlin, 2001). A number of proteins were identified that can bind copper, thus protecting it from the intracellular chelation, and deliver it to the respective targets. Most of the data about copper homeostasis in eucaryotic sells has emerged from the studies on *Saccharomyces (S.) cerevisiae*, although the mechanisms of Cu metabolism in the cell are believed to be highly conserved. Known copper trafficking routes are shown in Figure 1. Copper can be aquired by the cell via either high- or low-affinity transporter. In the first case, $Cu^{2+}$ from the cell environment is reduced to $Cu^{+}$ by Fre1p and Fre2p cell surface reducatses (Anderson et al., 1994; Georgatsou et al., 1997; Martins et al., 1998; De Freitas et al., 2003) and transported across the plasma membrane by Ctr1p and Ctr3p high affinity transporters. Expression of both of genes encoding these proteins as well as *FRE1* is under control of the transcriptional activator Mac1p (Labbe et al., 1997; Yamaguchi-Iwai et al., 1997).

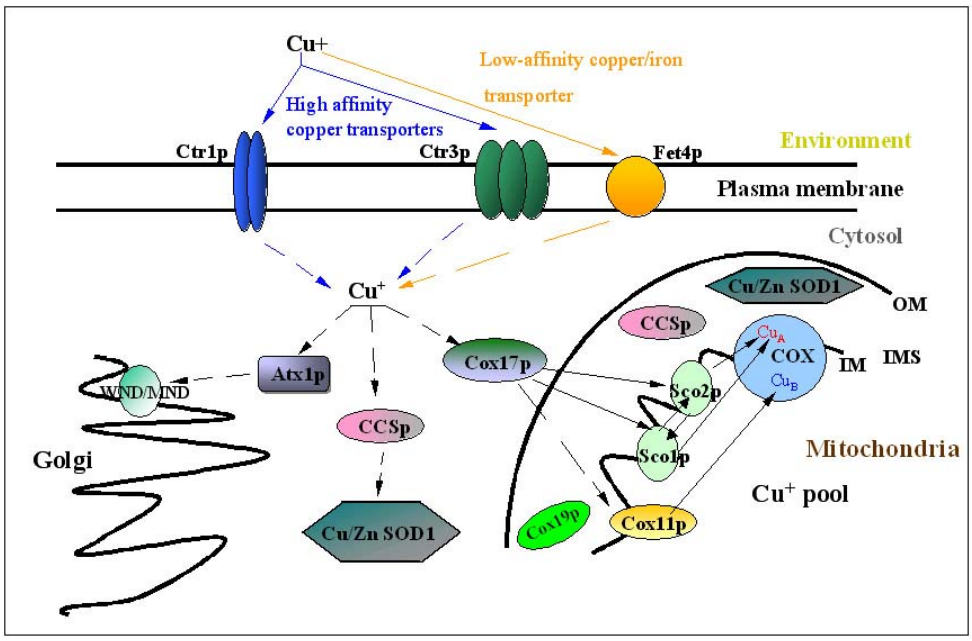



**Figure 1. Distribution of copper ions in the living cell.** A schematic that shows major distribution pathways for copper in the living cell (modified from Puig and Thiele, 2002).

Interestingly in *Schizosaccharomyces (S.) pombe* Ctr1p and Ctr3p are represented by only one protein – SpCtr4p that has similarity to both ScCtr1p and ScCtr3p (Labbe et al., 1999). Low-affinity Cu transport is provided by Fet4p that can transport both iron and copper ions, and Ctr2p, a protein similar to the Ctr-family which is presumably involved in the mobilization of intracellular Cu pools (Puig and Thiele, 2002; Rees et al., 2004). Following acquisition, copper ions can be routed on three different ways: to the secretory pathway, to cytosolic Cu,Zn-superoxide dismutase (SOD) and to mitochondria (Fig.1). It is still a point of discussion if cytosolic Cu pool exists (Finney and O'Halloran, 2003). One class of proteins that may be involved in Cu-storage are metallothioneins, small cysteine-rich proteins with high Cu-binding capacity, that are found in eukaryotes (Dameron and Harrison, 1998). In *S. cerevisiae* two isoforms are described, Cup1p and Crs5p. Expression of these two factors has been shown to depend on Cu concentration and is controlled by the Cu-containing transcription factor Ace1p, that also controls expression of Sod1p (Huibregtse et al., 1989; Evans et al., 1990; Culotta et al., 1994; De Freitas et al., 2003).

So far two types of proteins that represent copper delivery to the secretory pathway were identified, namely Atx1p (also known as Atox1p or Hah1p in other organisms) and the Cu-transporting P-type ATPase Ccc2p (also known as Atp7ap, Atp7bp, or Menkes and Wilson disease proteins). Atx1p can obtain copper directly from Ctr1p *in vitro*, however, it is believed that another pathways exists *in vivo* (Finney and O'Halloran, 2003). Hereby copper is transferred from Atx1p to its target protein Ccc2p, that delivers Cu ions into a trans-Golgi compartment. Delivered copper is utilized by a multi-copper oxidase Fet3p (ceruloplasmin in mammals) and other Cu-containing enzymes (Finney and O'Halloran, 2003). Another route of copper shuttling in the cytosol is represented by CCSp (Ccs1p), a metallochaperone required for the direct insertion of copper into the SOD (Sod1p) (Rae et al., 1999; Puig and Thiele, 2002; Finney and O'Halloran, 2003).

The third compartment that requires copper ions for normal function is the mitochondrion. Especially copper-requiring is cytochrome *c* oxidase (COX), one of the complex enzymes that form the respiratory chain, and together with ATPase complex, an OXPHOS system, where oxidative phosphorylation, the energy generating pathway (Rosamond, 1982) is located. The delivery of copper ions to mitochondria (mt) and their further distribution will be discussed in more details below.

### 1.2. Cytochrome *c* oxidase and its organization

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) is the terminal enzyme of the eukaryotic respiratory chain. The membrane-embedded complex, which acts as a dimer (Frey, 1994; Tsukihara et al., 1995), faces both the mt intermembrane space (IMS) and the mt matrix, slightly more emerging to the IMS side (Tsukihara et al., 1995; Carr and Winge 2003). COX plays a key role in the electron transport chain; it catalyzes the reduction of molecular oxygen to water and concomitantly the oxidation of reduced cytochrome *c*, and couples this redox reaction with the electrogenic transfer of protons across the inner mitochondrial membrane (IMM). Some bacterial COX homologues act as quinol oxidases and use quinol or ubiquinol as a substrate. Based on similarities of the primary structure between these quinol oxidases and COX both groups of enzymes are classified as the superfamily of

heme-copper terminal oxidases (reviewed by Ferguson-Miller and Babcock, 1996; Michel et al., 1998).

High-resolution structures of $aa_3$-type COX purified from bovine heart (Tsukihara et al., 1995; 1996; Yoshikawa et al., 1998), of bacterial homologues from *Paracoccus denitrificans* (Iwata et al., 1995; Ostermeier et al., 1997), and of $ba_3$-type COX (possessing heme *b* instead of heme *a*) from *Thermus thermophilus* (Williams et al., 1999; Soulimane et al., 2000) have been described. These data in combination with the data emerging from the genetic, immunological and biochemical studies provide a good basis for understanding the details of COX organization and function.

### *1.2.1. COX subunits*

Eukaryotic COX exist as high molecular weight complexes composed of 11 (*S. cerevisiae)* to 13 (mammals) subunits that are present in equimolar amounts in the assembled enzyme. The three largest proteins (Cox1p, Cox2p and Cox3p), which are encoded by the mt DNA, form the active core of the enzyme and represent the major part of the complex (Poyton and McEwen, 1996; Carr and Winge, 2003). The other subunits are encoded by the nuclear genome, translated on cytosolic ribosomes and transported into mitochondria, where assembly takes place. Figure 2A presents a simplified schematic version of mammalian COX, showing only subunits that contain cofactors or are engaged in early assembly steps of the enzyme. The crystal structure of the entire bovine enzyme is shown in Fig.2B.

Prokaryotic oxidases usually consist of 3 subunits (Capaldi, 1990), that share significant homology with the mt synthesized subunits (Keightley et al., 1995; Poyton and McEwen, 1996). Subunit Cox1p of both prokaryotic and eukaryotic COX coordinates heme *a* and a fused binuclear heme $a_3$-$Cu_B$ redox center. It also ligates a sodium ion by a site which also possesses affinity for Ca ions (Tsukihara et al., 1995; Kirichenko et al., 1998; Lee et al., 2002). The organization and function of these cofactors will be discussed later. The highly conserved Cox1p is the biggest (55-57 kDa) (Capaldi, 1990) and most hydrophobic protein of the core subunits. It spans the IMM with 12 transmembrane (TM) helices, which form hydrophobic pockets (Carr and Winge, 2003). Cox1p appears to be the key subunit both for assembly and function of COX. It is involved in proton pumping

by means of two proton translocating pores, the D- and K-channel. These are formed by hydrophilic residues which appear to be linked by a network of water molecules. The D-channel routes from the matrix side to a conserved glutamate residue, whereas the K-channel connects the matrix side with the $Cu_B$ center (Rich et al., 1998; Wikstrom et al., 1998; Gennis, 1998). Studies in yeast have shown that the absence of Cox1p has a deleterious effect on the assembly of the other two core-forming subunits, as well as of subunit Cox4p (Lemaire et al., 1998), which acts in the early steps of COX assembly (Nijtmans et al., 1998). Subunit Cox2p chelates the binuclear mixed valent $Cu_A$ center, which is exposed to the IMS. Cox2p is the smallest (26-27 kDa) and least hydrophobic core subunit with two TM domains. Cox2p participates in the docking of cytochrome *c* via the $Cu_A$ center (Poyton and McEwen, 1996). In human COX, subunit 2 together with Cox1p is necessary for the binding of heme $a_3$ (Rahman et al., 1999). In *S. cerevisiae cox2* mutants Cox3p and Cox4p are not detectable, indicating that their accumulation

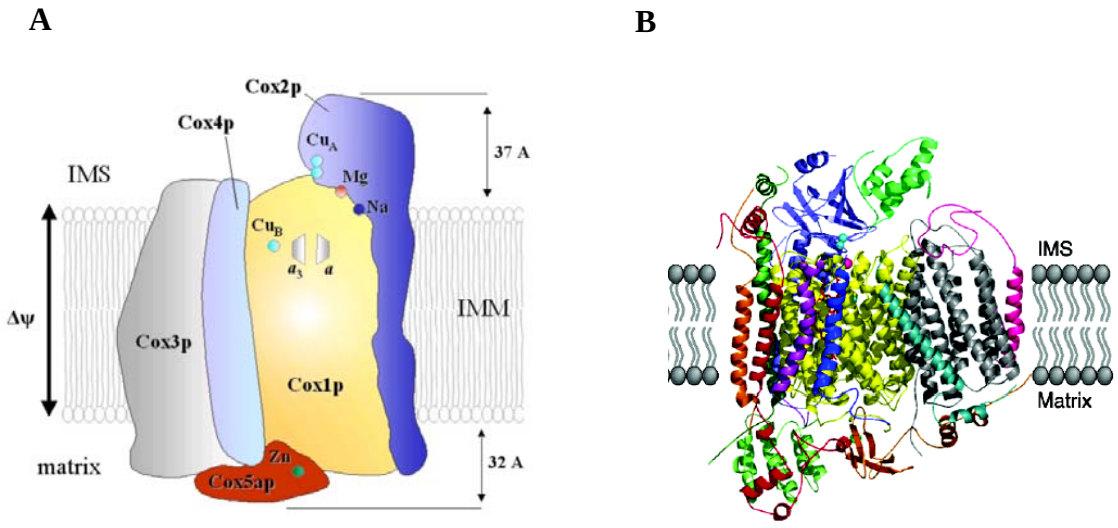



**Figure 2. Organization of COX.** (A) A schematic of selected mammalian Cox subunits. Only subunits containing cofactors or engaged in early assembly steps are shown. (B) Structure of the bovine COX (taken in part from Carr and Winge, 2003).

depends on the presence of Cox2p (Lemaire et al., 1998). Subunit Cox3p is a hydrophobic protein of about 30 kDa that spans the IMM 7 times. Its role in COX function remains to be clarified. It was proposed to be involved in the folding, action

and/or stability of COX (Lemaire et al., 1998; Meunier and Taanman, 2002), but it is not engaged in proton translocation (Capaldi 1990; Haltia et al., 1991; Poyton and McEwen, 1996). Based on studies of the *Rhodobacter sphaeroides* (Rs) COX homologue it was also concluded that Cox3p is not required for the binding of metal centers (Bratton et al., 2000). Originally, isolated active forms of *S. cerevisiae* COX were reported to contain 9 subunits. Table 1 summarizes the subunit composition of yeast and mammalian COX and the respective genes. The nuclearly encoded subunits of the yeast enzyme are Cox4p, one of the Cox5p isoforms (Cox5ap/Cox5bp), Cox6p, Cox7p, Cox7ap and Cox8p (Power et al., 1984; Capaldi, 1990; Poyton and McEwen, 1996). A more complex yeast enzyme consisting of 11 subunits can be purified. It contains two additional subunits, Cox6ap and Cox6bp (Geier et al., 1995). However, these subunits appear not to have an effect on the catalytic activity, since the originally reported preparations with nine COX subunit are equally active (Burke and Poyton, 1998). Cox6ap is believed to carry the ATP-binding site. This feature appears to be important for modulation of the COX activity by ATP in response to the external ionic strength (Beauvoit et al., 1999; Beauvoit and Rigoulet, 2001). As deletion of *COX6a* results in respiratory deficiency, either this modulation of COX activity is essential *in vivo,* or Cox6ap fulfills an additional function in COX formation. As already stated above, all subunits are present in equimolar amounts in the assembled COX. This is also true for the mammalian oxidase, whose composition is similar to that of the yeast enzyme, except for the presence of two additional nuclearly encoded subunits: Cox7bp and Cox8p (not homologous to yeast Cox8p) (Capaldi, 1990). Notably, some subunits (Cox6ap; Cox7ap; Cox8p) are represented by different tissue-specific isoforms that are expressed in heart and skeletal muscle ("H-type" isoforms) or in liver ("L-type" isoforms). However, the number of isoforms sharing sequence identities of 50-65% varies between different mammalian species. Interestingly, their expression levels have been shown to differ during the developmental stages (Poyton and McEwen, 1996). All nuclearly encoded subunits are relatively small compared to the mt encoded proteins, with molecular masses ranging form 5.4 kDa to 14.9 kDa in yeast and from 5 kDa to 17.1 kDa in mammals (Capaldi, 1990). Several of them, represented merely by a single TM domain, are firmly attached to the core complex, whereas the others are extrinsic to the lipid bilayer (yeast Cox4p and Cox6p) (Tsukihara et al., 1995;

Glerum and Tzagoloff, 1997; Carr and Winge, 2003). Despite of the fact that the nuclearly encoded subunits rather play an auxiliary role in the catalytic function of COX, most of them are indispensable for the formation of the enzyme. *S. cerevisiae cox4*, *cox5*a and *cox5*b, *cox6a*, *cox7* and *cox9* deletion mutants are respiratory deficient and show COX deficiency accompanied by loss of hemes *a* and $a_3$ (Carr and Winge, 2003). Mammalian Cox4p has been reported to act in an early stage of the COX assembly and to play an important role in the modulation of holoenzyme assembly (Nijtmans et al., 1998). A similar function can be attributed to subunits Cox6p, Cox7p, Cox7ap, since the respective yeast deletion mutants show a similar phenotype as the yeast *cox4*Δ mutant, in that Cox2p and Cox3p are instable. In the case of *cox7*Δ mutation this effect appears to be even more severe resulting in the gross reduction of Cox1p levels (Calder and McEwen, 1991). In contrast the yeast *cox8*Δ mutation has only a modest effect on COX function. Cox8p appears to participate in the formation of the active COX dimer, contributing to the low-affinity electron transfer reaction (reviewed by Poyton and McEwen, 1996). The role of the Cox5p subunit isoforms, which exhibit 66 % sequence identity, has been studied extensively in yeast. Cox5ap is expressed during normal aerobic growth, whereas the Cox5bp isoform appears under low oxygen conditions. The turnover numbers of COX carrying the Cox5a isoform is low compared to the Cox5b-bearing COX. The function of Cox5p is to modulate the catalytic functions of Cox1p by affecting the ligand binding around the binuclear reaction center and by altering the environment around heme $a_3$. So far no oxygen-regulated isoforms of the nuclearly encoded subunits were reported for the mammalian COX. On the other hand there is suggestive evidence hinting at the existence of a hypoxic form of Cox4p (corresponding to yeast Cox5p) (Poyton and McEwen, 1996, Burke and Poyton, 1998). Analysis of a yeast *cox6a*Δ mutant demonstrated that the COX complex can be formed regardless of the presence of this protein (Taanman and Capaldi, 1993), but it seems to be important for mediating the effect of ATP on the enzyme activity (Beauvoit et al., 1999; Beauvoit and Rigoulet, 2001). Several studies on the yeast enzyme led to the conclusion that some of the nuclearly encoded subunits (Cox4p, Cox5ap, Cox6p and Cox9p) can assemble independently of the COX core subunits (Gavin et al., 2002; Carr and Winge, 2003).

However, these data could also reflect a high stability of these proteins against proteolytic degradation.

### *1.2.2. Functional/catalytic centers of COX*

The catalytic centers of all heme-copper oxidases including COX are highly conserved at the structural level (Ludwig et al., 2001). The electron flow through COX is schematically depicted in Fig. 3.

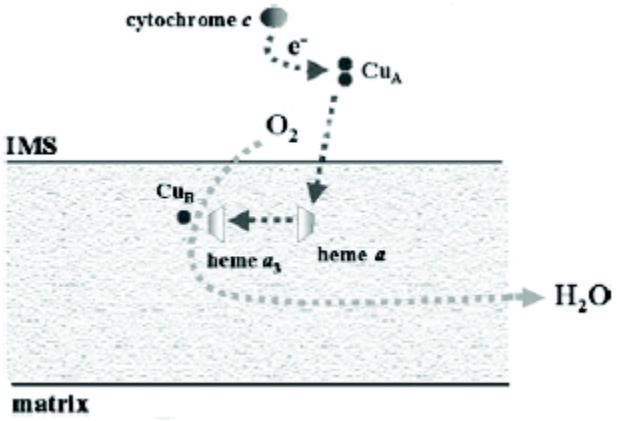



**Figure 3. Electron flow through the COX.** Schematic of the electron flow through COX. The IMM and the metal centers involved in electron transfer reactions are shown.

#### *1.2.2.1. $Cu_A$ center*

The $Cu_A$ center, which is located in the COX domain projecting to the outside of the IMM, is the entry point of the electrons from cytochrome *c*. This heme-containing soluble protein docks to a conserved domain in Cox2p adjacent to the $Cu_A$ site (Hill, 1991; Brzezinski et al., 1995; Zhen et al.,1999). The function of the $Cu_A$ center is to shuttle the electrons to the other redox centers located in Cox1p (Ramirez et al., 1995). Bacterial quinol oxidases lack a $Cu_A$ site, however it is possible to artificially engineer it there (so called "blue copper site") (Malmström and Aasa, 1993). Analysis of this construct by EPR spectroscopy revealed that $Cu_A$ exists as a $[Cu^{2+}/Cu^{1+}]$ complex of mixed valence. The unpaired electron is completely delocalized over the two copper nuclei consistent with $Cu^{1.5+}$-$Cu^{1.5+}$ (Kroneck et al., 1988; Malmström and Aasa, 1993;

Fee et al., 1995). The structure of the $Cu_A$ center of different COX homologues reveals that the binuclear copper center is coordinated by the $Hx_nCxExCGx_2Hx_2M$ motif, *i.e*. two cysteines, two histidines, one methionine and a carbonyl oxygen from the peptide backbone (Iwata et al., 1995; Tsukihara et al.,1995). According to the studies on *P. denitrificans* COX the cysteine residues appear to be most important for $Cu_A$ coordination as they are bridging ligands (Zickermann et al., 1997). Interestingly, the visible spectra of the $Cu_A$ site is recordable only in preparations of the soluble domain, while it is completely camouflaged by the heme absorptions in the entire COX (Ramirez et al., 1995). The study of the $Cu_A$ center of yeast COX confirmed the importance of the conserved aa residues bridging $Cu_A$ and demonstrated that the precise coordination environment is essential for the proper assembly and function of COX (Speno et al., 1995). Interestingly, one of the conserved glutamic acid residues that was shown to be important for the proper folding of the $Cu_A$ site is also involved in coordination of the $Mg^{2+}$ ion.

*1.2.2.2. $Cu_B$ center*

The $Cu_B$ site is a complex dimetallic motif formed by heme $a_3$ and copper moieties. It represents the oxygen binding site of COX. The copper atom, which is ligated by the imidazole nitrogens of three highly conserved histidines, is located 4.5 Å away from heme $a_3$. This myoglobin-type iron center is coordinated by another two histidine residues of the $Hx_3Yx_{44}HH$ motif (Shapleigh et al., 1992; Tsukihara et al., 1996; Yoshikawa et al., 1998). One of the distal histidines forms a covalent bond to the *ortho* carbon of the aromatic hydroxyl group of the adjacent tyrosine residue. This pair is believed to play an important role in the enzymatic action of COX (Yoshikawa et al., 1998). The tyrosine hydroxyl might also be involved in hydrogen bonding of the dioxygen molecule captured by heme $a_3$, and in the formation of water chains in the K-channel that supplies a proton to the $Cu_B$ center (Naruta et al., 2001; Cukier, 2005). The heme-copper site also appears to bind molecules other than dioxygen and water (e.g. a chloride ion), that might be involved in catalysis (Fabian et al., 2004 a,b). As already pointed above, the association of Cox1p and Cox2p subunits is necessary for the coordination of heme $a_3$ in the mammalian COX (Rahman et al., 1999). A similar

prediction can be made on the basis of the enzyme structure: the farnesyl group of heme $a_3$ has been shown to be located at the interface of Cox1p and Cox2p (Tsukihara et al., 1995). It should be noted that in contrast to the IMS-exposed $Cu_A$ site, the heme $a_3$-$Cu_B$ center is submerged 13 Å below the IMM surface (Tsukihara et al., 1995). It is still an open question how the copper ion reaches that position. This issue will be addressed below.

*1.2.2.3. Hemes*

The two heme *a* moieties of the COX complex, heme *a* (the low-spin heme) and heme $a_3$ (the high-spin heme), have already been described in part above. Heme *a* is a prosthetic group that is typical for eukaryotic and some bacterial COX. In general, most bacterial oxidases are of the $ba_3$ type and possess heme *b* (iron protoporphyrin IX or protoheme) which is ancestral to heme *a*. In heme *a* the vinyl group is replaced by hydroxyfarnesyl and the methyl group is substituted by a formyl group (Caughey et al., 1975). The farnesylation of heme may be important for the protein folding and packing (Carr and Winge, 2003). The low-spin heme *a* is coordinated by pyrrol nitrogens of two histidine residues (Trumpower and Gennis, 1994; Yoshikava et al., 1998 b). Heme *a* functions as the transition point in the transfer of the electron to the $a_3$-$Cu_B$ center. The electron is routed from the $Cu_A$ site by a combination of hydrogen-bonds and peptide backbone (Tsukihara et al., 1995; Ramirez et al., 1995). Heme *a* is located 19 Å away from the $Cu_A$ center and 14 Å from the iron atom of heme $a_3$. (Yoshikava et al., 1998). The properties and coordination of heme $a_3$ have been discussed above. The angle between two heme planes is 104° and they both are located in such a way that the hydroxyethylfarnesyl groups and the vinyl groups extend towards the matrix side, whereas the propionate moieties extend towards the IMS side (Yoshikava et al., 1998 b). The fully extended farnesyl group of heme *a* is tightly packed inside an α-helical bundle of Cox1p, while the U-shaped hydroxyethylfarnesyl of heme $a_3$ is sandwiched between Cox1p and Cox2p (Tsukihara et al., 1995; Yoshikava et al., 1998 b).

The finding that instability of yeast Cox1p is associated with the absence of heme *a* may indicate that heme is required for correct insertion and/or stability of this subunit (Nobrega et al., 1990). However, the absence of heme *a* could also be secondary to

Cox1p instability. In human fibroblasts deficient in heme *a* biosynthesis early COX assembly intermediates were not detected (Williams et al., 2004). In contrast, studies of COX from *R. sphaeroides* demonstrated that heme *a* is not essential for the insertion and association of the COX core complex subunits (Hiser and Hosler, 2001).

### *1.2.3. Other components*

Besides the structurally and functionally well characterized components, COX encompasses several elements whose roles in the catalytic activity are less evident. The $Mg^{2+}$ ion resides at the interface of Cox1p and Cox2p on the IMS side in close proximity to the propionate group of heme $a_3$. $Mg^{2+}$ is coordinated by conserved histidine and glutamic acid residues of Cox1p, a further glutamic acid of Cox2p, and a water molecule (Tsukihara et al., 1995). As already mentioned, the glutamic acid of Cox1p is also an important ligand for the $Cu_A$ site. $Mg^{2+}$ lies on the bottom of the proposed water channel (Tsukihara et al., 1996; Ostermeier et al., 1997). Bound $Mg^{2+}$ can easily be substituted by $Mn^{2+}$ without any changes in catalytic activity (Witt et al., 1997; Florens et al., 2001). This finding may hint at its direct involvement in the catalytic process.

Cox1p also coordinates one $Na^+$ ion, which is bound to the peripheral side of the protein (Tsukihara et al., 1995). The role of sodium in COX function remains unclear. The $Na^+$-ion can easily be displaced by calcium (Kirichenko et al., 1998), which causes distortion of the heme *a* spectrum (Lee et al., 2002). It can be speculated that these metals may have a modulating function, however, so far the significance of both ions is not clear. The structure analysis of bovine COX revealed one zinc ion coordinated at the matrix side of subunit Cox5bp (corresponding to yeast Cox4p) (Tsukihara et al., 1995). The role of zinc in COX function remains to be clarified.

## 1.3. Assembly of COX

The assembly of COX is a sequential process that involves a number of accessory proteins (Nijtmans et al., 1998) (Table 1). Prior to assembly the mt-translated subunits need to be processed and inserted into the lipid bilayer of IMM. The nuclearly encoded subunits must be translocated to the site of assembly in the IMM. Only after these processes are successfully accomplished can assembly occur.

*1.3.1. Assembly of mitochondrially encoded subunits*

The three mt encoded subunits are synthesized on mt ribosomes which are associated with the IMM (Green-Willms et al., 2001; Jia et al., 2003; Szyrach et al., 2003). Indeed, it is important for the organelle to keep the newly synthesized highly hydrophobic mt proteins in close proximity to the lipid bilayer. Based on the observation that the soluble mt protein Var1p is also synthesized next to the IMM, Fox and co-workers proposed the existence of distinct sites of assembly for mt ribosomes and cytochrome *c* oxidase (Fiori et al., 2003).

A couple of proteins with different functions have been shown to be involved in positioning translationally active mitoribosomes in proximity to the IMM. These proteins include translational activator proteins and factors involved in translocation of mt-encoded COX subunits. The mt mRNAs are recruited to the IMM by a set of specific membrane-bound translational activators that in yeast interact with the 5' untranslated region (UTR) of COX transcripts (Naithani et al., 2003). Translation of *COX1* mRNA is activated by Mss51p and Pet309p (Perez-Martinez et al., 2003; Manthey and McEwen, 1995), *COX2* by Pet111p (Mulero and Fox, 1993 a,b) and *COX3* by Pet54p, Pet122p, and Pet494p (Costanzo and Fox, 1988; Brown *et al.*,1994). Studies on translational activators have been mainly confined to fungi; their existence in other eukaryotic organisms remains to be analysed. In case of Pet111p only fungal homologues have been identified (Green-Willms et al., 2001; Carr and Winge, 2003).

Upon or during synthesis the mt translated proteins undergo the translocation/insertion step to attain their proper orientation in the IMM. This process involves the export of protein segments across the membrane. Translocation of all mt synthezised Cox subunits requires the function of Oxa1p, that transiently interacts with the nascent polypetides during their translation (Hell et al., 2001). However, only Cox2p export strictly depends on Oxa1p. The other subunits can be inserted into the IMM in the absence of Oxa1p,

Table 1. Proteins involved in yeast COX biogenesis*.

| Protein | Description |
|---|---|
| Pet309p | Translational activator of *COX1* mRNA |
| Pet111p | Translational activator of *COX2* mRNA |
| Pet54p | Translational activator of *COX3* mRNA |
| Pet122p | Translational activator of *COX3* mRNA |
| Pet494p | Translational activator of *COX3* mRNA |
| Mss51p | Involved in Cox1p translation and insertion |
| Cox14p | Required for Cox1p expression and assembly |
| Cox16p | Required for COX assembly, exact role is not clear |
| Pet100p | Assembly facilitating protein, facilitates the formation of COX complex |
| Pet117p | Required for COX assembly, exact role is not clear |
| Pet191p | Required for COX assembly, exact role is not clear |
| Oxa1p | IMM translocase, mediates insertion of mt Cox subunits |
| Mba1p | Involved in translocation of mt Cox subunis |
| Cox18p | Required for translocation of the C-terminal part of Cox2p precursor protein |
| Pnt1p | Required for translocation of Cox2p precursor |
| Mss2p | Required for translocation of Cox2p precursor |
| Cox10p | Farnesyltransferase, catalyzes the first step of heme *a* biosynthesis |
| Cox15p | Heme *a* synthase, catalyzes the second step of heme *a* biosynthesis |
| Yah1p | Ferredoxin, required for heme *a* biosynthesis |
| Arh1p | Ferredoxin reductase, required for heme *a* biosynthesis |
| Shy1p | Facilitates insertion of heme *a* into Cox1p |
| Yfh1p | Frataxin, regulates mt iron accumulation, heme *a* biosynthesis and insertion |
| Cox17p | Metallochaperone, involved in copper transfer to COX |
| Cox19p | Metallochaperone, required for COX assembly |
| Cox23p | Required for COX assembly, may be involved in mt copper metabolism |
| Sco1p | Metallochaperone, presumably provides copper for Cox2p |
| Sco2p | Similar to Sco1p and may have a redundant function |
| Cox11p | Metallochaperone, presumably provides copper for Cox1p |
| Mia40p | Essential IMS metal-binding protein, involved in import and assembly of IMS proteins |

* Proteins mutations in which are known to cause pleotropic defects are not considered.

albeit with a much lower efficiency (Hell et al., 2001; Herrmann and Neupert, 2003). Obviously translocation of these subunits is only partially dependent on Oxa1p function (Stuart, 2002). The exact way of the translocation of Cox1p and Cox3p still has to be clarified.

Cox2p is an integral membrane protein which spans the IMM two times and possesses an $N_{out}$-$C_{out}$ topology. It is synthesized as a precursor (pCox2p) with an extended N-terminal part that represents a cleavable presequence. Successful insertion of the pCox2p into the IMM has been shown to depend on the presence of mt membrane

potential (Herrmann et al., 1995; Stuart, 2002). Whereas both the N- and C-terminal part of pCox2p are translocated with the help of Oxa1p (Hell et al., 1997; He and Fox, 1997), translocation of its C-terminal part in addition depends on Cox18p (Saracco and Fox, 2002), Pnt1p (He and Fox, 1999) and Mss2p (Broadley et al., 2001). Cox18p (= Oxa2p) is a distant homologue of Oxa1p. It possesses a similar topology and acts as a post-translational translocase in contrast to the co-translationally acting Oxa1p (Saracco and Fox, 2002; Preuss et al., 2005). Cox18p associates with Pnt1p and Mss2p, possibly cooperating in translocation of pCox2p. Also Mba1p, a protein believed to represent Oxa1p-independent insertion route may be involved in translocation of Cox subunits. However, its role remains elusive (Preuss et al., 2001).

Upon emerging in the IMS, the N-terminus of pCox2p is processed yielding the mature form of Cox2p that can be inserted into COX (Hell et al., 2000; Stuart, 2002). The cleavage step is performed by Imp1p/Imp2p protease residing on the outer surface of IMM (Nunnari et al., 1993; Stuart, 2002; Gakh et al., 2002). Interestingly, the unprocessed form of Cox2p does not assemble into the COX complex. Following translocation pCox2p associates with Cox20p, a membrane-bound chaperone required for processing (Hell et al., 2000). In contrast to yeast, in mammalian cells there is no precursor form of Cox2p in mammalian cells. This possibly implies that Cox2p insertion in mammalian mitochondria involves an alternative pathway. On the other hand, Oxa1p and Cox18p homologues are conserved among eukaryotes (Carr and Winge, 2003). Also the mammalian Imp1p/Imp2p homologues were identified recently (Petek et al., 2001, Gakh et al., 2002).

Cox1p insertion appears to depend on the function of Mss51p, a protein originally described as translation activator (Decoster et al., 1990; Siep et al., 2000). It interacts with the nascent polypeptide chain and is thought to facilitate the Cox1p insertion (Perez-Martinez et al., 2003). Recently, it has been shown that both Cox1p and Mss51p interact with Cox14p, a protein required for stability of Cox1p (Glerum et al., 1995). The Cox1p/Mss51p/Cox14p complex is important for Cox1p assembly (Barrientos et al., 2004). The same authors proposed that Shy1p, which is necessary for COX assembly (Mashkevich et al., 1997) may cooperate with the Cox1p/Mss51p/Cox14p complex during Cox1p insertion, but it seems not to interact with the latter. Homologues of

Mss51p and Cox14p were found only in fungi (Fiori et al., 2000; Barrientos et al., 2004), whereas Shy1p shows high degree of conservation between eukaryotes (Zhu et al., 1998; Poyau et al., 1999). Therefore it is not clear whether the same mechanism holds true for mammalian Cox1p. Only very few data are available concerning the assembly of the core subunits. It is still debatable whether assembly occurs near the sites of TOM/TIM translocation complexes, where assembly intermediates may be accessible to the imported nuclearly encoded subunits, or whether the site of assembly is located elsewhere along or within the IMM boundary (Carr and Winge, 2003). Nijtmans and co-workers (1998) have shown that assembly of mammalian COX initiates with the association of Cox1p with the nuclearly encoded Cox4p. This is in agreement with the structure of COX, where these two subunits are in tight contact. A similar situation was reported for the yeast homologues (Lemaire et al., 1998). The association may enhance the stability of Cox1p (Nakai et al., 1994). In the next step assembly of Cox2p and Cox3p as well as of most of nuclearly encoded subunits occurs.

### *1.3.2. Assembly of nuclearly encoded subunits*

The nuclearly encoded COX subunits are imported into the mitochondria in the same way as most of the other mt proteins of nuclear origin. The proteins are targeted to mitochondria by their N-terminal presequences, or less defined internal targeting signals that bind to the receptor subunits of the translocase of outer membrane (TOM) complex. The other components of TOM facilitate the transport of precursor proteins across the OMM. In the IMS, targeting sequences interact with TIM22 or TIM23 (TIM, for translocase of inner membrane) complex (for review see Neupert and Brunner, 2002, Truscott et al., 2003). Proteins following the membrane potential-dependent TIM23 pathway are inserted into the IMM, their presequences are removed by intramembrane protease Imp1p/Imp2p (Gakh et al, 2002). This allows the proteins to obtain the folding state, that is necessary for assembly and function. Usually the assistance of different molecular chaperones is required. The mature proteins may be arrested in the TIM23 complex and move laterally in the lipid bilayer to the site of COX assembly (so-called "stop transfer" mechanism). Some presequence-containing proteins undergo the "conservative sorting" pathway, which leads to the insertion of the protein from the

matrix side of IMM. This pathway involves an additional export step, sometimes involving a further cleavage by mt processing peptidase (MPP, Poyton et al., 1992; Gakh et al, 2002). The alternative route involving the TIM22 complex facilitates the insertion of proteins without N-terminal presequences (Sirrenberg et al., 1996; Koehler et al., 1998). In mammals, as already noted, the first nuclearly encoded subunit in the sequential assembly steps of COX is subunit Cox4p. Most of the other nuclear-born subunits, among them Cox5ap, Cox5bp, Cox6bp, Cox6cp, Cox7cp and Cox8p, are attached to the Cox1p/Cox4p sub-complex, likely concomitantly with subunits Cox2p and Cox3p. Cox6ap, Cox7ap and Cox7bp appear to be the last incorporated subunits of the complex (Nijtmans et al., 1998). As pointed above, in yeast Cox4p, Cox5a, Cox6p and Cox9p can form a complex independent of the COX core (Gavin et al., 2002; Carr and Winge 2003). Cox6ap has been reported to be dispensable for the assembly of the remaining COX complex (Taanman and Capaldi, 1993). Interestingly, a sub-complex containing Cox7p, Cox7ap and Cox8p can be formed, and is believed to be incorporated into the holo-COX by means of Pet100p, a membrane-anchored chaperone indispensable for COX assembly (Church et al., 1996; Forsha et al., 2001; Church et al., 2005).

Several additional chaperones with less evident functions have been described as "assembly facilitators". Among them are Pet117p, Pet191p (McEwen et al., 1993, Poyton and McEwewn, 1996) and Cox16p (Carlson et al., 2003), however, their role in COX assembly is still unclear.

### *1.3.3. Formation and assembly of heme a*

Heme *a* is generated by the sequential conversion of protoheme (heme *b*). The conversion begins with a farnesylation step of the C2 vinyl group of the porphyrin ring. This process is catalyzed by Cox10p, a membrane-bound farnesyltransferase that displays a high degree of conservation between eukaryotes and some bacteria (Nobrega et al., 1990; Tzagoloff et al., 1993; Glerum and Tzagoloff, 1994). The farnesylation reaction yields heme *o* (Saiki et al., 1993), a biosynthesis intermediate (Mogi et al., 1994), that is also found as a final cofactor in some bacterial oxidases (Puustinen and Wikstrom., 1991). In the next step, the C8 methyl group of heme *o* is oxidized to aldehyde, resulting in heme *a*. The complex oxidation process involves two discrete monooxygenase steps (Brown et

al., 2002) that are catalyzed by Cox15p, a membrane-anchored heme *a* synthase, mt ferredoxin Yah1p and ferredoxin reductase Arh1p (Glerum et al., 1997; Barros et al., 2001; Barros et al., 2002). Yah1p and Arh1p are presumably involved in the electron supply to Cox15p. Interestingly, these two proteins are also involved in the assembly of iron-sulfur clusters (Mühlenhoff et al., 2003; Alves et al., 2004).

Cox15p homologues were reported in other fungi as well as in mammals (Petruzzella et al., 1998; Barros et al., 2001). The involvement of Yah1p in Cox15p catalysis became apparent by the finding that *S. pombe* Cox15p homologue is fused to the Yah1p-like counterpart (Barros et al., 2001; Bureik et al., 2002). Such fusions have been proposed to hint at possible interacting partners in the other species (Sali et al., 1999; Enright and Ouzounis, 2001).

Little is known about the insertion of heme *a*. It appears that newly synthesized heme *a* is inserted into Cox1p at an early assembly step, perhaps prior to association with Cox2p and Cox3p (Nijtmans et al., 1998). Possibly the insertion occurs in a co-translational manner (Carr and Winge, 2003). Up to now no specific protein required for the insertion step has been identified. The synthesis of heme *a* is believed to be regulated either by subunit assembly or by assembly of COX sub-complexes, since distortion of a number of assembly factors results in loss of the heme *a* spectrum (Barros and Tzagoloff, 2002, Carr and Winge 2003). Recently, a link between insertion of heme $a_3$ and Surf1p (homologue to yeast Shy1p), a protein required for COX assembly, became evident: *R. sphaeroides* Surf1p was shown to facilitate the insertion of heme $a_3$ into the heme $a_3$-$Cu_B$ center (Smith et al., 2005). As Shy1p cooperates with the above mentioned factors required for the insertion of Cox1p, this observation may hint at a co-translational mode of heme *a* insertion. Smith et al. (2005) proposed that the formation of the bimetallic center may be one of the limiting steps in association of Cox1p and Cox2p.

### *1.3.4. Delivery of metal ions and formation of metal centers*

The delivery and insertion of metals into COX is still not well understood despite of many available data. During the past decade a number of mt proteins, that are able to bind metals have been identified (Fig. 4). Depletion of most of them results in respiratory deficiency and often in misassembly of Cox subunits. These data imply that the

successful delivery of metals to the site of COX assembly and their proper insertion are important steps in the complex formation.

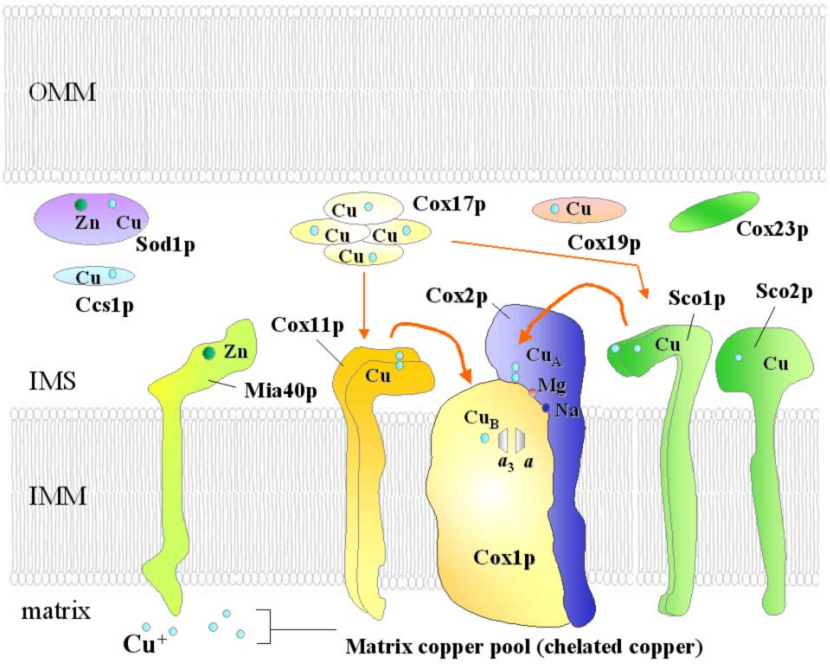



**Figure 4. Overviw of the factors involved in mitochondrial copper metabolism.** A schematic of IMS-located factors involved in mitochondrial copper metabolism and COX metallation.

*1.3.4.1. Delivery and insertion of copper*

The mt copper-binding proteins involved in COX assembly and their properties were studied most extensively. It has to be noted that the amount of free cellular copper is extremely low in living cells (Rae et al., 1999). Cu ions are mainly present in the cell as reduced $Cu^+$, bound either to proteins or to low-molecular weight water-soluble peptides (Harris, 2000; Puig and Thiele, 2002). A number of proteins engaged in mt copper metabolism have been identified. We will focus on those proteins engaged in COX formation that are either exclusively localized to mitochondria or possess a dual localization in the cytosol and mt IMS.

*1.3.4.1.1. Cox17p*

Yeast *COX17* was originally identified as one of the *PET* genes, whose depletion causes respiratory deficiency (Glerum et al., 1996). *cox17* mutants lack heme *a*, but the Cox subunits are stable, though Cox2p level is decreased (Glerum et al., 1996; Punter and Glerum, 2003). Suprisingly, the phenotype of *cox17*Δ can be partially rescued by the addition of exogenous copper (Glerum et al., 1996). Cox17p is a small (8 kDa) hydrophilic protein with dual localization in the cytosol and the mt IMS (Beers et al., 1997). Homologues were found in eukaryotes, but not in prokaryotes (Amaravadi et al., 1997; Horvath et al., 2000; Arnesano et al., 2005). Recently, however, a $Cu^+$-binding protein found in some bacteria was suggested to be the bacterial Cox17p ortholog (Banci et al., 2005). Site-directed mutagenesis and spectroscopy of Cox17p have demonstrated that $Cu^+$ is ligated by the CCxC motif. The $Cu^+$ ion is bound in a labile cuprous-thiolate polycopper cluster. This coordination is achieved by oligomerization of the protein, presumably involving the CCxC motif (Sirinivasan et al., 1998; Heaton et al., 2000; 2001). Oligomerization is necessary for protein function. Mutant Cox17p proteins affected in copper-binding are not mislocalized (Heaton et al., 2000; 2001).

Different stochiometries of bound $Cu^+$ were reported for Cox17p. The recombinant yeast protein can bind two (Beers et al., 1997) or three (Heaton et al., 2000) copper ions. Recently, purified native mammalian Cox17p was reported to coordinate four $Cu^+$ ions (Palumaa et al., 2004). Only a small subset of random mutations introduced into yeast *COX17* leads to respiratory deficient mutants. Based on this finding it was concluded that Cox17p may be an intrinsically unstructured protein (Punter and Glerum, 2003). The solution structure of yeast Cox17p revealed two antiparallel α-helices following the extensive unstructured N-terminal part, thus confirming that it is intrinsically unfolded (Abajian et al., 2004).

On the basis of NMR studies it was predicted that one copper ion is ligated by the flanking cysteines of the CCxC motif in a two-coordinate complex. Interestingly, the copper-binding segments become more structured in Cu-loaded Cox17p (Abajian et al., 2004). The finding of the dual localization of Cox17p led to the suggestion that it acts as a copper shuttle between the cytosol and the mt IMS, where it could cooperate with downstream acting Cu-binding proteins (Beers et al., 1997). In view of the recent result

that tethering of Cox17p to the IMM does not cause any respiration defect (Maxfield et al., 2004), this proposal probably has to be revised. In addition, *cox17*Δ mutants show no decrease in mt Cu levels (Carr and Winge, 2003; Cobine et al., 2004). Nevertheless, Cox17p remains one of the key players in copper delivery to COX, possibly acquiring copper ions in the IMS. Kako and co-workers (2004) have shown that binding of copper to mammalian Cox17p is a stringently regulated process that is important for COX activation. Very recently Arnesano et al. (2005 b) demonstrated the existence of at least two different copper-bearing conformers of yeast Cox17p: the $Cu_1$-Cox17p conformer, that is formed via the disulfide isomerization and the polycopper form of Cox17p that binds a copper cluster stably coordinated in an oligomeric structure. These two forms may fulfill different functions in copper distribution ($Cu_1$-Cox17p) or in IMS Cu-storage (polycuprous-Cox17p) (Arnesano et al., 2005 b). NMR studies reveal that the C-terminal part of apo-Cox17p possesses a twin $Cx_9C$ motif stabilized by intramolecular disulfide bonds. This structural motif is also found in several other IMS proteins involved in COX assembly, namely Cox19p, Cox23p and Mia40p (see below). Interestingly, this specific fold is also found in Cox6bp which docks to Cox2p close to the $Cu_A$ site-forming stretch (Arnesano et al., 2005 b).

*1.3.4.1.2. Cox19p and Cox23p*

Cox19p (11.1 kDa) is another soluble protein that exhibits features similar to those of Cox17p (Nobrega et al., 2002). Cox19p also possesses a dual localization in the cytosol and the IMS and shares significant sequence similarity in its C-terminal part with Cox17p. Deletion of *COX19* results in the absence of heme *a* and COX-deficiency, but this phenotype cannot be rescued by addition of exogenous copper (Nobrega et al., 2002). Although Cox19p lacks the CCxC motif, its recombinant form was reported to bind copper (Cobine et al., 2004). Interestingly, there is no decrease in mt Cu content in either *cox19*Δ mutant or *cox17*Δ *cox19*Δ double mutant (Cobine et al., 2004). As noted above Cox19p as well as Cox23p possesses a twin $Cx_9C$ motif and may form oligomers like Cox17p. Therefore, copper transfer in the IMS may involve the formation of hetero-dimers between these two proteins and Cox17p or other coiled-coil proteins like Cox6bp or Mia40p (Arnesano et al., 2005 b).

A second Cox17p-like protein, Cox23p, has been identified very recently (Barros et al., 2004). The small soluble Cox23p is localized both to the cytosol and the IMS. Homologues are found in fungi, mammals and plants. Its C-terminal part shares sequence similarity with the respective Cox17p counterpart. Interestingly, there is no sequence similarity between Cox23p and Cox19p. Like *cox17*Δ and *cox19*Δ mutants, *cox23*Δ mutants are COX deficient. *cox23*Δ mutants can be rescued by the addition of exogenous copper, albeit only with concomitant *COX17* overexpression. These findings suggest that Cox17p acts downstream of Cox23p, whereas Cox19p may represent another part of the $Cu^{+}$ distribution pathway (Barros et al., 2004).

*1.3.4.1.3. Sod1p and the matrix copper pool*

A small fraction of Cu/Zn superoxide dismutase (Sod1p) and of its specific metallochaperone Ccs1p is located in the IMS (Sturtz et al., 2001). Translocation to the IMS of the mainly cytosolic enzyme occurs in its unloaded apo-form and its uptake depends on Ccs1p. This implies that the metallation steps may occur in the IMS. This suggestion appears to be quite reasonable in the light of the recent finding that yeast mt matrix contains a non-proteinaceous copper pool (Cobine et al., 2004). The bulk of this mt copper is present as soluble, anionic, low molecular weight complexes. Neither proteins nor mt DNA contribute to the chelating of copper. Possibly $Cu^{+}$ is ligated by small peptides or organic molecules. A possible candidate for such a chelator may be a representative of the chalkophore group, that was recently described in methane-oxidizing bacteria (Kim et al., 2004; 2005; D. R. Winge, personal communication). The copper pool was shown to be labile and dynamic and to respond to changes in the exogenous copper content (Cobine et al., 2004). So far it is not clear how copper ions enter the matrix and can be eventually recruited later to the IMS. It appears that neither Cox17p nor Cox19p contribute to these processes (Cobine et al., 2004).

*1.3.4.1.4. The Sco protein family*

The mt copper binding protein Sco1p (Schulze and Rödel, 1988; 1989) appears to cooperate with Cox17p in the COX assembly process. Deletion of the *SCO1* gene results in loss of the heme *a* spectrum and rapid degradation of newly synthesized Cox1p and

Cox2p subunits (Schulze and Rödel, 1988; Krummeck and Rödel, 1990) The mature Sco1p is a 28.7 kDa membrane protein with $N_{in}$-$C_{out}$ topology, anchored in the IMM by one TM domain in the N-terminal part (Buchwald et al., 1991; Beers et al., 1997; Beers et al., 2002). On the basis of the observation that *SCO1* is the high-copy suppressor of a *cox17* missense mutation, it was suggested that Sco1p may be involved in the copper transfer to COX and to act downstream of Cox17p (Glerum et al., 1996b). This idea was supported by the identification of the metal-binding CxxxC motif in the C-terminal part of the protein. This motif is essential for protein function, mutations of any of the conserved cysteines result in respiratory deficiency (Rentzsch et al., 1999). Nittis et al. (2001) demonstrated that the purified soluble part of Sco1p binds copper, involving the CxxxC motif. Extended X-ray absorption fine structure (EXAFS) analysis indicates that $Cu^+$ is coordinated by the two sulfurs of the cysteines of the CxxxC motif and by the nitrogen of the adjacent conserved histidine residue. Mutations in any of these residues impair copper binding and cause COX deficiency. The solution structure of the *Bacillus subtilis* apo-Sco1p homologue (Balatri et al., 2003) reveals that the copper ligands are derived from two flexible loop regions of the protein. Very recently Horng et al. (2004) demonstrated by *in vitro* experiments and a yeast cytosolic expression system, that Cox17p can directly transfer copper to Sco1p. However, a stable interaction of both proteins was not detected. Several lines of evidence argue that Sco1p determines the formation of the $Cu_A$ site in Cox2p. Biochemical and genetic studies on yeast Sco1p demonstrated its ability to directly interact with Cox2p (Lode et al., 2000; Dickinson et al., 2000). In addition, the CxxxC motif also constitutes the copper-binding site in Cox2p (Coruzzi and Tzagoloff, 1979). As an alternative function of Sco1p it was proposed that it might be involved in the reduction of the CxxxC cysteines in Cox2p thus allowing $Cu^+$ incorporation. This proposal is based on the similarity of Sco1p with the peroxiredoxin protein family (Chinenov, 2000). In favour of the idea of a possible redox role of Sco1p in the formation of $Cu_A$ center are the data of Ye and co-workers (2005) reporting a redox switch effect in *B. subtilis* Sco1p. Depending on the oxidized or reduced state of protein, the local conformation in the vicinity of the CxxxC motif-containing loop changes. Similarly, the human Sco1p homologue has been proposed to act as a redox sensor in the IMS (Williams et al., 2005). Gene neighborhood analysis of prokaryotic genomes

revealed that Sco1p homologues can be linked to the variety of the cuproenzymes, distinct from COX. Therefore Sco1p may act as a functional linker connecting a number of biological processes (Arnesano et al., 2005). Combining these data it is still not possible to give a clear answer as to the exact role of Sco1p in the mt copper distribution and insertion. Maybe Sco1p is one of the key players in COX assembly fulfilling both proposed functions. Sco1p belongs to a conserved protein family with pro- and eukaryotic members. In *S. cerevisiae* a second member (Sco2p) with 53.8% identity to Sco1p was identified. The exact role of Sco2p is unclear. In contrast to *sco1* mutations, deletion of *SCO2* does not affect respiratory growth (Lode et al., 2002). Sco2p is anchored in the membrane by a single TM domain in the N-terminal part, and presumably possesses the same topology as Sco1p (Glerum et al., 1996b). Overexpression of *SCO2* cannot substitute for the function of Sco1p (Glerum et al., 1996b), but can rescue the respiratory deficiency of a *cox17* mutant, albeit less efficiently and only in the presence of higher concentrations of exogenous copper. The C-terminal part of Sco1p can be replaced by the respective Sco2p portion, the resulting chimeric proteins are functional and able to overcome the respiratory defect of *sco1*Δ (Rentzsch et al., 1999). Overall, the function of Sco2p remains to be elucidated, however the finding that it is indispensable for the presence of residual levels of Cox2p in *sco1Δ* strain (Lode et al., 2002) suggests a role in COX assembly or in COX subunits stabilization.

Sco homologues are detected in mammals and prokaryotes, often in more than one copy (Arnesano et al., 2005). Interestingly, in *S. pombe* only one *SCO* homologue has been found. In human cells, two homologues were identified. Due to the almost identical degree of identity to the yeast Sco proteins, it is hard to assign the human homologues to either *SCO1* or *SCO2*. Contrary to the situation in *S. cerevisiae*, both genes are important for COX function. Mutations of the human homologue, which was designated *HsSCO1* (Petruzzella et al., 1998), result in lactic acidosis and severe liver failure as a consequence of COX deficiency (Valnot et al., 2000; Paret et al., 2000; Horvath et al., 2000; Hamza and Gitlin, 2002). Hs*sco1*-deficient fibroblasts were reported to accumulate an early COX assembly intermediate containing Cox1p, Cox4p and Cox5ap subunits (Williams et al., 2004). Biochemical characterization of HsSco1p was

performed. Like its yeast counterpart it is an integral membrane protein with $N_{in}$-$C_{out}$ topology that can form a dimer (Paret et al., 1999; Leary et al., 2004).

Mutations in *HsSCO2* appear to be more frequent than in *HsSCO1*. They result in various tissue-specific clinical features, associated with COX misassembly and selective degradation of mt Cox subunits (Papadopoulou et al., 1999; Jaksch et al., 2000, 2001; Hamza and Gitlin, 2002). Jaksch et al. (2001) have shown that HsSco2p-deficient fibroblasts exhibit a dramatic increase in copper uptake, and COX activity can be completely restored by the addition of exogenous Cu-histidinate. However, it is not clear whether the excess of copper can overcome the mutation or is required for direct loading of Cox2p. Foltopoulou et al. (2004) reported that recombinant mutant forms of HsSco2p are affected in copper binding accompanied by disturbance of the conformational state of the Cu-ligating protein stretch.

Recently, Leary and co-workers (2004) proposed that HsSco1p and HsSco2p have independent cooperative functions in Cu delivery to mammalian COX. According to the model of the authors, HsSco2p plays the key role in the $Cu_A$ formation, whereas HsSco1p assists HsSco2p function.

*1.3.4.1.5. Cox11p*

Another important member of the mt copper transfer pathway is the 28 kDa copper-binding protein Cox11p (Tzagoloff et al., 1990). It appears to be required for $Cu^+$ supply of Cox1p. Yeast *COX11* was originally identified as a *PET* gene, important for respiration and normal COX activity (Tzagoloff et al., 1990). *cox11*Δ mutants are characterized by impaired COX activity due to the degradation of Cox1p subunit and unstable heme *a* (Tzagoloff et al., 1990). Interestingly, the *cox11* including *cox11*Δ mutations confer high sensitivity to N-nitrosodiethylamine (NDEA) and 8-hydroxyquinoline (8HQ). These chemicals are metabolized via redox cycling, thereby yielding hydroxialamine radicals and reactive oxygen species (Pungartnik et al., 1999; 2002). This observation may hint at an additional role of Cox11p in oxidative stress response. Curiously, the *COX11* gene was identified in two independent screens indicating an involvement of Cox11p in cell wall biosynthesis (Lussier et al., 1997; Tong et al., 2004). A link between mt function and cell wall biogenesis has also been reported

for some other mt proteins with distinct functions (Lussier et al., 1997; Zhong et al., 2005). The molecular basis of this observation is presently unclear.

Similarly to Sco1p, Cox11p possesses an $N_{in}$-$C_{out}$ topology and spans the IMM by a single TM helix located in the N-terminal part of the protein (Tzagoloff *et al.,* 1990; Khalimonchuk et al., 2005; Carr et al., 2005). The understanding of Cox11p function emerged from studies of the *R. sphaeroides* counterpart. Rs*cox11* null mutants lack $Cu_B$, but not $Cu_A$ and have an impaired $Mg^{2+}/Mn^{2+}$ site. In addition it was reported that the environment of heme $a_3$, but not heme *a* is disturbed (Hiser et al., 2000). These findings led to the conclusion that RsCox11p is important for the formation of the $Cu_B$ site. More recently, a recombinant soluble truncated form of yeast Cox11p was shown to bind copper (Carr et al., 2002). Cox11p acts as a dimer and binds one $Cu^+$ per monomer via a CxC motif located in the IMS-exposed C-terminal part of protein. A third conserved cysteine near the IMS-side of TM helix, not engaged in copper binding, may be involved in dimer formation. EXAFS data indicate that each $Cu^+$ is ligated by three sulfurs of conserved cysteine residues, and the Cu-Cu distance is 2.71 Å (Carr et al., 2002; Carr and Winge, 2003). The solution structure of the soluble part of the Cox11p homologue dimer from *Sinorhizobium meliloti* suggests that two ligands for a single $Cu^+$ derive from the CxC motif, and the third ligand is donated by the conserved cysteine of the CxC motif of the other monomer (Banci et al., 2004). Interestingly, the metal-binding motif resides in an immunoglobulin-like ß-barrel structure, which might also mediate interaction(s) with the other proteins (Banci et al., 2004). Replacement of any of the conserved cysteines precludes copper binding and results in COX deficiency (Carr et al., 2002). As for Sco1p, the direct transfer of copper from Cox17p to Cox11p was shown recently (Horng et al., 2004). However, as in the case of Sco1p, physical interaction between these proteins could not be detected. Guo et al. (2005) identified in a two-hybrid screen rat Cox11p as an interacting partner of ACDP4, a member of the ancient conserved domain protein family (Wang et al., 2003). Ectopic expression of both genes in HEK293 cells results in metal ion toxicity, suggesting a functional coupling between these proteins. Contrary to earlier reports linking the yeast ACDP4 homologue, a $Mg^{2+}$-binding protein (Mam3p) to mitochondria, it was recently shown that it resides in the vacuolar membrane and has a

function unrelated to mitochondria (Yang et al., 2005). Therefore the significance of the observed interaction appears to be less clear in yeast.

Cox11p homologues were described and characterized in a number of eukaryotes and prokaryotes (Cao et al., 1992; Petruzzella et al., 1998; Hiser et al., 2000; Leary et al., 2004; Bengtsson et al., 2004; Arnesano et al., 2005). In prokaryotic genomes proteins engaged in the same overall process are often encoded in a common operon. The detection of genes next to each other by genome neighboring analysis can provide clues to the function of these proteins. Such an analysis of bacterial genomes revealed that the function of Cox11p seems to be solely confined to the COX assembly process (Arnesano et al., 2005). Two *COX11* homologues have been detected in the human genome (*HsCOX11)*, one of which, however, is predicted to be a pseudogene (Petruzella et al. 1998). So far no diseases associated with mutations in *HsCOX11* were reported. Interestingly, in the protozoon *Reclinomonas americana* the *COX11* homologue is encoded by the mt genome (Lang et al., 1997; Gray et al., 1998).

The genome sequence of the fission yeast *S. pombe* also revealed the presence of two homologues of *COX11* (*cox11*$^{+}$, *cox11b*$^{+}$) (Khalimonchuk et al., 2005). Both *S. pombe* proteins (SpCox11p, SpCox11bp) contain long N-terminal extensions of more than 500 aa, that exhibit a significant degree of identity to *S. cerevisiae* Rsm22p (Carr and Winge, 2003; Carr et al., 2005). This protein is a component of the small subunit of the mt ribosome and necessary for respiratory growth (Saveanu et al. 2001; Gan et al. 2002). Carr et al. (2005) showed recently that an artificial *COX11-RSM22* fusion in *S. cerevisiae* mimicking the situation in *S. pombe* is able to rescue deletion mutants lacking *COX11* or *RSM22*. The link between Cox11p and a ribosomal protein may hint at a co-translational manner of Cu insertion into Cox1p and thus explain how the metal can be transfered to a site located 13Å below the IMM surface.

Therefore, as outlined above, topogenesis and topological properties of Cox11p from *S. cerevisiae* as well as from *S. pombe* will be characterized in order to get a clue how the $Cu_B$ site is formed.

# Chapter 2

# Materials and Methods

## 2.1 Equipment

### 2.1.1 Consumables and equipment

| | |
|---|---|
| Pipets (P1000, P200, P20, P10) | Gilson |
| Pipet tips (1000 µl, 200 µl, 10 µl) | TPP |
| Reaction tubes (0.5 ml, 1.5 ml, 2.0 ml) | Böttger |
| Reaction tubes (15 ml, 50 ml) | Greiner or TPP |
| Centrifuge tubes (5 ml, 50 ml, 500 ml) | Beckman |
| Erlenmeyer flasks (50 ml, 100 ml, 250 ml, 500 ml, 1L, 2L, 3L, 5L) | Schott or Simax |
| X-ray films | GE Healthcare or Kodak |
| Gel blotting paper | Schleicher & Schuell |
| Immobilon™-P PVDF Membranes | Millipore |
| Dialysis membranes (0.025 µm, VS type) | Millipore |
| Petri dishes | Greiner |
| Dounce homogeniser | Wheaton |
| Glass beads | Roth |

### 2.1.2 Devices

| | |
|---|---|
| Centrifuges: | |
| Biofuge pico | Haereus |
| Biofuge fresco | Haereus |
| Centrifuge 5417R | Eppendorf |
| Sigma 3K30 | Sigma |
| Avanti™ J25 | Beckman |
| Optima ™ MAX Ultracentrifuge | Beckman |
| Thermo cyclers: | |
| Primus | MWG-Biotech |
| Cyclone 96 | PeqLab |

| | |
|---|---|
| LI-COR DNA sequencer 4000/4200 | MWG-Biotech |
| Water bath | GFL |
| Incubators | Binder or Heraeus |
| Ultrospec 3000 | Pharmacia Biotech |
| Gradient maker | Hoefer |
| Concentrator 5301 | Eppendorf |
| Gene Pulser® II | Bio-Rad |
| Heating blocks | Eppendorf or Kleinfeld |
| Vortexer | IKA-Works Inc. |
| Blotter | Biometra |
| Power supplies | Bio-Rad or Pharmacia Biotech |
| DNA/Protein gel chambers | PeqLab/Hoefer |
| Gel-Doc system | MWG-Biotech |

## 2.2 Reagents

### 2.2.1 Chemicals and reagents

| | |
|---|---|
| Tris base | Roth or Merck |
| Raffinose | Fluka |
| EDTA | Roth |
| PMSF | Roth |
| AEBSF | AppliChem |
| Dextrose | Roth |
| Yeast nitrogen base (with or without Ammonium sulphate) | Invitrogen |
| Glycerol | Roth |
| Agarose | BioZym |
| Digitonin | Sigma |
| TEMED | Roth |
| Tween 20 | Roth |
| Dithiohtreitol (DTT) | Roth |
| Protease inhibitors, EDTA-free | Roche |

| | |
|---|---|
| Water (HPLC grade) | Roth |
| Essential amino acids (salts) | Roth or Sigma |
| Yeast extract | Roth |
| Agar | Roth |
| Sodim chloride (NaCl) | Roth |
| Peptone/Tryptone | Roth |
| Sodium dodecyl sulphate (SDS) | Merck |
| ß-mercaptoethanol | Sigma or Roth |
| Ethanol | Roth |
| Acetone | Roth |
| Ampicillin | Roth |
| Geneticin | Gibco-BRL |
| Ammonium persulphate (APS) | Merck |
| Mannitol | Roth |
| Sucrose | Merck |
| Magnesium sulphate ($MgSO_4$) | Sigma or Merck |
| HEPES | Serva |
| Acrylamide/Bisacrylamide | Roth |
| Glycine | Roth |
| Methanol | Roth |
| Coomassie® Brilliant blue G250 | Merck |
| Rnase-OFF™ | AppliChem |
| Ponceau S | Roth |
| Acetic acid | Roth |
| λ phage DNA | Invitrogen |
| Herring sperm DNA | Invitrogen |
| dNTP's | Invitrogen |
| Ammonium sulphate | Merck or Roth |
| Boric acid | Roth |
| Bromphenol blue | Serva |
| Skimmed milk powder | Lasana |

| | |
|---|---|
| ATP | Sigma |
| BenchMark™ Prestained Protein Ladder | Invitrogen |
| Iodine resublimed | Merck |
| NADH | Sigma |
| Valinomycin | Sigma |
| Creatine phosphate | Sigma |
| Ethidiumbromide | Sigma |
| Primers | MWG |
| 5'-IRD 800 labeled primers | MWG |
| Imidazol | Roth |
| Sorbitol | Roth |
| Calcium chloride ($CaCl_2$) | Merck |
| IgG matrix | Sigma |
| Calmodulin matrix | Stratagene |
| Anti-HA Affinity Matrix | Roche |
| 6-aminocaproic acid | Fluka |
| Sodium carbonate ($Na_2CO_3$) | Sigma |
| Hydrochloric acid (HCl) | Roth |
| Potassium hydroxide (KOH) | Roth |
| Sodium hydroxide (NaOH) | Roth |
| Malt extract | Merck |
| Biotin | Merck |
| PEG-3350 | Merck |
| Litium acetate (LiOAc) | ICN |
| Potassium acetate (KOAc) | Roth |
| Sodium acetate (NaOAc) | Roth |
| Magnesium acetate (MgOAc) | Merck |
| Trichloroacetic acid (TCA) | Roth |
| Manganese chloride ($MnCl_2$) | Merck |
| Lactic acid | AppliChem |
| Triton X-100 | Roche or Roth |

| | |
|---|---|
| Isopropanol | Roth |
| Puromycin | Sigma |
| Chloramphenicol | Roth |
| EGTA | Sigma |
| MOPS | Roth |
| BSA | Serva |
| Potassium chloride (KCl) | Merck |
| Potassium dihydrogenphosphate ($KH_2PO_4$) | Merck |
| Phenol/chlrophorm/isoamyl alcohol | Roth |
| Spermidin | Sigma |

### 2.2.2 Kit systems

| | |
|---|---|
| Jetquick PCR Purification Spin Kit | Genomed |
| Nucleospin® Extract Kit | Macherey-Nagel |
| Nucleospin® Plasmid Quick Pure Kit | Macherey-Nagel |
| Wizard® SV Gel and PCR Clean-Up Kit | Promega |
| Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP | Amersham Biosciences |
| ECL-Plus Kit™ | Amersham Biosciences |
| High-molecular-weight Gel-filtration Calibration Kit | Amersham Biosciences |
| $D_C$ Protein Assay | Bio-Rad |

### 2.2.3 Enzymes

| | |
|---|---|
| Restriction enzymes | Invitrogen or MBI Fermentas |
| CombiZyme polymerase | Invitek |
| *Pfx*-Platinum polymerase | Invitrogen |
| T4 DNA ligase | Promega |
| Rnasin | Promega |
| RNase Out | Invitrogen |
| Proteinase K | Roth |

| | |
|---|---|
| RNase A | Roth |
| Zymolyase 20T | ICN |
| Lysing enzymes | Sigma |
| TEV protease | Invitrogen |
| SP6 polymerase | Promega |
| Creatine kinase | Sigma |

### 2.2.4 Antibodies

For Western blot analysis the antibodies were diluted as indicated in TBS-T buffer (section 2.2.5.2) containing 5% (w/v) skimmed milk powder. All listed antibodies were raised against respective yeast proteins unless otherwise specified.

Primary antibodies:

| | |
|---|---|
| Mouse-anti-HA (Roche) | 1:5000 |
| Mouse-anti-cMyc (Roche) | 1:5000 |
| Mouse-anti-Cox2p (Molecular Probes) | 1:500 |
| Mouse-anti-Cox3p (Molecular Probes) | 1:1000 |
| Mouse-anti-EcAtp5p (Molecular Probes) | 1:1000 |
| Rabbit-anti-TAP (Open Biosystems) | 1:2000 |
| Rabbit-anti-Aco1p (kind gift of R. Lill, Marburg) | 1:2000 |
| Rabbit-anti-MrpL36p (kindly provided by J.M. Herrmann, Munich) | 1:250 |
| Rabbit-anti-Oxa1p (kindly provided by J.M. Herrmann, Munich) | 1:100 |
| Rabbit-anti-Tim50p (kindly gifted by D. Mokranjac, Munich) | 1:500 |
| Rabbit-anti-Pet123p (kind gift of T.D. Fox, Ithaca, NY) | 1:600 |
| Rabbit-anti-Sco1p (Buchwald et al., 1991) | 1:3000 |
| Rabbit-anti-Adh1p (kindly gifted by C. Walch-Solimena, Dresden) | 1:5000 |

Secondary antibodies:

| | |
|---|---|
| Sheep-anti-mouse IgG-HRP-coupled (Amersham Biosciences) | 1:5000 |
| Donkey-anti-rabbit IgG-HRP-coupled (Amersham Biosciences) | 1:5000 |

### 2.2.5 Buffers

*2.2.5.1 Commercial buffers and stocks*

| | |
|---|---|
| Buffers for restriction enzymes | Invitrogen or Fermentas |
| Ligation buffer (2x) | Promega |
| PCR buffer, $Mg^{2+}$ free (10x) | Invitek |
| PCR buffer, $Mg^{2+}$ free (10x) | Invitrogen |
| $MgCl_2$ stock [50 mM] | Invitek |
| $MgSO_4$ stock [50 mM] | Invitrogen |

*2.2.5.2 Standard buffers*

*TBE (10x):* 0.9 M Tris base, 0.9 M Boric acid, 25 mM EDTA

*TBS (10x):* 0.2 M Tris-HCl (pH 7.4), 1.37 M NaCl

*TBS-T:* TBS-buffer (x1), 0.1% (v/v) Tween 20

*Breaking buffer for isolation of genomic/plasmid DNA from yeast:*
10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1mM EDTA, 1% (w/v) SDS, 2% (v/v) Triton X-100

*TE:* 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)

*Yeast transformation buffer:* 0.1 M LiOAc, 10 mM Tris-HCl (pH 5.5), 1 mM EDTA (pH 8.0), 40% PEG 3350

*DNA loading buffer (x6):* 0.25% (w/v) Bromophenol blue, 30% (w/v) Glycerol

*TES:* 1.5 M sorbitol, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA.

*2.2.5.3 Buffers for preparation of mitochondria*

*2.2.5.3.1 from S. cerevisiae*

*MTE:* 0.65 M Mannitol, 20 mM Tris-HCl (pH 7.1), 1 mM EDTA, 1 mM PMSF or AEBSF (freshly added)

*Buffer A:* 0.1 M Tris-$H_2SO_4$ (pH 9.4), 1.54 mg DTT/ml Buffer A (freshly added)

*Buffer B:* 1.2 M Sorbitol, 20 mM $KH_2PO_4$ (pH 7.4), 0.3 mg Zymolyase 20T/ml Buffer B (freshly added)

*Buffer C:* 1.2 M Sorbitol

*Buffer D:* 0.65 M Mannitol, 10 mM Tris-HCl (pH 7.4), 1 mM PMSF or AEBSF (freshly added), Protease inhibitors mix EDTA-free (1x)

*2.2.5.3.2 from S. pombe*

*Washing buffer 1:* 0.1 M Tris-HCl (pH 9.3), 0.5 M ß-mercaptoethanol (freshly added)

*Washing buffer 2:* 0.5 M KCl, 10 mM Tris-HCl (pH 7.0)

*Cell lysis buffer:* 1.35 M Sorbitol, 1 mM EGTA, 10 mM citrate/phosphate (pH 5.8), 2 mg Zymolyase 20T/ml Lysis buffer (freshly added), 2 mg Lysing enzymes/ml lysis buffer (freshly added)

*Washing buffer 3:* 0.75 M Sorbitol, 0.4 M Mannitol, 10 mM MOPS (pH 6.8), 0.1% (w/v) BSA, 1 mM PMSF /AEBSF (freshly added), Protease inhibitors mix EDTA-free (1x) (freshly added)

*Washing buffer 4:* 0.65 M Mannitol, 2 mM EGTA, 10 mM MOPS (pH 6.8), 0.5% (w/v) BSA, 1 mM PMSF /AEBSF (freshly added), Protease inhibitors mix EDTA-free (1x) (freshly added)

*2.2.5.4 Buffers for in vitro transcription*

*TC stock (10 x):* 400 mM Hepes-KOH (pH 7.4), 60 mM MgOAc, 20 mM Spermidin

*Premix:* TC stock (1x), 0.01% (v/v) BSA, 10 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM UTP

*2.2.5.5 Buffers for work with mitochondria*

*2.2.5.5.1 Buffers for breakage of mitochondria*

*Lysis buffer:* 20 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$, 1% (v/v) Digitonin, 4 mM AEBSF (freshly added), Protease inhibitors mix EDTA-free (1x) (freshly added)

*Sucrose gradient:* 20 mM Tris-HCl (pH 7.2), 0.1% Digitonin, 10 mM $MgSO_4$, 20 mM DTT (freshly added), 4 mM AEBSF (freshly added), Protease inhibitors mix EDTA-free (1x) (freshly added)

*Lysis buffer (TAP):* 0.6 M Sorbitol, 30 mM $MgSO_4$, 20% (w/v) Glycerol, 20 mM HEPES-KOH (pH 7.4), 1% (v/v) Digitonin, 40 units of RNase Out inhibitor, 4 mM AEBSF (freshly added), Protease inhibitors mix EDTA-free (1x) (freshly added)

*Cleavage buffer (TAP):* Lysis buffer, 1 mM DTT (freshly added), 2 mM $CaCl_2$, 1 mM Imidazole, 10 mM β-mercaptoethanol (freshly added)

*Lysis buffer (for Blue Native-PAGE):* 50 mM NaCl, 5 mM 6-aminocaproic acid, 50 mM Imidazol-HCl (pH 7.0), 1% (v/v) Digitonin, 1 mM AEBSF (freshly added), 40 units of RNase Out inhibitor

*2.2.5.5.2 Buffers for mitochondrial import*

*Import buffer:* 3% BSA (fatty acids free), 0.5 M Sorbitol; 80 mM KCl, 10 mM MgOAc, 2 mM $KH_2PO_4$ (pH 7.4), 2.5 mM $MnCl_2$,

*H buffer:* 20 mM HEPES (pH 7.4)

*SH buffer:* 0.6 M Sorbitol, 20 mM HEPES (pH 7.4)

*SHKCl buffer:* 0.6 M Sorbitol, 20 mM HEPES (pH 7.4), 80 mM KCl

*MPP-processing buffer:* 20 mM HEPES-NaOH (pH 7.3), 100 mM NaCl

*2.2.5.6 Buffers for separation of proteins*

*Separating gel:* 375 mM Tris-HCl (pH 8.8), 12% or 16% (w/v) Acrylamide, 0.32% or 0.4% (w/v) Bisacrylamide, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED

*Stacking gel:* 125 mM Tris-HCl (pH 6.8), 4% (w/v) Acrylamide, 0.1% (w/v) Bisacrylamide, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED

*Running buffer:* 25 mM Tris-base, 192 mM Glycine, 0.1% (w/v) SDS

*Protein loading buffer (6x):* 300 mM Tris-HCl (pH 6.8), 30% (w/v) Glycerol, 10% (w/v) SDS, 0.1% (w/v) Bromophenol blue, 600 mM DTT or 5% (v/v) β-mercaptoethanol (freshly added)

*Coomassie staining buffer:* 42% (v/v) Methanol, 17% (v/v) Acetic acid, 0.1% (w/v) Coomassie brilliant blue G250

*Destaining buffer:* 30% (v/v) Methanol, 7% (v/v) Acetic acid

*Transfer buffer (Western Blot):* 192 mM Glycine, 25 mM Tris-base, 5% (v/v) Methanol, 0.1% (w/v) SDS

*Ponceau S staining solution:* 0.2% (w/v) Ponceau S, 3% (w/v) TCA

### 2.2.6 Media

*2.2.6.1 E. coli media*

| | |
|---|---|
| *LB:* | 1.0% (w/v) Peptone / Tryptone |
| | 0.5% (w/v) Yeast extract |
| | 0.5% (w/v) NaCl |
| | 2.0% (w/v) Agar (for solid LB) |
| | 100 mg/L Ampicillin (for selection) |
| *SOC:* | 2.0% (w/v) Peptone / Tryptone |
| | 0.5% (w/v) Yeast extract |
| | 10 mM NaCl |
| | 2.5 mM KCl |
| | 10 mM $MgCl_2$ x $6H_2O$ |
| | 10 mM $MgSO_4$ x $7H_2O$ |
| | 20 mM Glucose / Dextrose |

*2.2.6.2 S. cerevisiae media*

| | |
|---|---|
| *YP:* | 1.0% (w/v) Yeast extract |
| | 2.0% (w/v) Peptone / Tryptone |
| | 2.0% (w/v) Agar (for solid YP) |
| *YPD:* | YP |
| | 2.0% (w/v) Dextrose / Glucose |
| *YPG:* | YP |
| | 3.0% (w/v) Glycerol |
| *YPGG:* | YP |
| | 3.0% (v/v) Glycerol |
| | 0.1% (w/v) Dextrose / Glucose |
| *YPEG:* | YP |
| | 3.0% (w/v) Glycerol |

| | |
|---|---|
| | 1.0% (v/v) Ethanol |
| *YPGalG:* | YP |
| | 3.0% (v/v) Galactose |
| | 0.1% (w/v) Dextrose / Glucose |
| *Lactate medium:* | 2.00% lactic acid |
| | 0.10% (w/v) Dextrose / Glucose |
| | 0.30% (w/v) Yeast extract |
| | 0.10% (w/v) $KH_2PO_4$ |
| | 0.10% (w/v) $NH_4Cl$ |
| | 0.05% (w/v) $CaCl_2$ x $2H_2O$ |
| | 0.06% (w/v) $MgCl_2$ x $2H_2O$ |
| | 0.05% (w/v) NaCl |
| | Adjust the pH to 5.5 with NaOH |
| *Minimal medium (WO):* | 0.17% (w/v) Yeast nitrogen base |
| | 0.50% (w/v) Ammonium sulphate |
| | 2.00% (w/v) Agar (for solid WO) |
| *WOG:* | WO |
| | 2.0% (w/v) Dextrose / Glucose |
| *WOGG:* | WO |
| | 3.0% (v/v) Glycerol |
| | 0.1% (w/v) Dextrose / Glucose |
| *WORaff:* | WO |
| | 2% (w/v) Raffinose |

Sterile minimal media were supplemented with the appropriate amino acids by addition of the respective stock solutions.

| | |
|---|---|
| Adenine (100x): | 6 g/L Adenine |
| Uracil (100x): | 3 g/L Uracil |
| L-Histidine (100x): | 6 g/L L-Histidine |
| L-Tryptophane (100x): | 8 g/L L- Tryptophane |
| L-Arginine (100x): | 2 g/L L-Arginine |
| L-Methionine (100x): | 2 g/L L-Methionine |
| L-Leucine (100x): | 8 g/L L-Leucine |
| L-Lysine (100x): | 3 g/L L-lysine |

| | |
|---|---|
| *Sporulation medium (SM):* | 1% (w/v) KOAc |
| | 2% (w/v) Agar (for solid SM) |

*2.2.6.3 S. pombe media*

| | |
|---|---|
| *Sporulation medium (MEA):* | 3% (w/v) Malt extract |
| | 100 mg/L L-Histidine |
| | 100 mg/L Uracil |
| | 50 mg/L Proline |
| | 50 mg/L L-Lysine |
| | 50 mg/L L-Leucine |
| | 75 mg/L Adenine |
| | 2% (w/v) Agar (for solid MMA) |
| | Adjust the pH to 6.5 with NaOH |

| | |
|---|---|
| *Solution A1(1000x):* | 8.09 mM $H_3BO_4$ |
| | 160 µM $CuSO_4$ x $5H_2O$ |
| | 602 µM KI |
| | 740 µM $FeCl_3$ x $6H_2O$ |
| | 2.37 mM $MnSO_4$ x $H_2O$ |
| | 247 µM $H_2MoO$ x $2H_2O$ |
| | 1.39 mM $ZnCl_2$ |

| | |
|---|---|
| *Solution A2 (10x):* | Solution A1 (10x) |

| | |
|---|---|
| | 73 mM $KH_2PO_4$ |
| | 5.00 g/L $MgSO_4$ x $7H_2O$ |
| | 1.0 g/L NaCl |
| | 5 mM $CaCl_2$ x $2H_2O$ |
| | 50.00 g/L $(NH_4)_2SO_4$ |
| *Solution A3a (1000x):* | 4.20 mM Calcium pantothenate |
| | 81.2 mM Nicotinamide |
| | 55.5 mM Meso-inositol |
| *Solution A3b (1000x):* | 40.8 µM Biotin |
| | 50% (v/v) Ethanol |
| *YEA:* | 3.0% (w/v) Dextrose / Glucose |
| | 0.5% (w/v) Yeast extract |
| | Solution A3a (1x) |
| | Solution A3b (1x) |
| | 50 mg/L Uracil |
| | 50 mg/L L-Proline |
| | 50 mg/L L-Leucine |
| | 50 mg/L L-Lysine |
| | 2.0% (w/v) Agar (for solid YEA) |
| | Adjust the pH to 6.5 with NaOH |
| | 100 mg/L Geneticin (for selection) |
| *Minimal medium (MM):* | Solution A2 (1x) |
| | Solution A3a (1x) |
| | Solution A3b (1x) |
| | 2.5% (w/v) Agar (for solid MM) |
| | Adjust the pH to 6.5 with NaOH |

| | |
|---|---|
| *MMG:* | MM |
| | 1% (w/v) Dextrose / Glucose |
| *MMGG:* | MM |
| | 3% (v/v) Glycerol |
| | 0.5% (w/v) Dextrose / Glucose |

Sterile minimal media were supplemented with the appropriate amino acids by addition of the respective stock solutions.

| | |
|---|---|
| Adenine: | 10 ml/L Adenine (100x) |
| Uracil: | 10 ml /L Uracil (100x) |
| L-Leucine: | 10 ml /L L-Leucine (100x) |

### 2.2.7 Strains

*2.2.7.1 E. coli strains*

| Strain | Genotype | Reference/Source |
|---|---|---|
| **DH5α** | *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 ($r_K^-$ $m_K^+$), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169 | Hanahan (1983)/BRL |
| **XL1-Blue** | *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 ($r_K^-$ $m_K^+$), *sup*E44, *rel*A1 [F'*::*Tn10 *pro*A+B+ *lac*$I^q$ Δ(*lac*Z)M15] | Stratagene |

*2.2.7.2 S. cerevisiae strains*

| Strain | Genotype | Reference/Source |
|---|---|---|
| **BY4741** | *MAT*a, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0, [rho$^+$]* | EUROSCARF |
| **W303-1A** | *MAT*a, *ade2-1*, *his3-1,15*, *leu 2,3,112*, *trp1-1*, *ura3-1, [rho$^+$]* | Muroff and Tzagoloff (1990) |
| **IL 933-5c** | *MAT*α, *ilv5*, *[rho$^0$]* | Wolf et al. (1973) |
| **KL14-4a** | *MAT*a, *his1, trp2, [rho$^0$]* | Wolf et al. (1973) |
| **Y06479** | *MAT*a, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *cox11::kanMX4, [rho$^+$]* | EUROSCARF |
| **Y15005** | *MAT*α, *his3Δ1*, *leu2Δ0*, lys*2Δ0*, *ura3Δ0, rsm22::kanMX4, [rho$^-$]* | EUROSCARF |
| **Y35005** | *MATa/α*, *his3Δ1/his3Δ1*, *leu2Δ0/leu2Δ0*, *lys2Δ0/LYS2*, *MET15/met15Δ0*, *ura3Δ0/ura3Δ0, rsm22::kanMX4/rsm22::kanMX4, [rho$^-$]* | EUROSCARF |
| **OK-R22** | *MATa/α*, *his3Δ1/his3Δ1*, *leu2Δ0/leu2Δ0*, *lys2Δ0/LYS2*, *MET15/met15Δ0*, *ura3Δ0/ura3Δ0, rsm22::kanMX4/RSM22, [rho$^+$]* | This work |

| | | |
|---|---|---|
| **SC1069** | *MAT*a, *ade2*, *arg4*, *leu2-3,112*, *trp1-289*, *ura3-52*, *MRP4::*(*MRP4-TAP*-tag-*URA3*), *[rho$^+$]* | CellZome AG |
| **YPH 499MO** | *MAT*a, *ura3-52*, *lys2-801$^{amber}$*, *ade2-101$^{ochre}$*, *trp1Δ63*, *his3Δ200*, *leu2Δ1*, *oxa1::*(*OXA1$^{1-317}{}_{\Delta C}$-3HA-HIS3*), *[rho$^+$]* | Szyrach et al.(2003) |
| **YSC1070-662427** | *MAT*α, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *COX11::*(*COX11-3HA-HIS3), [rho$^+$]* | Open Biosystems |
| **YSC1178-7501494** | *MAT*a, *his3Δ1, leu2Δ0*, *met15Δ0*, *ura3Δ0*, *RSM22:: (RSM22-TAP*-tag-*HIS3), [rho$^+$]* | Open Biosystems |

*2.2.7.2 S. pombe strains*

| Strain | Genotype | Reference/Source |
|---|---|---|
| **L972** | *h$^{-S}$* | Gutz et al. (1974) |
| **HE620** | *h$^{+S}$*, *leu1-32*, *ura4-D18* | K. Ostermann, Dresden |
| **HE665** | *h$^{+N}$*, *leu1-32*, *ura4-D18*, *ade6-M210* | K. Ostermann, Dresden |
| **HE639** | *h$^{-S}$*, *leu1-32*, *ura4-D18*, *ade6-M216* | K. Ostermann, Dresden |
| **OK2n-1** | *h$^{+N}$/h$^{-S}$*, *leu1-32/leu1-32*, *ura4-D18/ura4-D18, ade6-M210/ade6-M216*, *cox11/cox11::ura4$^+$* | This work |
| **OK2n-2** | *h$^{+N}$/h$^{-S}$*, *leu1-32/leu1-32*, *ura4-D18/ura4-D18, ade6-M210/ade6-M216*, *cox11b$^+$/cox11b::ura4$^+$* | This work |
| **OK1** | *h$^{-S}$, leu1-32, ura4-D18, ade6-M216, cox11::ura4$^+$* | This work |
| **OK2** | *h$^{-S}$, leu1-32, ura4-D18, ade6-M216, cox11b::ura4$^+$* | This work |
| **OK2n-3** | *h$^{+N}$/h$^{-S}$*, *leu1-32/leu1-32*, *ura4-D18/ura4-D18, ade6-M210/ade6-M216*, *cox11$^+$/cox11::ura4$^+$*, *cox11b$^+$/cox11b::kanMX4* | This work |

### 2.2.8 Oligonucleotides

The melting and annealing temperature (Tm and Ta) of oligonucleotides were calculated using the formula provided by the supplier (MWG):

Tm = 69.3°C + 0.41 x (GC-content %)- 650 / primer length

Ta = Tm – 5°C

Primers used in this study are summarized in the table below. Sequences of the respective restriction enzymes are underlined. Overlapping sequences are shown in bold. Introduced mutations and deletion sites are depicted in red. 5'-IRD 800 labeled primers are marked by an asterisk.

| No. | Sequence (5' to 3') | Direction | Introduced restriction site |
|---|---|---|---|
| 1 | TAT TTA GGA TCC ATG ATA AGA ATA TGT CCC ATT G | Forward | *Bam*HI |
| 2 | TAT TTA CTC GAG TTA ATT TGA GTT GTC TTT CCT TG | Reverse | *Xho*I |
| 3 | **ACA AGG AAA GAC AAC TCA AAT** CTG GTT CCG CGT GGA | Forward | ----- |
| 4 | TCC ACG CGG AAC CAG **ATT TGA GTT GTC TTT CCT TGT** | Reverse | ----- |
| 5 | TAT TTA TCT AGA ATG ATA AGA ATA TGT CCC ATT GTT AGA TCT AAG GTT | Forward | *Xba*I |
| 6 | TAT TTA CTC GAG CTA TTA GCG GCC GCA CTG AGC AGC | Reverse | *Xho*I |
| 7 | TCT TAT TTC TTC AAC **GCC ATT TGT GCT CGT** | Forward | ----- |
| 8 | **ACG AGC ACA AAT GGC** GTT GAA GAA ATA AGA | Reverse | ----- |
| 9 | TAT TTA TCT AGA ATG CTG AAG TTG TCA AGA AGT | Forward | *Xba*I |
| 10 | **GGC AAT CTT TTA ACT AAA CTC AAC** CTG GTT CCG CGT GGA | Forward | ----- |
| 11 | TCC ACG CGG AAC CAG **GTT GAG TTT AGT TTA AAG ATT GCC** | Reverse | ----- |
| 12 | TAT TTA CTC GAG ATG CCC ATT CTA ACA TGC AGA TAT AAA ATT CTG | Forward | *Xho*I |
| 13 | ATT ATT CCA TGG CTA TTA GCG GCC GCA CTG AGC AGC | Reverse | *Nco*I |
| 14 | TAT TTA GGA TCC ATG ATG AAA AGA TGC TTC AGT ATC CTA CCA | Forward | *Bam*HI |
| 15 | TAT TTA CTC GAG CTA TTT TCT ATT TAC ATG TTG TAA AAA ATC GTT GCC | Reverse | *Xho*I |
| 16 | TAT TTA GGA TCC ATG CCC ATT CTA ACA TGC AG | Forward | *Bam*HI |
| 17 | TAT TTA CTC GAG TCA GTT GAG TTT AGT TAA AAG ATT G | Reverse | *Xho*I |
| 18 | TAT TTA GGA TCC ATG GAG GGC AAA GTT CAA AGT ACT TCT CCA | Forward | *Bam*HI |
| 19 | TAT TTA CTC GAG TCA GCC CTC TAA CGG AAA CAA GTC | Reverse | *Xho*I |
| 20 | ACT GCT AAG AAT ACG TCC GAC CAT GAC | Forward | ----- |
| 21 | GTC ATG GTC GGA CGT ATT CTT AGC AGT | Reverse | ----- |
| 22 | GAT GCT CAT GAA GAA GTA GAC TTG CCT | Forward | ----- |

| | | | |
|---|---|---|---|
| 23 | AGG CAA GTC TAC TTC TTC ATG AGC ATC | Reverse | ----- |
| 24 | GAT GCT CAT GAA AAA GTA GAC ATG CCT GTG | Forward | ----- |
| 25 | CAC AGG CAT GTC TAC TTT TTC ATG AGC ATC | Reverse | ----- |
| 26 | GAT GCT CAT GAA GAA GTA GAC ATG CCT | Forward | ----- |
| 27 | AGG CAT GTC TAC TTC TTC ATG AGC ATC | Reverse | ----- |
| 28 | **GTA CCA CTC TAT AGA** CTT TTC TGT TCG | Forward | ----- |
| 29 | CGA ACA GAA AAG **TCT ATA GAG TGG TAC** | Reverse | ----- |
| 30 | **CCG TGG AAA TTC GTT** CCC CAG CAG CGT GAA | Forward | ----- |
| 31 | TTC ACG CTG CTG GGG **AAC GAA TTT CCA CGG** | Reverse | ----- |
| 32 | ACC ATA ATG AGC TCT **AAA AAA AGT ATA GCT** | Forward | ----- |
| 33 | **AGC TAT ACT TTT TTT** AGA GCT CAT TAT GGT | Reverse | ----- |
| 34 | GTC CCT GAG AGG CGG GTG G | Forward | ----- |
| 35 | ATG GAA TTA AGC TGA ACC CAT GCC TGC | Reverse | ----- |
| 36 | TAT TAT AGA TCT ATG GGA GGG CTC TGG CGT CC | Forward | *Bgl*II |
| 37 | TAT TAT CTC GAG TCA ATT ATA TCC TGG AAC TGG CAA CTT GTG C | Reverse | *Xho*I |
| 38 | **ACA AGG AAA GAC AAC TCA** AAT GTC CCA AGA GTA ATC | Forward | ----- |
| 39 | GAT TAC TCT TGG GAC ATT **TGA GTT GTC TTT CCT TGT** | Reverse | ----- |
| 40 | TAT TTA CTC GAG TCA ATT CAA GTC TTC TTC TGA | Reverse | *Xho*I |
| 41 | **CAT ACA TTT GAT ATT TCA** AAA TTT AAA GAT CGT | Forward | ----- |
| 42 | ACG ATC TTT AAA TTT **TGA AAT ATC AAA TGT ATG** | Reverse | ----- |
| 43 | **TAT TCA GTG AAC AGC** AAA TTT AAA GAT CGT ACG | Forward | ----- |
| 44 | CGT ACG ATC TTT AAA TTT **GCT GTT CAC TGA ATA** | Reverse | ----- |
| 45 | **GAG AGA AAA TTT AAA GAT CGT** GCC ATT GCT CTA TTC | Forward | ----- |
| 46 | GAA TAG AGC AAT GGC **ACG ATC TTT AAA TTT TCT CTC** | Reverse | ----- |
| 47 | **TAT GCA GCG GCC CCA CTC TAT AGA** | Forward | ----- |
| 48 | **TCT ATA GAG TGG GGC CGC TGC ATA** | Reverse | ----- |
| 49 | **GCA GCG GTA GCC CTC TAT AGA** | Forward | ----- |
| 50 | **TCT ATA GAG GGC TAC CGC TGC** | Reverse | ----- |
| 51 | TAT TTA GCA TGC ATG ATA AGA ATA TGT CCC ATT GTT | Forward | *Sph*I |

| | | | |
|---|---|---|---|
| 52 | TAT TTA GGA TCC TTA ATT TGA GTT GTC TTT CCT TGT | Reverse | *Bam*HI |
| 53 | TAT TTA GCA TGC TTA ATT TGA GTT GTC TTT CCT TGT | Reverse | *Sph*I |
| 54 | TAT TTA GAA TTC TCA GTT GAG TTT AGT TAA AAG ATT G | Reverse | *Eco*RI |
| 55 | TAT TTA GAA TTC ATG CCC ATT CTA ACA TGC AGA TAT AAA ATT | Forward | *Eco*RI |
| 56 | TAT TTA GGA TCC TCA GTT GAG TTT AGT TAA AAG ATT G | Reverse | *Bam*HI |
| 57 | GGT GTT TTG AGC ACA T **CCC ACT GGC TAT ATG TAT G** | Forward | ----- |
| 58 | **C ATA CAT ATA GCC AGT GGG** ATG TGC TGC TCA AAA ACA CC | Reverse | ----- |
| 59 | **GTC AGA AGG CAT TTA G** ATA AAA ATT AAG GGA CTA C | Forward | ----- |
| 60 | GTA GTC CCT TAA TTT TTA AT **CTA AAT GCC TTC TGA C** | Reverse | ----- |
| 61 | GTT AAC AAG GAA TGT TTC GTA AAT CGA AG | Forward | ----- |
| 62 | CTC TAT TTC ATT TCG TTA CTT TCA TGA C | Reverse | ----- |
| 63 | GTA ACA CCA TCT GCG GCC AC | Forward | ----- |
| 64 | CAT CCC CTC AGC TCT AGC TG | Reverse | ----- |
| 65 | GGT GTT TTG AGC ACA GCT TCG TAC GCT GCA | Forward | ----- |
| 66 | TGC AGC GTA CGA AGC TGT GCT CAA AAC ACC | Reverse | ----- |
| 67 | CGA ATT CAT CGA TGA TAT CAG ATC CAT AAA AAT TAA GGG ACT AC | Forward | ----- |
| 68 | GTA GTC CCT TAA TTT TTA TGG ATC TGA TAT CAT CGA TGA ATT CG | Reverse | ----- |
| 69 | GTC GGA AGA GGC ATA AAT TCC GTC | Reverse | ----- |
| 70 | CCC AAA GTT GAA AGA AAT ACA ACT GCA GAT CC | Forward | ----- |
| 71* | TTT ACC CGT CAA CTT CGC CG | Forward | ----- |
| 72* | TCC ACT GAA GGG AGA TGG AC | Forward | ----- |
| 73* | TTC CCG CAT CTA AGG AAT ATG ACC TC | Forward | ----- |
| 74* | TCA CTG GGT GAT GAC ACG CAA AAT TC | Forward | ----- |
| 75* | GCT ATA CCA AGC ATA CAA TC | Forward | ----- |
| 76* | GGA GGG CGT GAA TGT AAG CG | Reverse | ----- |
| 77* | GAC GGT AGG TAT TGA TTG TAA TTC TG | Forward | ----- |
| 78* | AGG AAT CCT GGC ATA TCA TC | Forward | ----- |

| | | | |
|---|---|---|---|
| 79* | TGC AGC TTG AAT GGG CTT CC | Reverse | ----- |
| 80* | GAT TTA GGT GAC ACT ATA G | Forward | ----- |
| 81* | TAA TAC GAC TCA CTA TAG GG | Forward | ----- |

### 2.2.9 Vectors and plasmids

*2.2.9.1 Vectors*

| Vector | Genetic markers | Reference/Source |
|---|---|---|
| p415ADH | Amp$^r$, *LEU2*, CEN6/ARSH4, *ADH*-promoter/MCS/*CYC1*-terminator | Mumberg et al. (1995) |
| p416ADH | Amp$^r$, *URA3*, CEN6/ARSH4, *ADH*-promoter/MCS/*CYC1*-terminator | Mumberg et al. (1995) |
| p425GPD | Amp$^r$, *LEU2*, *2μ*, *GPD*-promoter/MCS/*CYC1*-terminator | Mumberg et al. (1995) |
| p426GPD | Amp$^r$, *URA3*, *2μ*, *GPD*-promoter/MCS/*CYC1*-terminator | Mumberg et al. (1995) |
| pJR1-3XL | Amp$^r$, *LEU2*, f1 ori, *ars1*, *nmt1*-promoter (3X)/MCS/*nmt1*- terminator | Moreno et al. (2000) |
| pJR1-41XL | Amp$^r$, *LEU2*, f1 ori, *ars1*, *nmt1*-promoter (41X)/MCS/*nmt1*-terminator | Moreno et al. (2000) |
| pGEM®-3Z | Amp$^r$, *lacZ*', T7-promoter/MSC/SP6-promoter | Promega |
| pGEM®-4Z | Amp$^r$, *lacZ*', SP6-promoter/MSC/T7-promoter | Promega |

*2.2.9.2 Constructed plasmids*

| Plasmid | Vector | Template | Insert | Primers | Sequencing primers |
|---|---|---|---|---|---|
| p416*ScCOX11* | p416ADH | *ScCOX11*, *S. cerevisiae* genomic DNA | ScCox11p | #1, #2 | #75*, #76* |
| p416*SpCOX11* | p416ADH | *SpCox11*$^+$, *S. pombe* genomic DNA | SpCox11p | #16, #17 | #71*, #72*, #75*, #76* |
| p425*ScCOX11* | p425GPD | *ScCOX11*, p416ScCOX11 | ScCox11p | #1, #2 | #76*, #77* |
| p426*ScCOX11* | p426GPD | *ScCOX11*, p416ScCOX11 | ScCox11p | #1, #2 | #76*, #77* |
| p425*SpCOX11* | p425GPD | *SpCox11*$^+$, p416SpCOX11 | SpCox11p | #16, #17 | #71*, #72*, #76*, #77 |
| p426*SpCOX11* | p426GPD | *SpCox11*$^+$, p416SpCOX11 | SpCox11p | #16, #17 | #71*, #72*, #76*, #77 |

| | | | | | |
|---|---|---|---|---|---|
| p425*ScRSM22* | p425GPD | *ScRSM22*, *S. cerevisiae* genomic DNA | ScRsm22p | #14, #15 | #73*, #74*, #76*, #77 |
| p416*ScRSM22* | p416ADH | *ScRSM22*, p425*ScRSM22* | ScRsm22p | #14, #15 | #73*, #74*, #75*, #76* |
| p416*SpCOX11*C-term | p416ADH | *SpCox11*$^+$, *S. pombe* genomic DNA | SpCOX11p C-terminus | #17, #18 | #75*, #76* |
| p426*SpCOX11b*C-term | p426GPD | *SpCox11b*$^+$, *S. pombe* genomic DNA | SpCOX11bp C-terminus | #17, #18 | #76*, #77* |
| p425*SpCOX11*N-term | p425GPD | *SpCox11*$^+$, *S. pombe* genomic DNA | SpCOX11p N-terminus | #16, #19 | #71*, #72*, #76*, #77* |
| p416ch1 | p416ADH | *ScCOX11*/*Spcox11*$^+$, p416ScCOX11/ p416SpCOX11 | chimeric Sc/SpCox11p (CPC) | #1, #2, #30, #31, #32, #33 | #75*, #76* |
| p416ch2 | p416ADH | *ScCOX11*/*SpCox11*$^+$, p416ScCOX11/ p416SpCOX11 | chimeric Sc/SpCox11p (CP) | #1, #17, #30, #31 | #75*, #76* |
| p416ch3 | p416ADH | *ScCOX11*/*SpCox11*$^+$, p416ScCOX11/ p416SpCOX11 | chimeric Sc/SpCox11p (CPp) | #1, #17, #28, #29 | #75*, #76* |
| p416*SpCOX11b* | p416ADH | *SpCox11b*$^+$, *S. pombe* genomic DNA | SpCox11bp | #16, #17 | #71*, #72*, #75*, #76* |
| p416*HsCOX11* (L) | p416ADH | *HsCOX11*, liver cDNA library | HsCox11p | #34, #35, #36, #37 | #75*, #76* |
| p416*HsCOX11* (HeLa) | p416ADH | *HsCOX11*, HeLa HCT116 cDNA library | HsCox11p | #34, #35, #36, #37 | #75*, #76* |
| p416*ScCOX11*HA | p416ADH | *ScCOX11*, p416ScCOX11 | ScCox11p, HA-tagged | #3, #4, #5, #6 | #75*, #76* |
| pJR1-3XL*SpCOX11b*HA | pJR1-3XL | *Spcox11b*$^+$, *S. pombe* genomic DNA | SpCox11bp HA-tagged | #10, #11, #12, #13 | #71*, #72*, #78*, #79* |
| pJR1-41XL*SpCOX11b*HA | pJR1-41XL | *Spcox11b*$^+$, *S. pombe* genomic DNA | SpCox11bp HA-tagged | #10, #11, #12, #13 | #71*, #72*, #78*, #79* |
| pJR1-3XL *SpCOX11HA* | pJR1-3XL | *Spcox11*$^+$, *S. pombe* genomic DNA | SpCox11p HA-tagged | #10, #11, #12, #13 | #71*, #72*, #78*, #79* |
| p416ch1E$_{1992}$K (E$_{664}$K) | p416ADH | *ScCOX11*/*Spcox11*$^+$, p416ch1 | chimeric Sc/SpCox11p (CPC) with point mutation | #1, #2, #20, #21 | #75*, #76* |
| p416ch1K$_{2118}$E (K$_{706}$E) | p416ADH | *ScCOX11*/*Spcox11*$^+$, p416ch1 | chimeric Sc/SpCox11p (CPC) with point mutation | #1, #2, #22, #23 | #75*, #76* |

| | | | | | |
|---|---|---|---|---|---|
| p416ch1$L_{2127}$M ($L_{709}$M) | p416ADH | *ScCOX11*/*Spcox11*$^{+}$, p416ch1 | chimeric Sc/SpCox11p (CPC) with point mutation | #1, #2, #24, #25 | #75*, #76* |
| p416ch1$K_{2118}$E-$L_{2127}$M ($K_{706}$E, $L_{709}$M) | p416ADH | *ScCOX11*/*Spcox11*$^{+}$, p416ch1 | chimeric Sc/SpCox11p (CPC) with point mutations | #1, #2, #26, #27 | #75*, #76* |
| p416*SCO1*N-*COX11*CHA | p416ADH | *ScSCO1*/*ScCOX11*, pJR1-3XLScSco1/ p416ScCOX11HA | chimeric ScSco1/ScCox11p HA-tagged | #6, #7, #8, #9 | #75*, #76* |
| p415*SCO1*N-*COX11*CHA | p415ADH | *ScSCO1*/*ScCOX11*, p416SCO1N-COX11CHA | chimeric ScSco1/ScCox11p HA-tagged | #6, #7, #8, #9 | #75*, #76* |
| p416*ScCOX11*Myc | p416ADH | *ScCOX11*, p416ScCOX11 | ScCox11p, Myc-tagged | #5, #38, #39, #40 | #75*, #76* |
| p415*ScCOX11*Myc | p415ADH | *ScCOX11*, p416*ScCOX11*Myc | ScCox11p, Myc-tagged | #5, #38, #39, #40 | #75*, #76* |
| p416*ScCOX11*$_{\Delta 59\text{-}78}$Myc | p416ADH | *ScCOX11*, p416*ScCOX11*Myc | ScCox11p, Myc-tagged with deletion in the N-terminal part | #5, #40, #41, #42 | #75*, #76* |
| p416*ScCOX11*$_{\Delta 49\text{-}78}$Myc | p416ADH | *ScCOX11*, p416*ScCOX11*Myc | ScCox11p, Myc-tagged with deletion in the N-terminal part | #5, #40, #43, #44 | #75*, #76* |
| p415*ScCOX11*$_{TMSco1}$ Myc | p415ADH | *ScCOX11*, p416*ScCOX11*Myc | ScCox11p, Myc-tagged with the TM segment derived from ScSco1p | #5, #7, #8, #40, #45, #46 | #75*, #76* |
| p416*ScCOX11*$V_{104}$A Myc | p416ADH | *ScCOX11*, p416*ScCOX11*Myc | ScCox11p, Myc-tagged with point mutation | #5, #40, #47, #48 | #75*, #76* |
| p415*ScCOX11*$P_{105}$A Myc | p415ADH | *ScCOX11*, p416*ScCOX11*Myc | ScCox11p, Myc-tagged with point mutation | #5, #40, #49, #50 | #75*, #76* |

| | | | | | |
|---|---|---|---|---|---|
| pGEM3*ScCOX11* | pGEM3 | *ScCOX11*, p416*SpCOX11* | ScCox11p | #51, #52 | #81* |
| pGEM4*ScCOX11* | pGEM4 | *ScCOX11*, p416*SpCOX11* | ScCox11p | #1, #53 | #80* |
| pGEM3*SpCOX11* | pGEM3 | *Spcox11*$^{+}$, p416*SpCOX11* | SpCox11p | #16, #54 | #81*, #71*, #72* |
| pGEM4*SpCOX11* | pGEM4 | *Spcox11*$^{+}$, p416*SpCOX11* | SpCox11p | #55, #56 | #80*, #71*, #72* |

*2.2.9.3 Other plasmids*

| Plasmid | Vector | Insert | Reference/Source |
|---|---|---|---|
| pJR1-3XLScSco1 | pJR1-3XL | *S. cerevisiae SCO1* ORF | K. Ostermann, Dresden |

## 2.3 Methods

### 2.3.1 Genetic methods

#### 2.3.1.1 Crossing of the yeast strains and tetrad analysis

*2.3.1.1.1 Crossing and tetrad analysis of S. cerevisiae strains*

Cells of two different strains with different mating types (*MAT*a and *MAT*α) were mixed on a YPD plate (section 2.2.6.2) and after 2 days of incubation at 30°C transferred to sporulation medium (section 2.2.6.2). After 5 days at 30°C a sample of cell material was resuspended in 25 µl of sterile distilled water containing Zymolyase 20T and incubated for 30 min at 30°C to lyse the asci. The suspension was placed onto a fresh YPD plate, incubated for 4 h at 30°C and tetrads were dissected with the help of a micromanipulator (Singer).

*2.3.1.1.2 Crossing and tetrad analysis of S. pombe strains*

Crossing procedures were the same as for *S. cerevisiae*. Two different strains with different mating types ($h^{+S}$ and $h^{-S}$) were mixed on a YEA plate (section 2.2.6.3) and after 2 days of incubation at 30°C transferred to MEA medium (section 2.2.6.3). After 4 days at 30°C a sample of cell material was streaked out on a fresh YEA plate. In this case asci are self-lysed by incubation at 30° C for 30 min on the YEA plate. Tetrads were dissected with the help of micromanipulator (Singer).

#### 2.3.1.2 *Pet*–test

One of the reasons for non-complementation of the respective null-mutant by the certain construct and inability to grow on non-fermentable carbon sources may be the loss of mtDNA integrity due to the mutation. To test this possibility crosses with the respective *rho*$^0$ *PET* tester strain (IL933-5c or KL14-4A) were performed. If the mtDNA of the transformant is intact, the resulting diploid is able to grow on non-fermentable carbon sources (section 2.2.6.2).

### 2.3.2 Molecular biology methods

#### 2.3.2.1 Isolation of genomic DNA from yeast cells

*2.3.2.1.1 Isolation of genomic DNA from S. cerevisiae*

10 ml of *S. cerevisiae* culture were harvested at 3,500 x *g* for 5 min at RT and the cell pellet was resuspended in 200 µl of breaking buffer (section 2.2.5.2). 0.3 g of glass beads (Ø 0.45 mm) and 200 µl of phenol/chlrophorm/isoamyl alcohol (25:24:1) were added to the suspension. After vortexing for 3-4 min and addition of 200 µl TE buffer the mixture was centrifuged at 18,000 x *g* for 5 min (4°C). The upper phase was transferred to a new tube and 1/10 volume of 3M sodium acetate and 2 volumes of 99.8% ethanol were added and carefully mixed. The mixture was incubated at –20°C for 30 min to allow DNA precipitation. Precipitated genomic DNA was pelleted by centrifugation at 18,000 x *g* for 10 min at 4°C and washed with 70% ethanol, sedimented again (18,000 x *g* for 5 min at 4°C), air-dried and resuspended in 50 µl TE buffer containing 10 µl/ml RNase (DNase free). The mixture was incubated for 10-30 min at 37°C and DNA reprecipitated with sodium acetate and ethanol as described above in order to inactivate the enzyme.

*2.3.2.1.2 Isolation of genomic DNA from S. pombe*

Isolation of genomic DNA from *S. pombe* was performed according to Wright et al. (1986) with slight modifications. To obtain the protoplasts Lysing enzymes were used instead of Zymolyase 20T. Enzymatic treatment was performed in TES buffer (section 2.2.5.2).

**2.3.2.2 Polymerase chain reaction (PCR)**

DNA fragments were amplified by 30 cycles reaction in total volumes of 50 µl or 100 µl. Standard reaction mixture contained:

500-800 µM dNTPs mix

1-100 ng of template DNA

150 pM forward and reverse primers

PCR-buffer (1x)

2.5 mM $MgCl_2$ or 1.5 mM $MgSO_4$ (depending on the enzyme)

2.5 U polymerase (CombiZyme or *Pfx* Platinum)

For creation of modified DNA fragments overlap extension PCR was applied as described (Pogulis et al., 1996).

**2.3.2.3 Separation of DNA by agarose gel electrophoresis and its extraction from the gel**

DNA fragments were separated by gel electrophoresis in horizontal 1.5-2% (w/v) agarose gels in TBE buffer (section 2.2.5.2) at 80-120 V. For visualization of DNA 0.1 µg/ml of ethidum bromide was added to the gel. Length of DNA fragments was estimated with regard to the standard ladder obtained after treatment of λ phage DNA with *Eco*RI and *Bam*HI restrictases.

Following separation the fragments of interest were excised from the gel under UV light (wavelength 310 nm). DNA from the excised gel fragment was extracted using the Jetquick PCR Purification Spin Kit (Genomed) or Nucleospin® Extract Kit (Macherey-Nagel) or Wizard® SV Gel and PCR Clean-Up Kit (Promega) according to the manual provided by the manufacturer.

**2.3.2.4 Enzymatic digestion of DNA**

For enzymatic digestion DNA was mixed with sterile bi-distilled water, respective reaction buffer stock solution and appropriate enzyme(s) (1 U/µg DNA). Reaction mixture was incubated for 1.5-2.5 h at 37°C.

### 2.3.2.5 Ligation of DNA fragments

For ligation reaction the respective DNA fragment was mixed with vector DNA (molar ratio 3:1), ligase buffer and sterile bi-distilled water. 2-3 U of T4 DNA ligase were added and the reaction mixture was incubated overnight at 10°C or 15°C.

### 2.3.2.6 Transformation of *E. coli* cells

*2.3.2.6.1 Preparation of competent E. coli cells*

An *E. coli* overnight culture was diluted 1:100 by fresh LB medium (section 2.2.6.1). When the culture reached an $OD_{600}$ of 0.5-0.8 it was chilled on ice for 15 min and cells were harvested by centrifugation at 4,000 x *g* for 15 min at 4°C. Pelleted cells were washed twice with ice-cold sterile distilled water and once with sterile 10% (v/v) glycerol. After centrifugation cells were resuspended in 2 ml 10% Glycerol, aliquoted (40 µl), frozen and stored at –80°C.

*2.3.2.6.2 Electroporation*

6.5-7 µl of ligation mixture were dialysed on dialysis membrane (Millipore) against sterile distilled water for 10-15 min and gently mixed with 40 µl of *E. coli* electrocompetent cells and transferred to the chilled electroporation cuvette (2 mm gap). Parameters of electroporation were 25 µV, 200 Ω and 2.5 kV. Immediately after the pulse the transformation mixture was diluted in 1 ml of SOC medium (section 2.2.6.1) and incubated for 1 h at 37°C. The incubated mixture was suspended by centrifugation at 4,000 x *g* for 7 min at RT. Cells were resuspended in 100 µl and plated onto LB plates containing ampiciline (section 2.2.6.1) to screen for transformed cells. Plates were incubated overnight at 37°C.

### 2.3.2.7 Isolation of plasmid DNA from *E. coli* cells

Plasmid DNA from the transformed *E. coli* cells were prepared by the alkaline lysis method as described (Birnboim and Doly, 1979) from 2-5 ml of overnight culture. To obtain plasmid DNA of higher purity required for sequencing reaction Nucleospin® Plasmid Quick Pure Kit (Macherey-Nagel) was used according to the manual of the manufacturer.

#### 2.3.2.8 DNA sequencing

DNA sequencing was done using the dideoxy-chain termination method (Sanger et al., 1977) with 5'-IRD 800 labeled primers (MWG) and the "Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham Biosciences).

#### 2.3.2.9 Transformation of yeast cells

Yeast cells were transformed by the lithium acetate procedure according to Schiestl and Gietz (1989) with slight modifications. Cell material from 14-24 h-old culture (YPD plate) was picked and resuspended in 10 µl of carrier DNA (2 mg/ml herring sperm DNA). After addition of 5-9 µl DNA and 500 µl PEG/LiOAc, the mixture was vortexed and incubated for 15 min at 30°C. Incubation was followed by a heat-shock at 42°C for 10 min. Cells were centrifuged at 3,000 x *g* for 5 min at RT, resuspended in sterile distilled water and plated onto a selective minimal medium that contained respective supplements (section 2.2.6.2).

#### 2.3.2.10 Replacement of *cox11*$^+$ and *cox11b*$^+$ genes in *S. pombe*

A replacement cassette consisting of the *ura4*$^+$ gene, flanked on both sides with the 5'- and 3'-sequences of the *SpCOX11* reading frame was created to generate a *cox11*$^+$ knock-out mutant. Primers #57, #58, #59, #60, #61 and #62 were used. For the replacement a diploid strain was created by crossing *S. pombe* strains HE665 and HE639, diploids were selected and transformed with the respective construct. Uracil-positive clones were screened for the presence of the replacement cassette by means of PCR. To figure out which of the two *cox11*$^+$ versions was knocked-out a direct sequencing of the DNA stretches encompassing the divergent region were PCR-amplified. A second replacement cassette carrying the *Kan*MX4 gene (primers #61, #62, #65, #66, #67 and #68) was introduced into a diploid strain in which one of the genes is replaced by the *ura4*$^+$-cassette in order to exclude a possibility of a lethal effect of the deletion of both *cox11* genes. Candidate clones were selected by screening for both markers (uracil-prototrophy and G418-resistance. Transformants were sporulated and tetrad analysis performed.

### 2.3.3 Biochemical methods

#### 2.3.3.1 Isolation of *S. cerevisiae* mitochondria

*2.3.3.1.1 Large-scale isolation of mitochondria*

Yeast cells were grown at 30°C to the early stationary phase and mitochondria were prepared as described (Daum et al., 1982).

*2.3.3.1.2 Small-scale isolation of mitochondria*

50 ml of yeast culture was grown at 30°C and the cells were harvested by centrifugation at 3,500 x *g* (RT) for 5 min. Pelleted cells were washed in distilled water and resuspended in 500 µl of ice-cold MTE buffer containing respective protease inhibitors (section 2.2.5.3.1). 400-500 µl of sterile glass beads (Ø 0.2-0.45 mm) were added to the cell suspension and the cells were broken by extensive vortexing for 5 min. After sedimentation of the glass beads, the supernatant was placed into a new tube and the beads were washed with another 500 µl of ice-cold MTE buffer containing protease inhibitors. Upon combining the washing solutions intact cells and cell debris were removed by a short centrifugation step (4,000 x g (2°C) for 3 min). The cleared extract was centrifuged at 20,000 x g (2°C) for 15 min in order to pellet mitochondria. The cytosolic fraction (supernatant) was removed and the pellet resuspended in 50-100 µl of MTE buffer with protease inhibitors for further applications.

#### 2.3.3.2 Isolation of *S. pombe* mitochondria

*S. pombe* mitochondria were prepared according to Moore et al. (1992) with slight modifications. Cells grown in a rich medium to the late exponential phase were harvested by centrifugation at 2,000 x *g* (RT) for 5 min and washed twice in distilled water. Pelleted cells were incubated at 0.5 g/ml in washing buffer 1 (section 2.2.5.3.2) for 10 min at 30°C and centrifuged at 2,000 x *g* (RT) for 5 min. After centrifugation cells were washed three times in washing buffer 2 (section 2.2.5.3.2) and resuspended in cell lysis buffer (section 2.2.5.3.2). Spheroplasts were generated in two steps: first, 2 mg/ml Zymolyase 20T were added and cells were incubated for 15 min at 30°C. Then Lysing enzymes (Sigma) were added to 2 mg/ml and the mixture incubated for 10-15 min at 30°C. Spheroplasts were collected by centrifugation at 400 x *g* for 10 min at 2°C and

washed twice in washing buffer 3 (section 2.2.5.3.2). The washed spheroplasts were resuspended at 0.15 g/ml in washing buffer 4 containing protease inhibitors (section 2.2.5.3.2) and gently broken in a loose-fitting glass homgenizer. Unlysed cells and cell debris were removed by centrifugation at 1,000 x *g* for 10 min at 2°C. The mitochondrial pellet was obtained by centrifugation at 17,700 x *g* for 10 min (2°C) and resuspended in 30-40 ml of washing buffer 4 containing protease inhibitors. Residual contaminants were removed by additional centrifugation at 1,000 x *g* for 10 min at 2°C and mitochondria were harvested by centrifugation at 12,000 x *g* for 10 min (2°C), resuspended in 100-500 µl of washing buffer 4 with protease inhibitors, and shock-frozen in liquid nitrogen.

#### 2.3.3.3 Determination of protein concentrations

Protein concentrations were determined using the Lowry-based $D_C$ protein assay system (Bio-Rad) according to the manual provided by the manufacturer.

#### 2.3.3.4 Determination of cytochrome *c* oxidase activity

COX activities were determined spectroscopically as described (Tzagoloff et al., 1975). Commercial cytochrome *c* (Sigma) was reduced with sodium dithionite and oxidation of reduced cytochrome was measured spectrophotometrically. Reaction was started by the addition of cytochrome *c* to a concentration of 32 µM, then 100 µg of mt protein were added and the rate of oxidation was determined by following the decrease in absorbance at 550 nm. Activities were expressed in terms of the first-order velocity constant *k* calculated by the following formula: $k=\ln(\Delta E_0-\Delta E_1)$, where $\Delta E_0$ is the absorption difference between start and endpoints, and $\Delta E_1$ is the absorption difference between start and time point 1 min. Obtained *k* values were then compared. The *k* value obtained for wt was set as 100%.

#### 2.3.3.5 Separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of proteins by SDS-PAGE was performed as described by Laemmli (1970). A typical gel consisted of 12% or 16% separating gel and 4% stacking gel (section 2.2.5.6).

Gels with separated proteins were stained or subjected to electro-transfer procedure and then stained in Coomassie staining solution (section 2.2.5.6) for 20-60 min at RT and washed with the destaining solution (section 2.2.5.6) until the protein bands became visible.

**2.3.3.6 Western blot analysis**

Separation of the proteins by SDS-PAGE was followed by their electro-transfer to a PVDF or nitrocellulose membrane in Transfer buffer (section 2.2.5.6) by means of either semi-dry (1.5 mA/cm$^2$ for 1.5 h at RT) or tank-blot (3 mA/cm$^2$ for 30 min at 10°C) techniques. Transferred proteins were visualised by staining of the membrane in Ponceau S solution. Membranes were blocked in the TBS-T buffer containing 5% (w/v) fat-free skimmed milk overnight at 4°C or for 1 h at RT. Blocked membranes were incubated with the respective mono- or polyclonal antibodies or antisera for 1-2 h at RT. After intensive washing bound antibodies were detected for 30 min (RT) with horseradish peroxidase(HRP)-conjugated secondary antibodies raised against the immunoglobulines of host organism that generated the primary antibodies. Membranes were washed in TBS-T and detected proteins were visualized with the ECL$^{plus}$ chemiluminescence-based system according to the manufacturer's manual.

**2.3.3.7 Carbonate extraction of mitochondrial proteins**

Alkaline extraction of proteins was performed according to Fujiki et al. (1982) with slight modifications. 300-500 µg of mitochondrial protein were resuspended in 500 µl of 0.1 M sodium carbonate solution (pH 11.5), incubated on ice for 30 min and centrifuged at 165,000 x *g* for 1 h at 2°C. The supernatant and the pellet, which was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.5) were precipitated with 10% trichloroacetic acid (TCA), washed twice with ice-cold 80% acetone, dissolved in SDS-sample buffer, subjected to SDS-PAGE and analysed by immunoblotting.

**2.3.3.8 Proteinase K protection assay**

Mitochondria were resuspended in MTE buffer (section 2.2.5.3.1), diluted with 20 mM Tris-HCl (pH 7.5), 1 mM EDTA to a final manitol concentration of 0.1 M and incubated

on ice for 25 min. The resulting mitoplasts were spinned down at 12,000 x *g* for 10 min at 2°C, resuspended in 0.65 M manitol, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 µg aliquots were used for Proteinase K treatment.

1-200 µg of Proteinase K were added to mitoplasts in the presence or absence of 20 mM EDTA, pH 8.0 or 100 mM DTT. Addition of EDTA or DTT was immediately before Proteinase K treatment.

Samples were incubated on ice for 20 min and the reaction was stopped by addition of 4 mM PMSF or AEBSF. The pellets obtained after centrifugation at 20,000 x *g* for 10 min at 2°C were dissolved in SDS-sample buffer, subjected to SDS-PAGE and analysed by immunoblotting.

**2.3.3.9 Sucrose gradient centrifugation**

The analysis was performed according to Szyrach et al. (2003) with slight modifications. 500-1,500 µg of mitochondrial proteins were lysed in 1% digitonin, 20 mM Tris-HCl, pH 7.2, for 15 min at 4°C in the presence of 10 mM $MgSO_4$. The extracts were cleared by centrifugation for 10 min at 18,000 x *g* at 2°C, loaded onto continuous 20-40% sucrose gradients (5 ml) containing 20 mM Tris-HCl, pH 7.2, 0.1% digitonin, 20 mM DTT, 4 mM AEBSF and 10 mM $MgSO_4$, and centrifuged at 148.000 x *g* for either 1 h or 4 h at 2°C. 19 fractions of 270 µl were collected, 4 µl aliquots of each fraction were taken to determine the absorption at 260 nm to establish the mitoribosomal profile. The proteins were precipitated by 10% TCA and subjected to SDS-PAGE and Western blot analysis.

**2.3.3.10 Co-immunoprecipitation**

300 µg of mitochondrial proteins were isolated from the *cox11*Δ strain Y06479 transformed with p416Sc*COX11*HA and lysed with 1% digitonin, 20 mM Tris-HCl, pH 7.2, for 15 min at 4°C in the presence of 10 mM $MgSO_4$. The extract was cleared by centrifugation for 10 min at 18,000 x *g* at 2°C and incubated with an anti-HA Affinity Matrix (Roche) for either 1 h or overnight on a rotator at 4°C. The beads were washed according to the protocol of the manufacturer, resuspended in SDS-sample buffer, boiled, spinned down and the supernatant was subjected to SDS-PAGE and analysed by Western blotting.

### 2.3.3.11 Tandem affinity purification

The tandem affinity purification (TAP) was performed as described previously (Rigaut et al., 1999; Krause-Buchholz et al., 2004). 500-1,000 µg of total mt protein were lysed for 1 h in 1% digitonin, 30 mM $MgSO_4$, 20% glycerol, 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 40 units of RNase Out inhibitor. Lysis was performed in the presence or absence of 400 µM puromycin. Insoluble material was removed by centrifugation for 10 min at 18,000 x *g* at 2°C and the cleared lysate was incubated with IgG matrix (Sigma) for 2 h at 4°C. Cleavage with recombinant TEV-protease was done in lysis buffer with 1 mM DTT for 1 h at 23°C. $CaCl_2$ and imidazole (2 mM and 1 mM, respectively) as well as 10 mM β-mercaptoethanol were added to the supernatant prior to the incubation with a calmodulin matrix at 4°C in the presence of 0.1% digitonin for 1 h. Aliquots from each step of the TAP procedure were collected and subjected to SDS-PAGE and Western blot analysis.

### 2.3.3.12 Blue native polyacrylamide gel electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as described (Schägger 2001; Krause-Buchholz et al. 2004). Briefly, 200 µg of mt protein were lysed in 1% digitonin (detergent:protein ratio is 4:1), 50 mM NaCl, 5 mM 6-aminocaproic acid, 50 mM imidazol-HCl, pH 7.0, 1 mM AEBSF, with 40 units of RNase Out inhibitor added. The extract was cleared by centrifugation for 20 min at 20,000 x *g* at 2°C. 4 µl of a non-denaturing loading buffer (10% glycerol, 0.01% Ponceau S) were added. The samples were loaded onto 3–13% gradient gel. To determine the apparent molecular weights we used a high-molecular-weight gel-filtration calibration kit. Gel stripes were cut out and run in a second dimension in regular SDS-gel and analyzed by Western blot.

### 2.3.3.13 *In vitro* synthesis of [$^{35}$S]-labelled proteins

For synthesis of [$^{35}$S]-labelled proteins, the respective genes were cloned into pGEM-3Z or pGEM-4Z plasmid and transcribed by SP6-RNA polymerase. For that purpose 30 µl of Premix, 2.5 µl of 2.5 mM $m_7$G(5')ppp(5')G and 15 µl of plasmid DNA (300 ng/ml) were mixed, filled to 200 µl by sterile $H_2O$, and 1 µl RNasin (40 U/µl) und 1 µl SP6 RNA-polymerase (25 U/µl) added. The reaction mixture was incubated for 1 h at 37°C. The

resulting RNA was precipitated by addition of 5 µl of 10 M LiCl and 150 µl of ice-cold ethanol and incubation for 15 min. at –20°C followed by the centrifugation for 30 min. at 35000 x *g* (4°C). The resulting pellet was washed with ice-cold 70% ethanol and air-dried at RT. Obtained mRNA was dissolved in 30 µl of water containing 0,75 U/µl RNasin.
Next the respective mRNA was *in vitro* translated in the rabbit reticulocytes lysate system. The mixture containing 140 µl of rabbit reticulocytes lysate, 4 µl RNasin, 8 µl of amino acids mix (all protein-forming amino acids except methionine, 1 mM each), 16 µl [$^{35}$S]-methionine and 30 µl mRNA was incubated for 60 min. at 30°C. Then lysate was centrifugated for 30 min at 100,000 *g* (2°C), the clarified lysate was aliquoted, frozen in liquid $N_2$ and stored at –80°C.

#### 2.3.3.14 Protein import into isolated mitochondria

Import of [$^{35}$S]-labelled precursor proteins into isolated mitochondria was performed essentially as described previously (Hell, 1998). The import reaction contained 15-50 µg of mitochondria in a final volume of 300 µl. 2 mM NADH, 2 mM ATP, and an ATP-regenerating system containing 2.5 mM malate, 2.5 mM succinate, 1 mM creatine phosphate and 0.1 mg/ml creatine kinase was added during the import reaction to warrant a highly energized state of the mitochondria. 2% (v/v) of lysate were added and reaction performed for the indicated time at 25°C. The reaction was stopped by dilution (1:10) of reaction mixture by ice-cold SH (isotonic)or H (hypotonic) buffer. Non-imported precursor protein was removed by incubation with proteinase K (100 µg/ml) for 30 min on ice. The protease was inactivated by addition of 2 mM PMSF. Treated mitochondria were sedimented for 15 min. at 25,000 x *g* (4°), washed with SHKCl buffer, resuspended in protein loading buffer and subjected to SDS-PAGE.

### 2.3.4 Bioinformatics

Alignments were performed using the programs "MultAlign" (Corpet, 1988) and "BoxShade 3.21" (http://www.ch.embnet.org/software/BOX_form.html). pI values of the protein stretches were calculated using "Compute pI/Mw tool" software (http://us.expasy.org/tools/pi_tool.html). Organization of ScCox11p and SpCox11p was predicted by the program SMART program (Letunic et al., 2002). The charge distribution

in the protein sequences were predicted using "Helix Draw v1.00" program (http://www.bioinf.man.ac.uk/~gibson/ HelixDraw/helixdraw.html).

# Chapter 3

# Results

## 3.1 Organization of Cox11p

*S. cerevisiae* Cox11p was originally identified by Tzagoloff et al. (1990) as a product of a *PET* gene, whose mutation results in respiratory deficiency. It has been described as 28 kDa protein, however, the molecular mass calculated from the DNA sequence of the respective open reading frame (ORF) is 34 kDa. Bioinformatic analysis reveals a number of Cox11p homologues in different organisms from prokaryotes (here also known as CtaG) to higher eukaryotes. The C-terminal part of the protein demonstrates high conservation between different organisms (Appendix, Fig. 41). The highest degree of conservation is observed in the part (aa 158-258, *S. cerevisiae* numbering) which harbors the highly conserved CFC-motif involved in copper binding (Carr et al., 2000). The N-terminal part of the protein is less conserved. Cox11p is described as a mt protein, however the import presequence can not be easily defined. SMART analysis program (Letunic et al., 2002) reveals the presence of a single, relatively long TM domain (residues 85-105, *S. cerevisiae* numbering) in all Cox11p homologues. The C-terminal part of the TM segment is evolutionary well conserved.

Analysis of the *S. pombe* genome reveals the presence of two ORFs encoding Cox11p homologues (SpCox11p): *cox11*$^+$ and *cox11b*$^+$. Each ORF is part of a 5.7 kb duplicated region on chromosome 1. The distance between the duplicated regions is about 3637 kb. (The Sanger Institute, Cambridge, UK). Interestingly, the only difference between these two regions is inside the *cox11*$^+$ ORF, three nucleotides in positions 1659-1661, that give rise to two different aa residues (positions 553-554): NI in SpCox11p and KY in SpCox11bp, respectively. Carr et al. (2000) observed that SpCox11 proteins contain large N-terminal extensions of about 470 aa that share 25% identity with *S. cerevisiae* Rsm22p, a constituent of a small mitoribosomal subunit, that is required for the respiratory growth. No homologues of Rsm22p have been identified in bacteria (Saveanu et al., 2001). Interestingly, the Cox11p homologues from *Mycobacterium* and *Corynebacerium* species also possess long N-terminal extensions (Bengtsson et al., 2004), however these encompass multiple predicted TM domains and show no similarity to Rsm22p.

## 3.2 Complementation studies of *COX11*

### 3.2.1 Complementation of *S. cerevisiae cox11*Δ mutants

*COX11* genes from different organisms and their derivates were tested for their ability to complement the *cox11*Δ mutant of *S. cerevisiae*. As a positive control *S. cerevisiae COX11* (*ScCOX11*) was used in the complementation analysis. The ORF of *ScCOX11* was PCR amplified from genomic DNA of the respiratory-competent strain BY4741 and cloned into the expression vector p416 under the control of the *ADH1* promoter that provides moderate level of expression (Mumberg et al., 1995). The resulting construct was transformed into the *cox11*Δ strain Y06479. Transformants expressing *ScCOX11* are able to grow on non-fermentable carbon source (YPG, YPEG or YPGG) at all temperatures tested (23°C, 30°C and 37°C) (Fig. 5).

The ORF of the *S. pombe* homologue *Spcox11*$^{+}$ was PCR amplified using genomic DNA of the *S. pombe* strain L972 as a template. Because the two Sp*COX11* ORFs are part of 5.7 kb DNA duplication and the only difference between them is inside the *cox11*$^{+}$ ORF (three nucleotides in positions 1659-1661), direct sequencing of the PCR products was applied to prove the presence of both versions (Fig. 5). The same PCR products were used for the random cloning of *Spcox11*$^{+}$ or *Spcox11b*$^{+}$ gene into p416ADH, p425 or p426 vectors under control of the strong *GPD1* promoter. None of these constructs is able to restore respiration of strain Y06479 (Fig. 7). The mtDNA of transformants was proven to be intact by analysing the diploids obtained by crossing with the *rho*$^{0}$ strain IL933-5c (see Materials and methods, *pet*-test). To check whether the overexpresison of either *Spcox11*$^{+}$ or *Spcox11b*$^{+}$ causes a dominant-negative effect, each protein was expressed under control of the *GPD1* promoter in the respiratory competent strain BY4741. The results of replica-plating of the respective transformants on a media with non-fermentable carbon source did not reveal any obvious change in respiratory growth when compared to untransformed cells (Fig. 6).

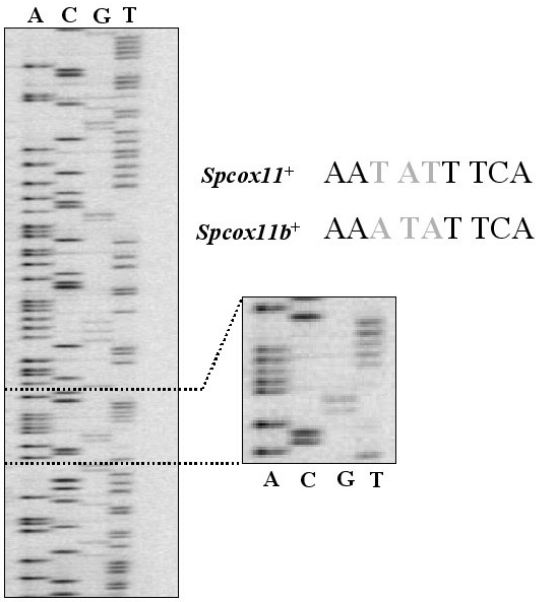



**Figure 5. Two versions of *cox11*$^+$ are present in the genome of *S. pombe*.** Direct sequencing reveals the polymorphism corresponding to the differences in the DNA sequences of *Spcox11*$^+$ and *Spcox11b*$^+$.

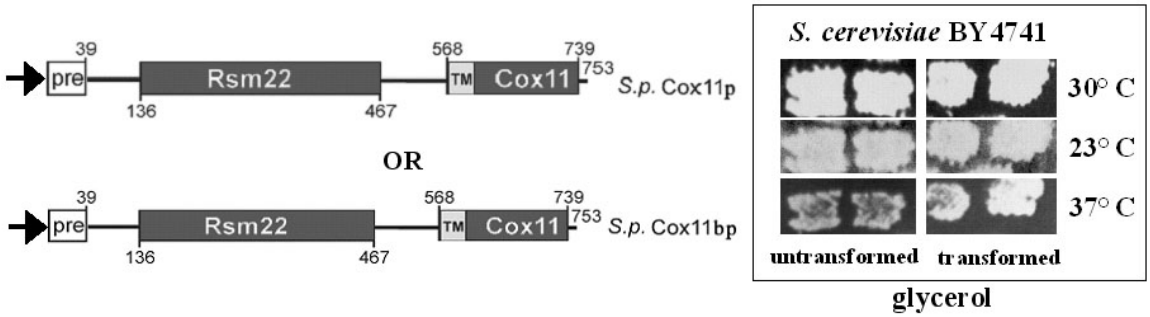



**Figure 6. Overexpression of *Spcox11*$^+$ or *Spcox11b*$^+$ in *S. cerevisiae* BY4741 has no dominant-negative effect on respiratory growth.** Strain BY4741 was transformed with high-expression plasmids encoding either SpCox11p or SpCox11bp. The transformants were replica-plated onto glycerol medium and grown for 3 days at 23°C, 30°C or 37°C.

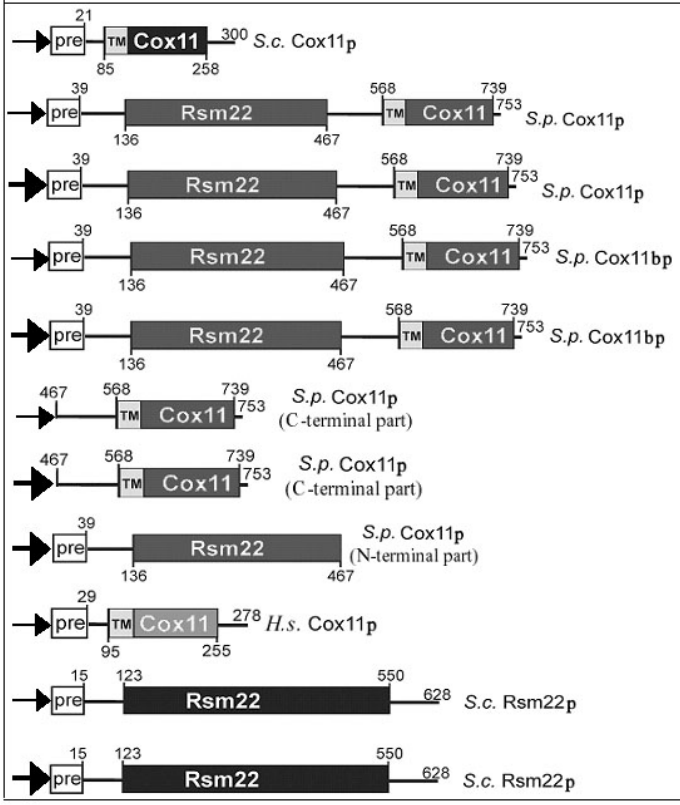
In order to clarify if the N- or C-terminal part of SpCox11p can substitute for ScCox11p shortened versions of the *S. pombe* protein were created. Part of *Spcox11*$^+$ ORF corresponding to the highly homologous C-terminal part of SpCox11p (aa residues 467-753) was amplified by PCR from genomic DNA and cloned into p416ADH or p426GPD

vectors. Sequence analysis of the picked clone revealed that the fragment cloned into p426GPD represented the C-terminal part of *Spcox11*$^{+}$. Similarly, a DNA fragment representing the N-terminal part of SpCox11p (aa residues 1-467) was obtained and cloned into the p425GPD vector.

Functional complementation of the *S. cerevisiae cox11* null mutant strain Y06479 was checked with all constructs. In no case complementation was observed. Deficiency of mt DNA could be excluded by *pet*-test (Fig. 7). These results show that neither the C- nor the N-terminal part of SpCox11p can substitute for ScCox11p.

Next a human homologue of the *COX11* gene was isolated using the commercial cDNA libraries from liver and HeLa HCT116 cell line, by means of nested PCR, and cloned into p416ADH vector. The second version of *HsCOX11* was not of interest for this study since it is described as a pseudogene (Petruzella et al., 1998). Upon transformation of p416HsCOX11 into strain Y06479, respiratory growth was tested. The construct failed to substitute for ScCox11p at all temperatures tested. Deletion of mtDNA could be excluded (Fig. 7).

BLAST search reveals a highly conserved region of about 100 aa between *S. cerevisiae* and *S. pombe* Cox11p. Due to the inability of the above described constructs to substitute for ScCox11p, a set of chimeras was created in order to identify a functionally conserved region of Cox11p. The first chimera consists of the highly conserved part of SpCox11p (aa 643-738) that harbours a predicted transmembrane (TM) domain and the copper-binding motif, flanked on both sides by sequences representing N- (aa 1-158) and C-terminal (aa 258-300) parts of ScCox11p. The second chimera was created by fusing the sequence representing the N-terminal part of ScCox11p (aa 1-158) and the C-terminal part of its *S. pombe* counterpart encompassing the highly conserved region (aa 643-753). The segment of ScCox11p adjacent to the TM domain possess a highly conserved cysteine residue ($C_{111}$). In order to find out the reason for non-complementation of *cox11* null mutant by chimera 2, chimera 3 encompassing the native cysteine of SpCox11p ($C_{595}$) was created (Fig. 9).

| Construct | Growth on glycerol at 23°C | Growth on glycerol at 30°C | Growth on glycerol at 37°C | *pet*-test |
|---|---|---|---|---|
|  | + | + | + | + |
| | - | - | - | + |
| | - | - | - | + |
| | - | - | - | + |
| | - | - | - | + |
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| | - | - | - | + |
| | - | - | - | + |



**Figure 7. Complementation of *S. cerevisiae cox11*Δ mutant by different constructs.** Schematic view of Cox11p from different organisms, their derivatives and ScRsm22p. The constructs were expressed in *S. cerevisiae cox11* knock-out strain Y06479 under control of the promoter that provides either moderate (*ADH1*, thin arrows) or high (*GPD1*, thick arrows) level of expression. Respiratory growth of the respective transformants was checked on glycerol-containing media (YPG, YPEG or YPGG) at 23°C, 30°C and 37°C. "+" indicates growth, "-" – its absence. The integrity of mtDNA was checked in *pet*-tests ("+"- intact mtDNA, "-"-integrity is affected).

Chimera 3 differs from chimera 2 in that the C-terminal SpCox11p-derived part is longer (aa 109-753) and starts immediately after the TM segment of the ScCox11p part (aa residues 1-109). The constructs were cloned into p416 expression vector under control of moderate *ADH1* promoter and transformed into *S. cerevisiae* strain Y06479. Surprisingly, the expression of neither of the chimeric proteins was able to confer respiratory growth to the *cox11* null mutant. The *pet*-tests indicate that mtDNA is intact (Fig. 9). The ability of

chimera 1 to substitute for Cox11p upon overexpression (*GPD1* promoter) was tested. The respective construct also failed to complement the *cox11* null mutant (data not shown).

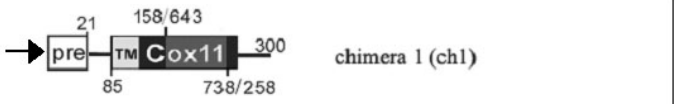
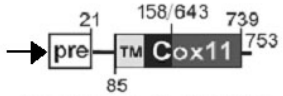
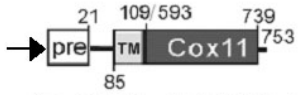
| Construct | Growth on glycerol at | | | *pet*-test |
|---|---|---|---|---|
| | 23°C | 30°C | 37°C | |
|  chimera 1 (ch1)<br>*S.c.* Cox11p (1-158)/*S.p.* Cox11p (643-738)/*S.c.* Cox11p (258-300) | - | - | - | + |
|  chimera 2 (ch2)<br>*S.c.* Cox11p (1-158)/*S.p.* Cox11p (643-753) | - | - | - | + |
|  chimera 3 (ch3)<br>*S.c.* Cox11p (1-109)/*S.p.* Cox11p (593-753) | - | - | - | + |

**Figure 8. Complementation of *S. cerevisiae cox11*Δ mutant by chimeric constructs.** Schematic view of Cox11p chimeras. The constructs were expressed in *S. cerevisiae cox11* knock-out strain Y06479 under control of the promoter that provides moderate (*ADH1*, thin arrows) level of expression. Respiratory growth of the respective transformants was checked on glycerol-containing media (YPG, YPEG or YPGG) at 23°C, 30°C and 37°C. "+" indicates growth, "-" – its absence. The integrity of mtDNA was checked in *pet*-tests ("+"- intact mtDNA, "-"-integrity is affected).

As already pointed above, the C-terminal part of Cox11p contains a stretch of about 100 aa that shows high degree of conservation between different organisms. Nevertheless there are a few positions within this region, that differ significantly. As the chimeric genes composed of *S. cerevisiae* and *S. pombe COX11* are not able to complement the *S. cerevisiae cox11*-null mutant, another set of constructs, based on chimera 1 was created by means of PCR-driven directed mutagenesis. As residues of the *S. pombe* segment that

differ significantly in their physicochemical properties from those in the respective *S. cerevisiae* counterpart may affect the function of chimeric protein in *S. cerevisiae* cells. In order to test this possibility the respective residues were replaced by those that correspond to the *S. cerevisiae* stretch. Thus, chimera 1 ($E_{664}K$), chimera 1 ($K_{706}E$), chimera 1 ($L_{709}M$) and chimera 1 ($K_{706}E$, $L_{709}M$) were created, cloned into p416 expression vector under control of moderate *ADH1* promoter, and transformed into strain Y06479. Surprisingly, expression of none of these constructs can confer the

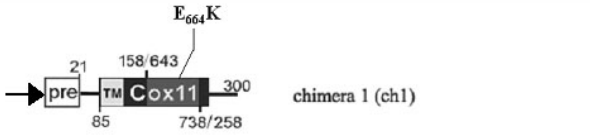
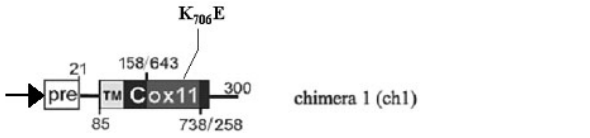
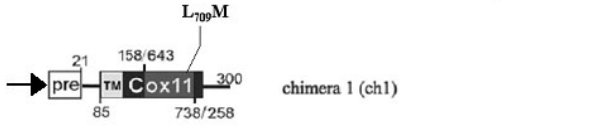
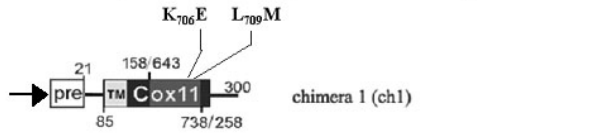
| Construct | Growth on glycerol at | | | *pet*-test |
|---|---|---|---|---|
| | 23°C | 30°C | 37°C | |
|  *S.c.* Cox11p (1-158)/*S.p.* Cox11p (643-738)/*S.c.* Cox11p (258-300) | - | - | - | + |
|  *S.c.* Cox11p (1-158)/*S.p.* Cox11p (643-738)/*S.c.* Cox11p (258-300) | - | - | - | + |
|  *S.c.* Cox11p (1-158)/*S.p.* Cox11p (643-738)/*S.c.* Cox11p (258-300) | - | - | - | + |
|  *S.c.* Cox11p (1-158)/*S.p.* Cox11p (643-738)/*S.c.* Cox11p (258-300) | - | - | - | + |

**Figure 9. Complementation of *S. cerevisiae cox11*Δ mutant by modified chimeric constructs.** Schematic view of mutated Cox11p chimeras The constructs were expressed in *S. cerevisiae cox11* knock-out strain Y06479 under control of the *ADH1* promoter. Respiratory growth of the respective transformants was checked on glycerol-containing media (YPG, YPEG or YPGG) at 23°C, 30°C and 37°C. "+" indicates growth, "-" – its absence. The integrity of mtDNA was checked in *pet*-tests ("+"- intact mtDNA).

respiratory competence to the *cox11* null mutant. The transformants were proven to be *rho*$^+$ in *pet*-tests (Fig. 9).

### 3.2.2 Complementation of *S. cerevisiae rsm22*Δ mutants

As already mentioned above, the N-terminal part of both versions of SpCox11p shows significant sequence identity (25%) to *S. cerevisiae* Rsm22p, a constituent of small subunit of mt ribosome. Interestingly, BLAST searches revealed that no other protein with significant homology to ScRsm22p is present in the proteome of *S. pombe*. In order to test whether SpCox11p is able to complement *rsm22* null mutant expression plasmids that provide different levels of expression and bear *cox11*$^+$ or *cox11b*$^+$ were transformed into the *S. cerevisiae rsm22* knock-out strain Y15005, and respiratory growth of the transformants was tested. Obviously SpCox11p is not able to functionally substitute for ScRsm22p. This inability is not a consequence of inadequate expression of the proteins (Fig. 10). Identical results were obtained when the N-terminal part of SpCox11p alone was introduced into strain Y15005 (Fig. 10).

*ScRSM22* gene was cloned under control of either *ADH1* or *GPD1* promoter and used as a control in the complemenation studies. As expected, over-expression of *ScRSM22* does not restore the respiration of *S. cerevisiae cox11*-null mutant (Fig. 6). Unexpectedly, however, the plasmid was also not able to complement the *S. cerevisiae Δrsm22* strain (Fig. 10). *pet*-tests provided evidence that the mtDNA of these transformants as well as of strain Y15005 is not intact. The likely explanation of this finding is the well-known observation that mutants affected in mt translation easily loose their mtDNA (Myers et al., 1985).

Similar results were obtained using spores from homozygous diploid strain Y35005 or from the heterozygous strain OK-R22 obtained by crossing strains Y15005 and BY4741. In no case a spore bearing the kanamycine resistance marker proved to harbour intact mtDNA as shown by *pet*-tests.

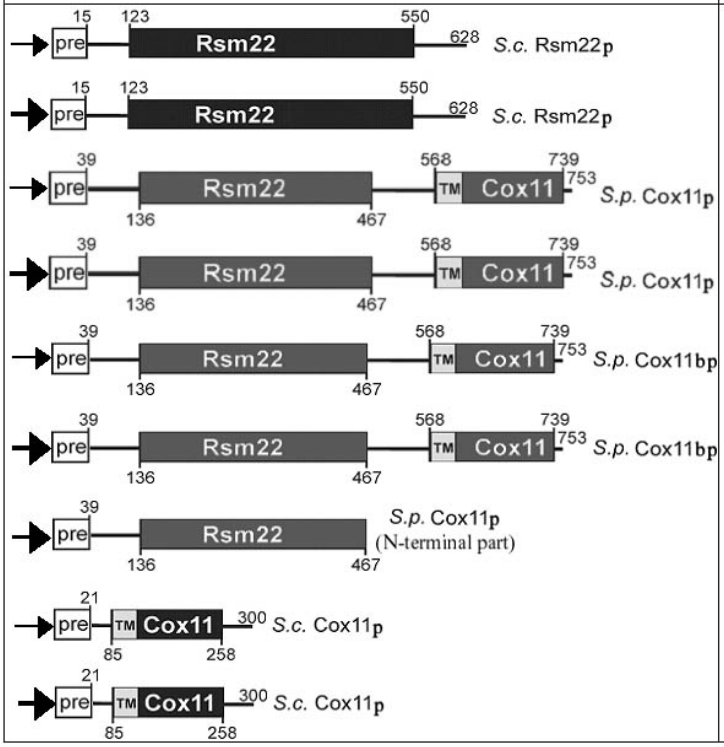
| Construct | Growth on glycerol at | | | *pet*-test |
|---|---|---|---|---|
| | 23°C | 30°C | 37°C | |
|  | - | - | - | - |
| | - | - | - | - |
| | - | - | - | - |
| | - | - | - | - |
| | - | - | - | - |
| | - | - | - | - |
| | - | - | - | - |
| | - | - | - | - |



**Figure 10. Complementation of *S. cerevisiae rsm22*Δ mutant by different constructs.** Schematic view of Rsm22p, Cox11p from different organisms and derivatives. The constructs were expressed in *S. cerevisiae rsm22* knock-out strain Y15005 under control of promoters that provide either moderate (*ADH1*, thin arrows) or high (*GPD1*, thick arrows) level of expression. Respiratory growth of the respective transformants was checked on glycerol-containing media (YPG, YPEG or YPGG) at 23°C, 30°C and 37°C. "+" indicates growth, "-" – its absence. The integrity of mtDNA was checked in *pet*-tests ("-"-integrity is affected).

## 3.3 Biochemical studies of Cox11p

### 3.3.1Topological studies of Cox11p

As outlined in the Introduction, Cox11p has been reported to be firmly associated with the inner mt membrane (Tzagoloff et al., 1990). In line with this observation the structure of the *S. meliloti* Cox11p homologue revealed the presence of a TM domain (Banci et al., 2004). Bioinformatic analysis of ScCox11p predicts a single TM helix at the position of aa 85-105. The method of alkaline extraction (Fujiki et al., 1982, see Material and

Methods) was applied to define whether Cox11p is an integral membrane protein or loosely associated with the membrane. Mitochondria of strain YSC1070-662427 expressing Cox11p-3HA from its authentic promoter were treated with 0.1 M sodium carbonate and separated in a soluble and insoluble protein fraction by centrifugation.. The mt matrix protein aconitase (Aco1p), which served as a soluble control protein, is exclusively present in the supernatant (Fig. 11, middle panel), whereas the integral membrane protein Sco1p was exclusively detected in the pellet fraction (Fig. 11, lower panel). Similarly, Cox11p is detected in the pellet fraction (Fig. 11, upper panel). The presence of a tiny amount of the protein in the soluble fraction has also been reported for the human Cox11p homologue (Leary *et al.*, 2004). These results unequivocally demonstrate that Cox11p is an integral membrane protein.

A Proteinase K protection assay was performed to experimentally prove the predicted $N_{in}$-$C_{out}$ topology (see Introduction). Mitochondria were purified from strain YSC1070-662427, converted to mitoplasts and treated with increasing amounts of Proteinase K as described in Materials and Methods. Even the lowest amount of Proteinase K added (1 µg per 1 mg mt protein) was sufficient to significantly reduce the signal intensity of Cox11p-3HA compared to untreated mitoplasts (Fig. 12A, upper panel). A similar profile was observed for Sco1p which is known to possess an $N_{in}$-$C_{out}$ topology (Krummeck, 1992; Beers et al., 1997) (Fig. 12A, lower panel).

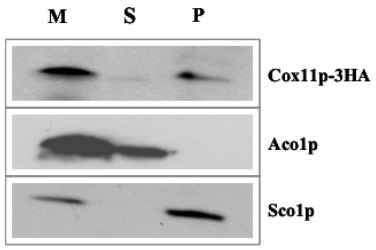



**Figure 11. Cox11p-3HA is an integral membrane protein.** Mitochondria (M) isolated from strain YSC1070-662427 expressing Cox11p-3HA were treated with 0.1 M $Na_2CO_3$. Pellet (P) and supernatant (S) fractions were collected after centrifugation. Western blot analysis was performed with antibodies directed against the HA-epitope, the soluble matrix protein aconitase (Aco1p) and the integral membrane protein Sco1p.

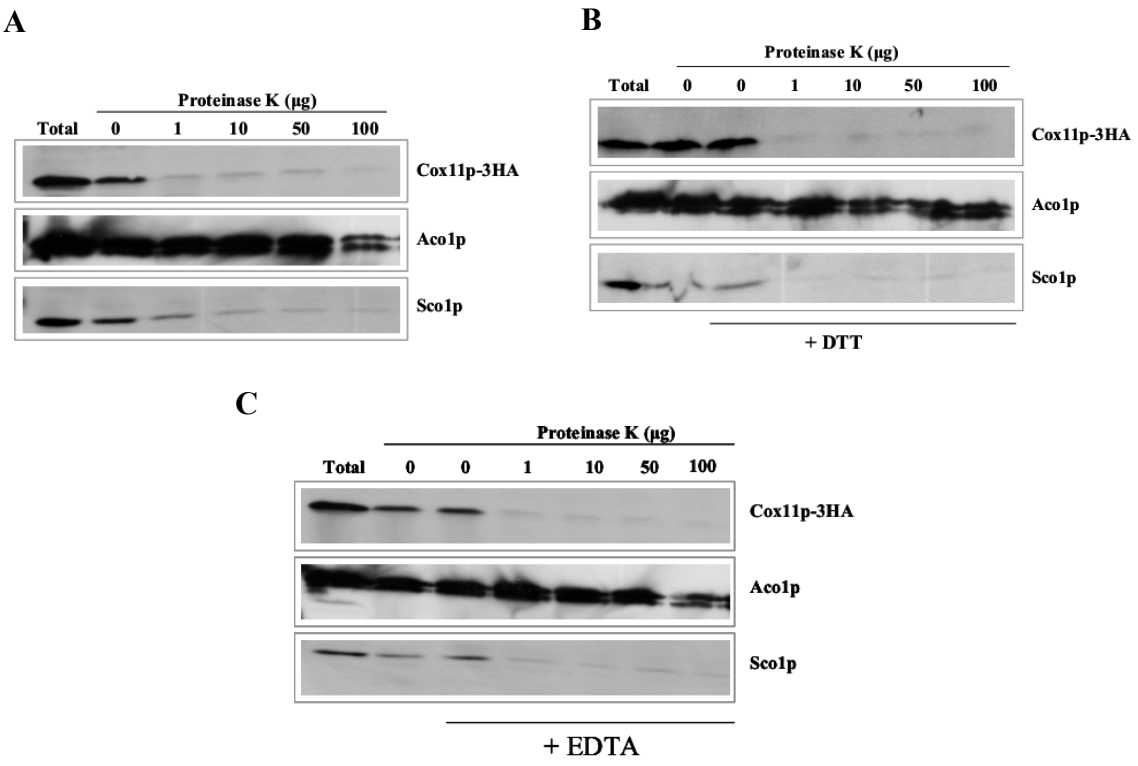



**Figure 12. The C-terminal domain of Cox11p-3HA is exposed to the IMS.** Mitochondria isolated from strain YSC1070-662427 expressing Cox11p-3HA were subjected to hypo-osmotic treatment and then treated with increasing amounts of Proteinase K (A). In addition the same treatment was applied in the presence of 100 mM DTT (B) or 20 mM EDTA (C). Aliquots were subjected to SDS-PAGE and analyzed by Western blot using antibodies directed against HA-epitope, the matrix protein aconitase (Aco1p) and the integral membrane protein Sco1p.

In contrast, aconitase (Aco1p) remained protected even in the presence of up to 100 µg per 1 mg mt protein of Proteinase K (Fig. 12A, middle panel). These results prove the $N_{in}$-$C_{out}$ topology of Cox11p and show that the C-terminal copper-binding site is exposed to the IMS.

In order to clarify whether the accessibility for Proteinase K of Cox11p-3HA is influenced by bound copper the Proteinase K protection assay was performed in the presence of either 100 mM DTT or 20 mM EDTA. The results were identical to that obtained in the absence of DTT (Fig. 12B) or EDTA (Fig. 12C). Thus Proteinase K sensitivity of Cox11p-3HA is not affected by the bound copper.

### 3.3.2 Cox11p co-fractionates with mitochondrial ribosomes in sucrose gradients

The mechanism of how Cox11p can transfer the Cu(I) ion into the $Cu_B$ site of COX, which is buried within the IMM, is not clear. As mentioned in the Introduction, the fusion of Cox11p in *S. pombe* with a protein bearing significant homology to *S. cerevisiae* Rsm22p (Saveanu et al., 2001, Carr et al., 2002) may hint at a link between the translation of Cox1p and its loading with copper by Cox11p (Sali, 1999, Carr and Winge, 2003). To clarify the potential association of ScCox11p with the mt protein-synthesizing machinery we separated mt ribosomes by ultra-centrifugation in continuous sucrose gradients and tested Cox11p for co-sedimentation with mitoribosomal proteins. Mitochondria were isolated from strain Y06479 expressing Cox11p-3HA, lysed with digitonin in the presence of 10 mM $MgSO_4$ (thus favouring the assembled forms of ribosomes), and the cleared lysate was subjected to high velocity centrifugation in a sucrose gradient. 270 µl fractions of the gradient were analysed by measuring the absorption at 260 nm and by imunoblotting (Fig. 13 A). The distribution of mitoribosomes was followed by detection of the small mitoribosomal subunit protein Pet123p (McMullin et al., 1990) and of the large mitoribosomal subunit protein MrpL36p (Williams et al., 2004). The distribution of the matrix protein Aco1p and of the membrane protein Cox2p served as controls (Fig.13 A).

Aconitase (Aco1p) was mainly detected in the low density top fractions, whereas the membrane protein Cox2p was mainly present in fractions of higher density. The highest concentration of Cox2p was observed in fraction 5, which may reflect the assembled COX. MrpL36p and Pet123p were detected in fractions of low and intermediate density (fractions 2-4, 7-9) as well as of the highest density (fractions 16-18). The distribution probably reflects the free proteins (top fractions) as well as disassembled (intermediate fractions) and assembled ribosomes (bottom fractions), indicating that the conditions used do not allow to clearly differentiate between fully assembled ribosomes and the subunits.

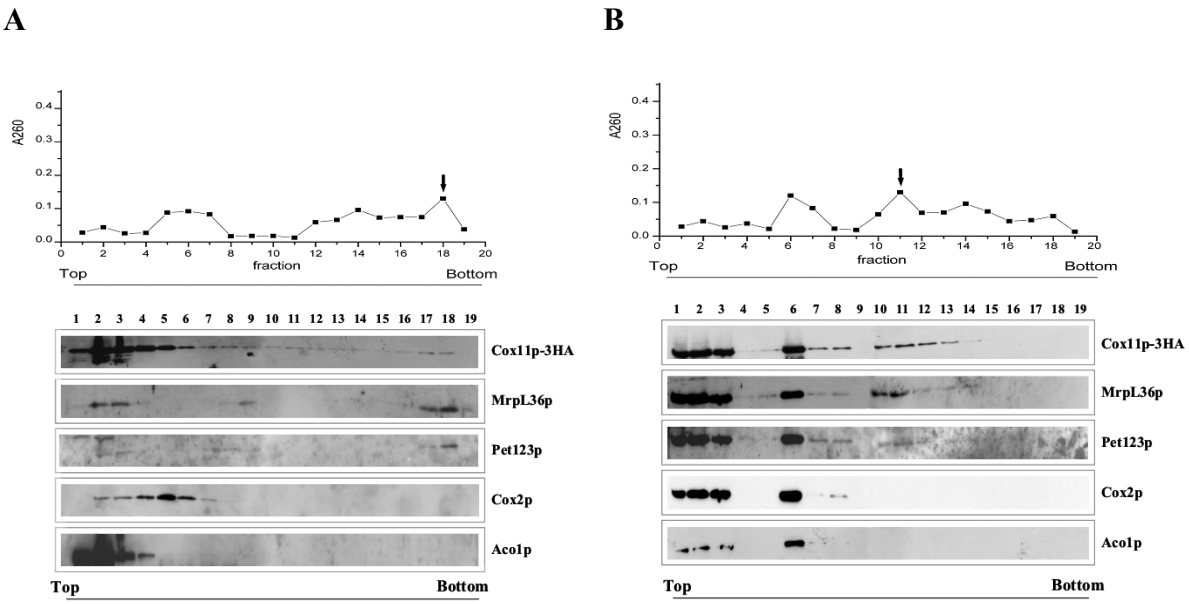



**Figure 13. Cox11p-3HA partly co-sediments with mt ribosomes in sucrose gradients.** Mitochondria isolated from the *cox11*Δ strain Y06479 expressing Cox11p-3HA were lysed with 1% digitonin in the presence of 20 mM $MgSO_4$. The cleared extract was loaded onto a continuous 20-40% sucrose gradient. After centrifugation for either 16 h (A) or 1 h (B) 19 aliquot fractions were collected, precipitated with TCA and subjected to SDS-PAGE. The distribution of Cox11p-3HA, the mitoribosomal proteins MrpL36 and Pet123p, as well as Aco1p and Cox2p was analysed by Western blot. An aliquot from each fraction was used to determine the absorption at 260 nm. Fractions representing mitoribosomes are marked by arrows.

The distribution of Cox11p-3HA was very similar to that of MrpL36p, with the highest concentration present in the top fractions and a significant portion in the bottom fractions. As this profile differs from that observed for Cox2p, it can be excluded that the presence of Cox11p in the high density fractions is not due to contamination by membrane fragments.

In order to prove that the observed distribution is not due to the protein aggregation we modified the gradient conditions by shortening the time of centrifugation to 1 h (Fig. 13 B). Again, the distribution of Cox11p-3HA was similar to that one of MrpL36p and Pet123p (Fig. 13 B). The accumulation of the various proteins in fraction 6 might result from membrane contamination in this preparation.

The finding of a fraction of Cox11p co-migrating with mitoribosomes suggests a direct or indirect interaction of the copper chaperone with the translation machinery.

### 3.3.3 Co-immunoprecipitation of Cox11p and MrpL36p

To test the interaction of Cox11p with mt ribosomes we performed a co-immunoprecipitation experiment with mt lysate from strain Y06479 expressing Cox11p-3HA. Immunoprecipitation was performed with the HA-specific antibodies covalently bound to agarose beads. These antibodies recognize no proteins in the mt lysate of the parental strain that does not express Cox11p-3HA (data not shown). The result of the immunoprecipitation is presented in Figure 14.

Cox11p-3HA can be detected in the lysate, unbound material after incubation with the matrix and in the precipitated fraction (upper panel, lanes 2,3 and 7), but not in the negative control (non-incubated beads, lane 1). Antibodies directed against aconitase (Aco1p), Cox2p and Cox3p were used as controls. Apparently none of these proteins interacts with Cox11p, because in no case a signal could be detected in lane 7. Incubation with antibodies directed against MrpL36p, however, yielded a faint signal of the respective molecular weight (20 kDa) in lane 7. A stronger signal was obtained after overnight incubation (Fig.14, right panel). The detection of Pet123p yielded an extremely weak signal detectable only upon long-time exposure (data not shown). The result is in favour of the proposal that Cox11p interacts directly or indirectly with the mt protein synthesis machinery.

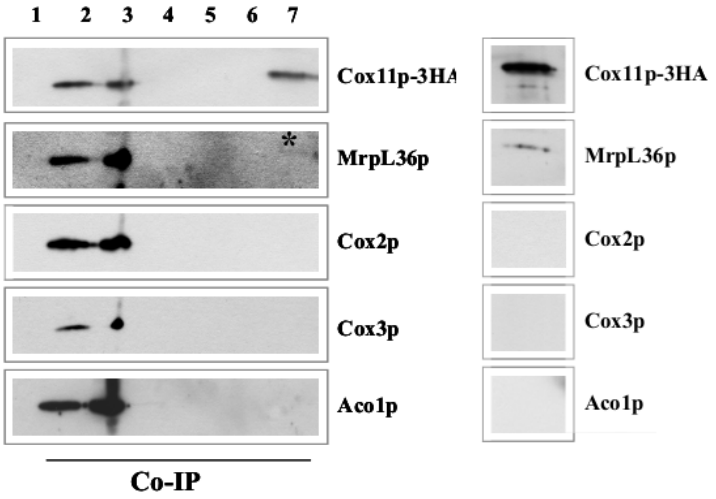



**Figure 14. Co-immunoprecipitation of Cox11p with mitoribosomal proteins.** Mitochondrial lysate of strain Y06479 expressing Cox11p-3HA was incubated with Anti-HA Affinity Matrix (AM) for 1 h (left) or overnight (right), and analyzed in a Western blot with antibodies against the HA-epitope, MrpL136p, Aco1p, Cox2p and Cox3p. Lane 1, non-incubated AM (negative control); lane 2, mitochondrial lysate applied to AM; lane 3, unbound material; lanes 4-6, washing steps; lane 7, immunoprecipitated proteins. The faint protein band in panel 2 corresponding to MrpL36p is marked by an asterisk.

### 3.3.4 Cox11p is associated with mt ribosomes

Despite of the observed interaction of Cox11p with the large mitoribosomal subunit protein MrpL36p, proof for an interaction with the entire mitoribosome is missing. To clarify this issue we applied a TAP procedure (Rigaut et al., 1999). Unfortunately, fusion of the TAP-tag to Cox11p interferes with the protein´s function. Instead Cox11p-3HA was expressed in strain SC1069 carrying TAP-tagged version of Mrp4p, a constituent of the small mitoribosomal subunit (Davis et al. 1992; Gan et al. 2002). Isolated mitochondria were lysed with digitonin under conditions favoring the assembled forms of mitoribosomes, and cleared lysate was used for subsequent affinity purification steps on IgG and Calmodulin (Cal) resins. Mrp4p-TAP is detectable in the lysate and after the first step of purification (IgG beads), but not in the last step (calmodulin beads) due to processing of the TAP-tag (Fig. 15A). Detection of MrpL36p and Pet123p, a constituent of small ribosomal subunit (McMullin et al., 1990) in the final eluate (Cal) indicates the

successful purification of the mitoribosomes. The failure to detect Cox2p in the IgG and Cal fractions excludes contamination by membrane fragments. Part of Cox11p-3HA can be detected in eluate (Fig. 15A), similar to Oxa1p that is known to be associated with mitoribosomes (Szyrach et al., 2003).

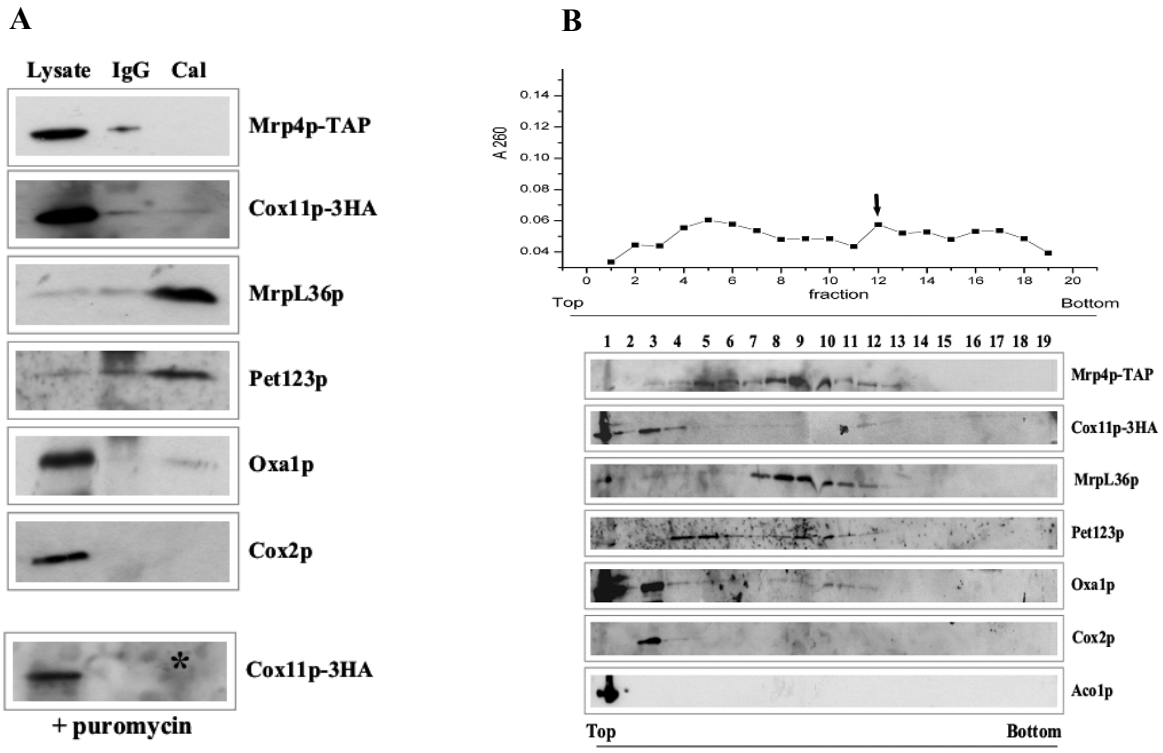



**Figure 15. Cox11p-3HA co-purifies with mitoribosomes.** (A) Mitochondria isolated from strain SC1069 co-expressing Mrp4p-TAP and Cox11p-3HA were lysed with 1% digitonin in the presence of 30 mM $MgSO_4$. The cleared extract (lane 1, 2% of total) was first incubated with the IgG matrix and afterwards with calmodulin matrix. Part of the lysate was subjected to the purification in the presence of 400 µM puromycin (lower panel) yielding a faint band in the Cal eluate (marked with the asterisk). Proteins bound to the IgG (lane 2, 10% of total) and calmodulin (lane 3) resins were analyzed by Western blot with antibodies directed against TAP-tag, HA-epitope, MrpL36p, Pet123p, Oxa1p and Cox2p. (B) Mitochondria isolated from strain SC1069 co-expressing Mrp4p-TAP and Cox11p-3HA were lysed with 1% digitonin in the presence of 20 mM $MgSO_4$. The cleared extract was loaded onto a continuous 20-40% sucrose gradient. After centrifugation for 4 h 19 aliquot fractions were collected and analysed as described in Fig. 13.

Next the effect of puromycin on the association of Cox11p with the mitoribosomes was analysed. This antibiotic causes release of the nascent polypeptide chains from the ribosomes. Therefore, a TAP with Mrp4p-TAP as a bait was performed in the presence of 400 μM puromycin. Interestingly, part of Cox11p-3HA remains associated with mitoribosomes (Fig. 15A, lower panel). The partial loss of Cox11p-3HA in the Cal eluate may hint that Cox11p is not permanently and directly associated with mitoribosomes, but rather associates with the components that are transiently bound to the mt ribosome during the translation process.

The results of the Co-IP study were confirmed by sucrose gradient centrifugation of the mt lysate from the strain SC1069 expressing Cox11p-3HA (Fig. 15B). The distribution of Cox11p-3HA as well as the control proteins is very similar to that obtained in the experiments shown in Fig. 13.

### 3.3.5 Cox11p is present in high molecular weight complexes

Recently Krause-Buchholz et al. (2004) described a modification of the blue-native (BN)-PAGE conditions, where mitoribosomes can be resolved *in toto* as high molecular weight complexes. We applied this method to gain a further line of evidence for the association of Cox11p with mt ribosomes. Mitochondria of strain Y06479 expressing Cox11p-3HA was lysed in 1% digitonin and the cleared lysate was subjected to the BN-PAGE. Taking into account the observation that the complexes with the molecular weight higher than 1,500 kDa are not able to enter the separating gel (Nijtmans et al. 2002), both the stacking and separating gel was used for the Western blot analysis.

Cox3p (or Cox2p) that is detected in two subpopulations reflecting assembled COX (around 400 kDa) and COX supracomplex (in the range of 1,000-1,300 kDa), respectively, as well as Atp2p, detected in the two distinct forms corresponding to the monomer (500 kDa) and dimer (1,000 kDa) of ATPase complex were used as molecular weight markers (Fig 16 A). Antibodies directed against MrpL36p and Pet123p revealed that the mitoribosomal proteins are present in high molecular weight complexes of 1,300 kDa and more, possibly reflecting assembled ribosomes. Detection of Cox11p-3HA

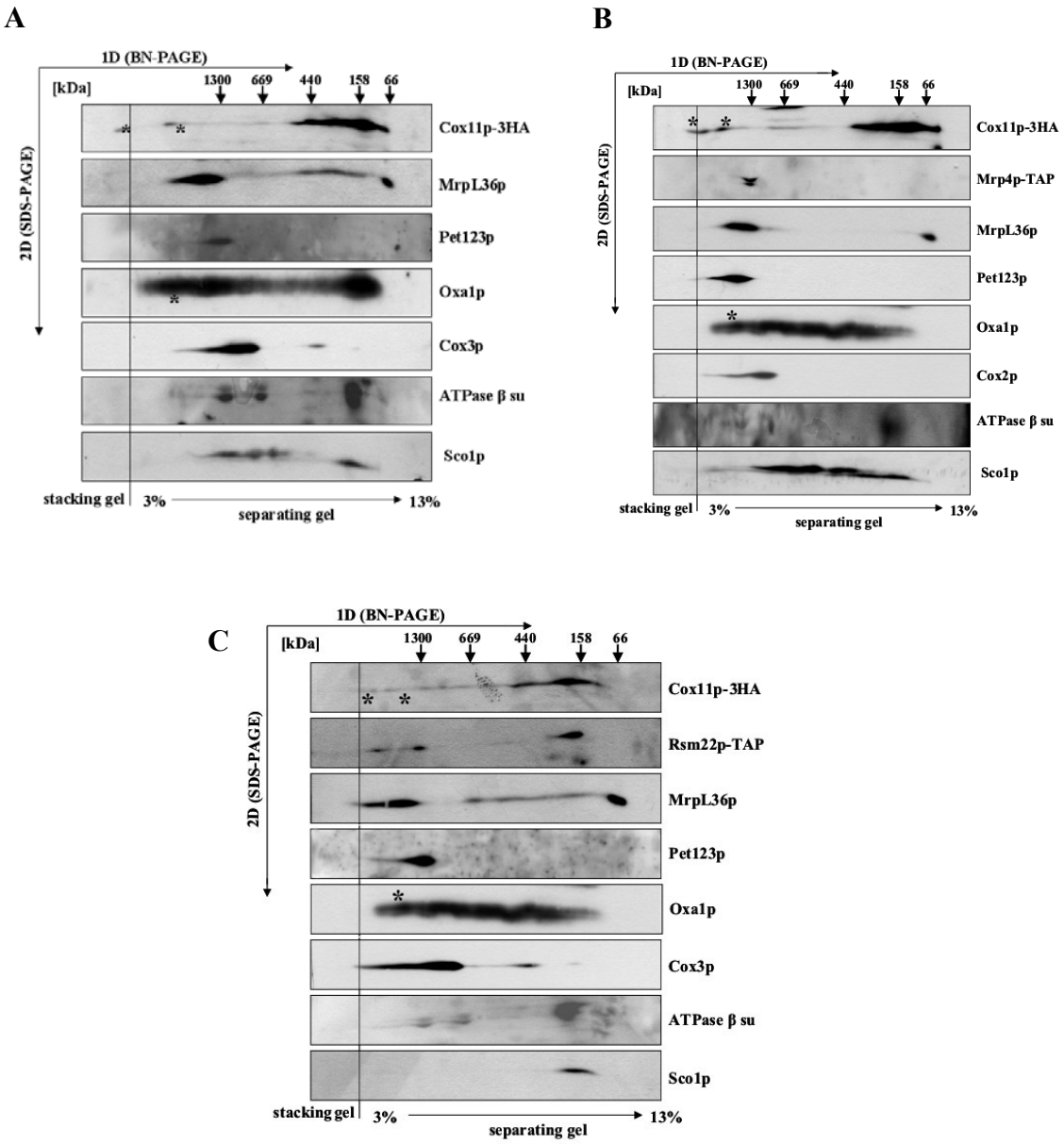



**Figure 16. Part of Cox11p is detected in high molecular weight complexes.** Mitochondria isolated from strain Y06479 expressing Cox11p-3HA (A), strain SC1069 co-expressing Mrp4-TAP and Cox11p-3HA (B), or strain YSC1178-7501494 expressing Cox11p-3HA (C) were lysed and analyzed by BN-PAGE at conditions maintaining the assembled forms of mitoribosomes. Western blot analysis was performed using the antibodies raised against HA-epitope, TAP-tag, MrpL36p, Pet123, Oxa1p, Cox2p or Cox3p, *E. coli* Atp5p (cross-reacts with yeast Atp2p), and Sco1p.

demonstrates a continuous tale-shaped signal with relatively high intensity in the range similar to that of the mitoribosomal subunits. Notably Oxa1p can also be detected in complexes of similar sizes (Fig.16 A, marked by the asterisks). Interestingly, Sco1p also migrates in high molecular weight complexes, however their size is smaller, compared to these ones observed for Cox11p.

Similar results were obtained with mitochondria isolated from strains SC1069 and YSC1178-7501494, respectively, both expressing Cox11p-3HA (Fig. 16 B, C). These results confirm the finding that Cox11p is associated with mitoribosomes similarly to the Oxa1p.

### 3.3.6 *S. cerevisiae* Cox11p is processed during its import into mitochondria

Most proteins that are imported into mt undergo a proteolytic processing step that removes the N-terminal targeting sequence. To test whether Cox11p is processed during its import an *in vitro* import analysis was performed. Radiolabeled Cox11p was produced by *in vitro* translation of RNA-transcripts that were generated by *in vitro* transcription of expression plasmids pGEM3 or pGEM4 carrying *COX11* ORF as an insert. Cox11p was added to MPP buffer with (+) or without (-) recombinant *S. cerevisiae* MPP. Upon incubation with MPP two bands of about 28 kDa and 5 kDa appear, probably reflecting the mature form and the processed presequence of Cox11p (Fig. 17, left panel). Processing of radiolabeled Oxa1p, a well studied process, was used to control the MPP activity (Fig. 17, right panel). As expected the Oxa1p precursor was processed in the presence of MPP thereby yielding the mature form of Oxa1p with a molecular weight of about 40 kDa.

Radiolabeled Cox11p precursor was also incubated with mt isolated from strain W303-1A as described in Materials and Methods, and the samples were taken at different time points for analysis. Part of the samples was treated with Proteinase K (PK) in order to remove non-imported protein, and the other part was converted to mitoplasts and treated with PK in order to degrade IMS-located or IMS-exposed proteins. Clearly a PK-resistant band corresponding to the mature form of Cox11p (around 28 kDa) can be detected, that does not appear when import is blocked by the addition of 2 µM valinomycin (Fig. 18). The lower bands probably represent degradation products that appear due to

compromised integrity of mitochondria during the import. When the mitochondria are swollen and IMS becomes accessible for PK, the band corresponding to the mature form of Cox11p is degraded, as expected from the topological studies (see section 3.3.1). Interestingly, in the course of the membrane potential-dependent import an additional band of slightly higher molecular weight appears that might represent an import intermediate. This band can also be seen upon treatment with PK suggesting that this protein has entered the mt matrix.

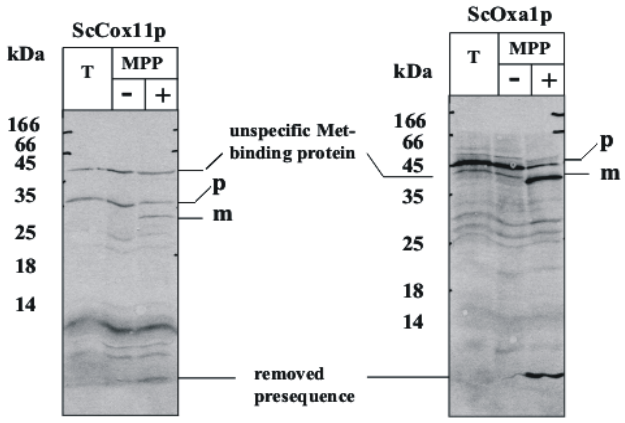



**Figure 17. Cox11p is processed by the recombinant MPP similarly to Oxa1p.** Radiolabeled lysates (total 40%, T) containing either Cox11p (left panel) or Oxa1p (right panel) precursor proteins were incubated with purified recombinant MPP expressed in *E. coli* cells. After incubation samples were resolved by SDS-PAGE and analysed by autoradiography.

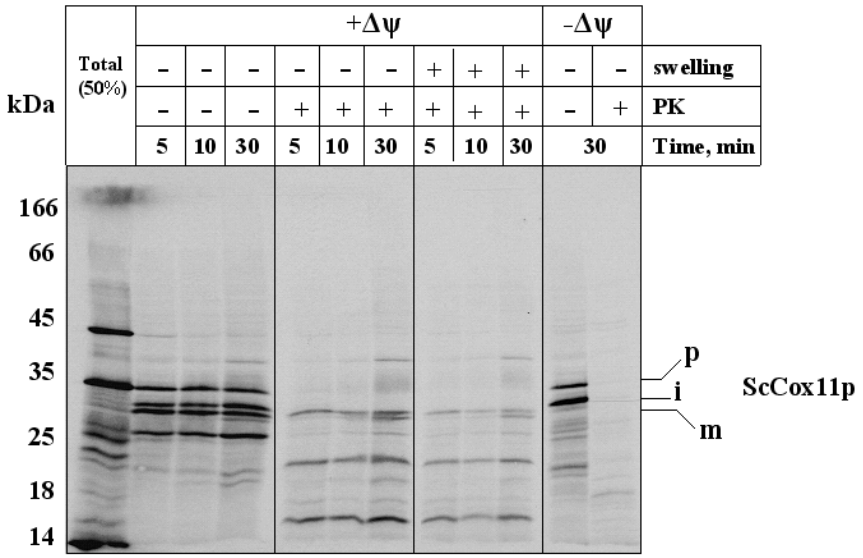



**Figure 18. Cox11p is processed during import into mitochondria.** Radiolabeled lysate (50% of total) containing Cox11p precursor protein was incubated with isolated mitochondria from *S. cerevisiae* strain W303-1A as described in Material and Methods. Samples were taken after 5, 10 and 30 min., treated with PK and analysed by SDS-PAGE and autoradiography.

## 3.4 Studies on modified forms of Cox11p

### 3.4.1 Replacement of the N-terminal part of Cox11p

The N-terminal part of Cox11p possesses a highly charged amino acid stretch adjacent to the TM helix (Fig. 19 A). Such stretches have been reported previously to participate in protein-protein interactions, particularly with mitoribosomes (Szyrach et al., 2003). To test whether this is also true for Cox11p, the N-terminal moiety of Cox11p including the TM domain was replaced by the respective Sco1p counterpart (Fig. 19 B). The resulting construct was placed under control of the *ADH1* promoter and transformed into strain Y06479. The *cox11*Δ transformants that express this chimeric protein (Sco1N-Cox11Cp-3HA) are respiratory deficient at all temperatures tested (23°C, 30°C and 37°C), demonstrating that the N-terminal part of Cox11p is important for function (Fig. 19 C and data not shown). To check whether the inability of Sco1N-Cox11Cp-3HA to complement the *cox11*Δ mutation might be due to defective mt import we analyzed the intracellular distribution of Sco1N-Cox11Cp-3HA. The protein is predominantly present in mitochondria and not in the cytosolic fraction, thus excluding that mislocalization can be

the reason for the inability to complement (Fig. 19 D). The presence of the protein in the mt membrane was assessed by alkaline extraction. Aco1p, that served as a soluble control protein, is exclusively present in the supernatant (Fig. 19 E, middle panel), whereas the integral membrane protein Sco1p is exclusively detected in the pellet fraction (Fig. 19 E, lower panel). Similarly, Sco1N-Cox11Cp-3HA is present in the pellet fraction (Fig. 19 E, upper panel), demonstrating that the N-terminally modified Cox11p is an integral mt membrane protein.

Results of sucrose gradient centrifugation show that the distribution profile of the Sco1N-Cox11Cp-3HA differs from that of the native Cox11p in that it does not co-migrate with mitoribosomes. The distribution profile of mitoribosomal proteins (MrpL36p and Pet123p), Oxa1p, Cox2p and Aco1p is similar to that obtained in a previous experiments (Fig. 20 A).

In order to get further evidence for the inability of Sco1N-Cox11Cp-3HA to associate with mitoribosomes the TAP procedure was performed using strain SC1069 expressing Sco1N-Cox11Cp-3HA. In contrast to Cox11p, Sco1N-Cox11Cp-3HA can not be detected in final eluate (Fig. 20 B), while the Mrp4-TAP, Mrpl36p, Pet123p, Cox2p and Oxa1p demonstrate the same distribution as in TAP procedures described above.
Thus, either the N-terminal part, or the TM domain, of Cox11p, or both are important for its function. These results are in line with the idea that the N-terminal part of Cox11p, including TM segment, defines a site which is crucial for the observed association with mitoribosomes.

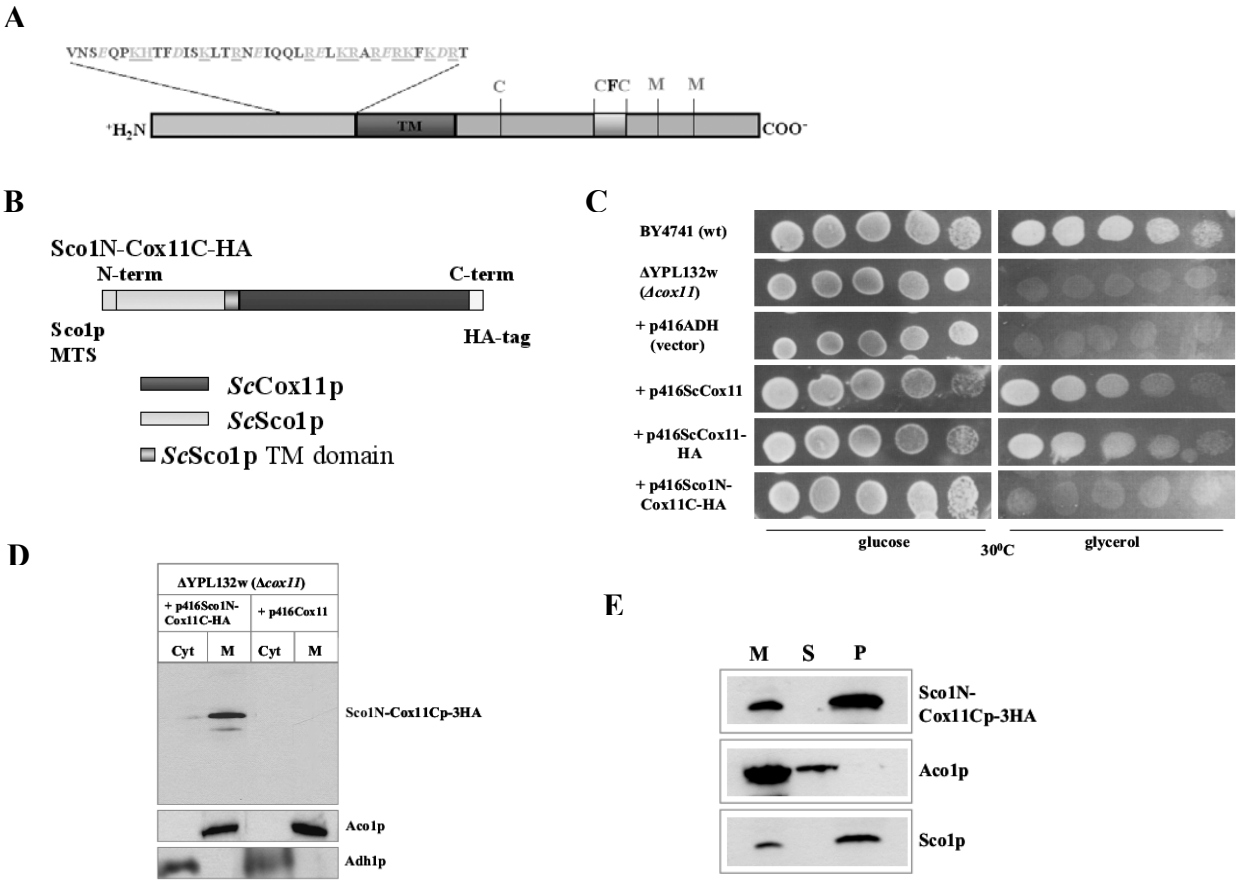



**Figure 19. Replacement of the N-terminal moiety (including TM domain) of Cox11p.** (A) Schematic view of yeast Cox11p. Conserved aa residues involved in copper binding are shown in grey. Positively (underlined) or negatively (italics) charged residues in the stretch adjacent to the TM helix are shown in light grey. (B) Schematic view of the N-terminally modified Cox11p. (C) Complementation analysis of the *cox11* mutant. To assess the growth on variable carbon sources, cells from the *cox11*Δ strain Y06479 harboring the respective plasmids were diluted to equal cell density and serial dilutions were plated on selective media containing glucose or glycerol. Growth was monitored after 3 days at 30°C. (D) Mitochondria (M) and cytosolic (Cyt) fractions were isolated from the strain Y06479 expressing either Sco1N-Cox11Cp-3HA or Cox11p, subjected to SDS-PAGE and analyzed by Western blot with antibodies raised against the HA-epitope, the mt protein Aco1p and the cytosolic protein Adh1p. (E) Mitochondria (M) isolated from strain Y06479 expressing Sco1N-Cox11Cp-3HA were treated with $Na_2CO_3$. Pellet (P) and supernatant (S) fractions were collected, precipitated with TCA and resolved by SDS-PAGE. Western blot analysis was performed using antibodies directed against the HA-epitope of Sco1N-Cox11Cp-3HA, the soluble matrix protein Aco1p and the integral membrane protein Sco1p.

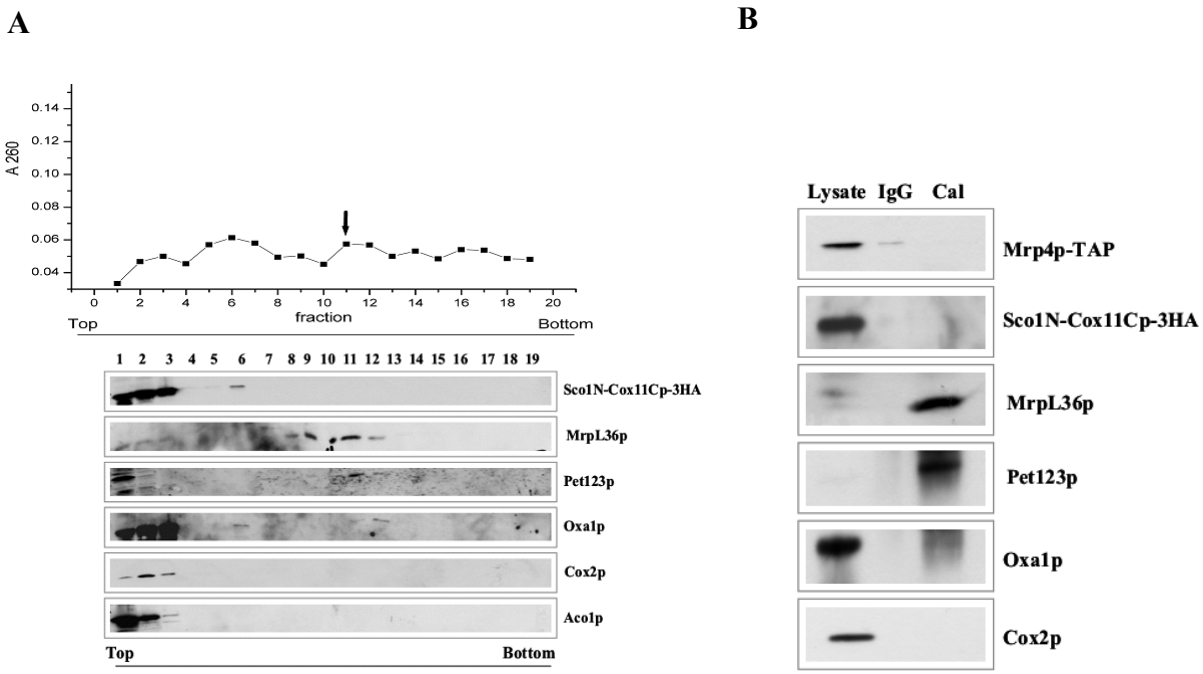



**Figure 20. Sco1N-Cox11Cp-3HA does not associate with mitoribosomes.** (A) Mitochondria isolated from the *cox11*Δ strain Y06479 expressing Sco1N-Cox11Cp-3HA were lysed and applied to a continuous sucrose gradient as described above. Following centrifugation for 4 h at 148.000 x *g*, 19 aliquot fractions were collected and analysed as described in Fig. 13. (B) Mitochondria isolated from strain SC1069 co-expressing Mrp4p-TAP and Sco1N-Cox11Cp-3HA. were lysed and subjected to the TAP procedure as described above. The cleared extract (lane 1, 2% of total), proteins bound to the IgG (lane 2, 10% of total) and calmodulin (lane 3) resins were analyzed by Western blot with antibodies directed against TAP-tag, HA-epitope, MrpL36p, Pet123p, Oxa1p and Cox2p.

Distribution of Sco1N-Cox11Cp-3HA was also analyzed by BN-PAGE and subsequent Western blot. The distribution of Cox2p, Atp2p, Mrpl36p, Pet123p and Oxa1p is similar to that observed in previous BN-PAGEs. Surprisingly, part of Sco1N-Cox11Cp-3HA is still detected in high molecular weight complexes of sizes comparable with mitoribosomes (Fig. 21). This unexpected result may be explained by the finding that the calculated pI values for the replaced charged stretch adjacent to the TM domain of Cox11p and the replacing Sco1p counterpart are both very basic and almost identical (Fig. 21, right block). This issue will be discussed more detailed in the Discussion chapter.

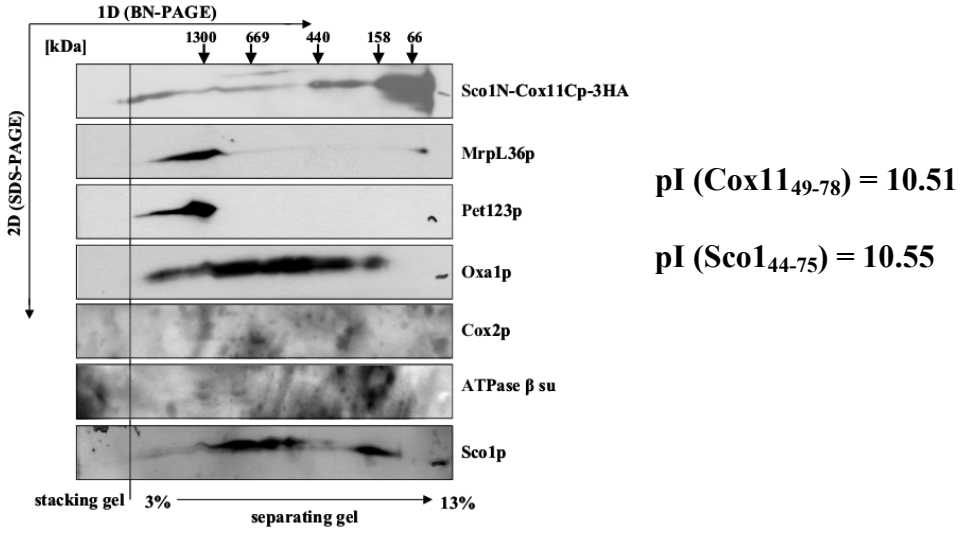



**Figure 21. Sco1N-Cox11Cp-3HA is present in high molecular weight complexes.** Mitochondria isolated from strain Y06479 expressing Sco1N-Cox11Cp-3HA were lysed and analyzed by BN-PAGE under conditions favoring the assembled forms of mitoribosomes. Western blot analysis was performed as described in Fig. 16. Calculated pI values for the charged stretches adjacent to the TM of Cox11p and Sco1p, resepctively, are shown on the right hand side.

### 3.4.2. The N-terminal part of Cox11p excluding the TM is dispensable for function and association with ribosomes.

As outlined above, the N-terminal part of Cox11p possesses a highly charged stretch of aa adjacent to the TM domain. Interestingly, BLAST analysis reveals that this stretch includes a motif which is highly similar to a sequence in a probable translation elongation factor of *S. pombe* (Fig. 22, right panel). A similar conserved motif has been reported recently to define a site of interaction of the two signal recognition particles (SRPs), cpSRP43 and cpSRP45, in *Arabidopsis thaliana* chloroplasts (Funke et al., 2005). However, Carr et al. (2005) showed that a large portion of the Cox11p N-terminal part (residues 55-75) can be replaced by the respective Sco1p part without affecting Cox11p function. To clarify which part of Cox11p is required for association with ribosomes a set of Cox11p derivatives with different modifications in the N-terminal part was created (Fig. 22, left panel). Cox11$_{\Delta 59-78}$p-5Myc and Cox11$_{\Delta 49-78}$p-5Myc contain deletions of 19 and 29 aa, respectively, of the charged stretch of the matrix-protruding portion of the

protein. Surprisingly, *cox11*Δ transformants expressing Cox11$_{\Delta 59\text{-}78}$p-5Myc or Cox11$_{\Delta 49\text{-}78}$p-5Myc are respiratory competent, albeit their growth on non-fermentable carbon sources is less efficient compared to that of transformants expressing Cox11p-5Myc (Fig. 23). This observation holds true for all temperatures tested (23°C, 30°C and 37°C) (data not shown).

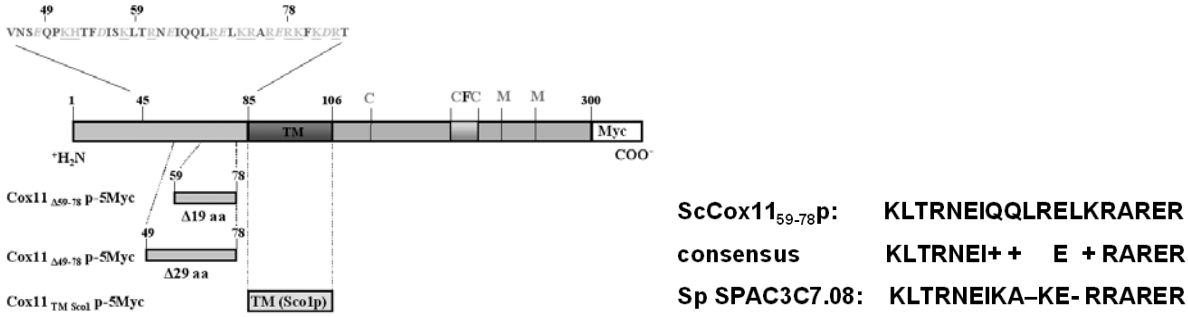



**Figure 22. Modular structure of *S. cerevisiae* Cox11p and its modifications.** Scheme of yeast Cox11p. The copper-binding motif CFC as well as conserved aa residues in the C-terminal part of Myc-tagged Cox11p are indicated. Position and sequence of a charged aa stretch adjacent to the TM helix are shown. Positively charged residues are underlined, negatively charged aa are shown in italics. Sequences that were either deleted (Δ19 aa or Δ29 aa) or replaced (TM(Sco1p)) are given in the lower part. Consensus sequence revealed by BLAST alignment of the charged stretch of ScCox11p and the homologous sequence of the *S. pombe* ORF SPAC 3C7.08 is given in the right part.

Transformants expressing the mutant proteins were grown on supplemented raffinose minimal medium and used for preparation of mitochondria. Both Cox11p mutant proteins are efficiently imported into mitochondria (Fig. 24 A, 25 A) and - as shown by their resistance to carbonate extraction - inserted into the mt membrane. Similarly, both Cox11$_{\Delta 59\text{-}78}$p-5Myc and Cox11$_{\Delta 49\text{-}78}$p-5Myc are present in the pellet fraction, demonstrating that the deletions in the N-terminal part have no effect on membrane anchoring (Fig. 24 B, 25 B). Next, a co-sedimentation analysis using mitochondria prepared from strain Y06479 expressing Cox11$_{\Delta 59\text{-}78}$p-5Myc or Cox11$_{\Delta 49\text{-}78}$p-5Myc, respectively, was performed. Mitochondria were lysed with digitonin, and the lysate was applied onto a continuous sucrose gradient and fractionated upon high velocity

centrifugation. The distribution of Cox11$_{\Delta 59\text{-}78}$p-5Myc, Cox11$_{\Delta 49\text{-}78}$p-5Myc, MrpL36p, Pet123p, Oxa1p, Cox2p and Aco1p was assessed by immunoblotting (Fig. 24 C, 25 C).

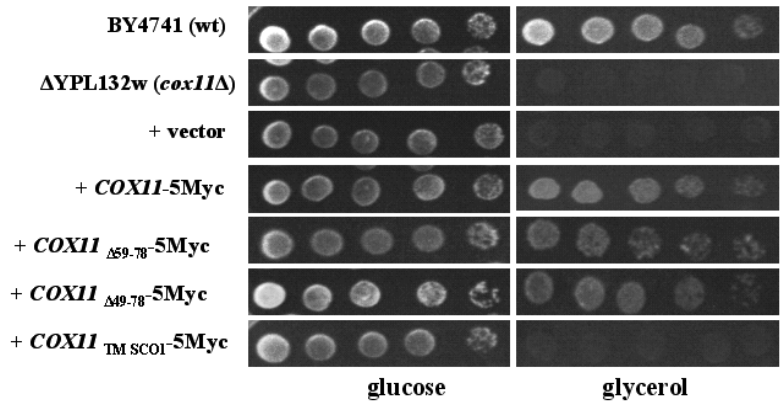



**Figure 23. Complementation analysis of *S. cerevisiae cox11*Δ mutant.** Cells of the *cox11*Δ strain Y06479 bearing an empty vector or the indicated plasmids were serially diluted and growth was followed for 3 days on selective media containing glucose or glycerol as the sole carbon source. Cells of wild-type BY4147 and of untransformed *cox11*Δ strain Y06479 were used as positive and negative controls, respectively.

The results obtained were in line with the previously reported data. Aco1p is exclusively present in the top fractions of low density, whereas Cox2p is present in the top fractions of low and the higher density. MrpL36p and Pet123p are detected in the upper fractions of low density as well as in the lower high-density fractions. Such a distribution is in agreement with the A 260 peaks. The distribution of Oxa1p and of the modified forms of Cox11p is very similar to that of MrpL36p and Pet123p, with the majority of the protein present in the top fractions and a significant portion in the bottom fractions. Thus, the N-terminal part of Cox11p appears to be dispensable for Cox11p function and its association with mitoribosomes.

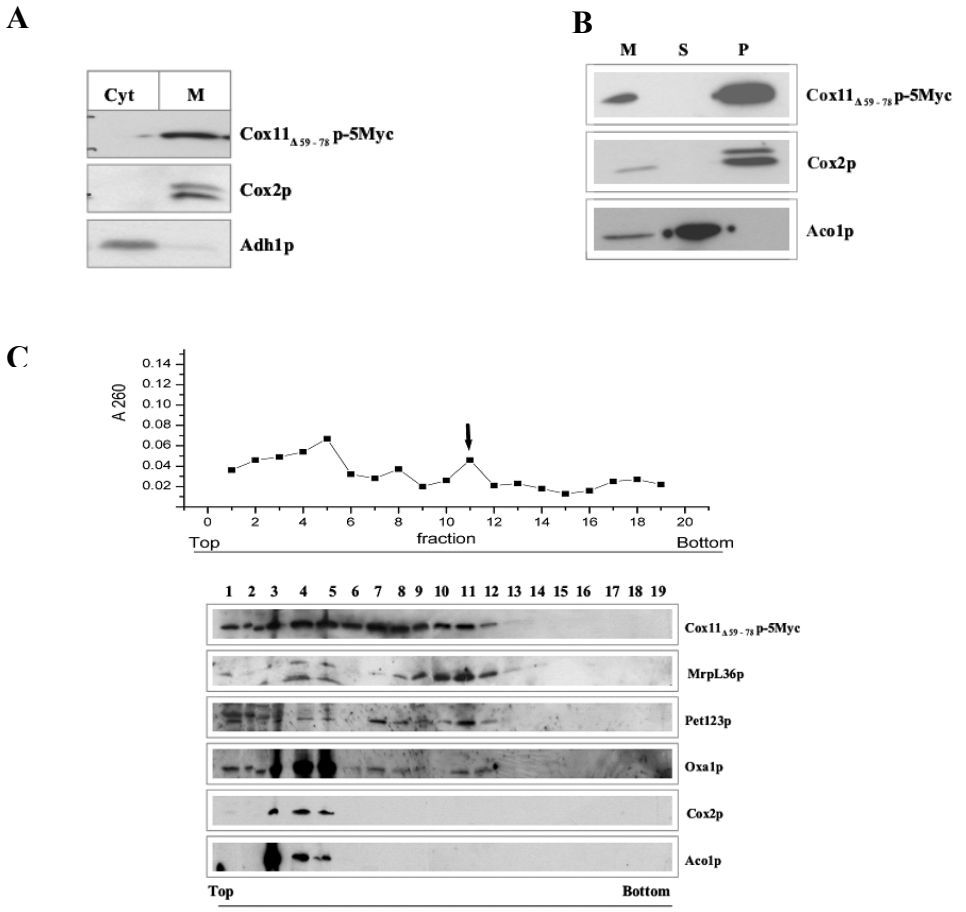



**Figure 24. Cox11$_{\Delta 59\text{-}78}$p-5Myc is localized to the mt membrane and co-sediments with mitoribosomes.** (A) Mitochondrial (M) and cytosolic (Cyt) fractions were isolated from the *cox11*Δ strain Y06479 expressing Cox11$_{\Delta 59\text{-}78}$p-5Myc, subjected to SDS-PAGE and analyzed by Western blot with antibodies against the Myc-epitope, the mt protein Cox2p and the cytosolic protein Adh1p. (B) The mt preparations were treated with 0.1 M $Na_2CO_3$. Pellet (P) and supernatant (S) fractions were collected, precipitated with TCA and resolved by SDS-PAGE. Western blot analysis was performed using antibodies against the Myc-epitope, the soluble matrix protein Aco1p and Cox2p. (C) Clarified mt lysates were applied onto a continuous 20-40% sucrose gradient and ultracentrifuged. Aliquot fractions of 270 µl were collected, precipitated with TCA and analyzed by immunoblotting using the antibodies against Myc-epitope, Oxa1p, MrpL36p, Pet123p, Cox2p and Aco1p. An aliquot of each fraction was used to determine the absorbance at 260 nm. The peak corresponding to the assembled forms of mitoribosomes is marked by an arrow.

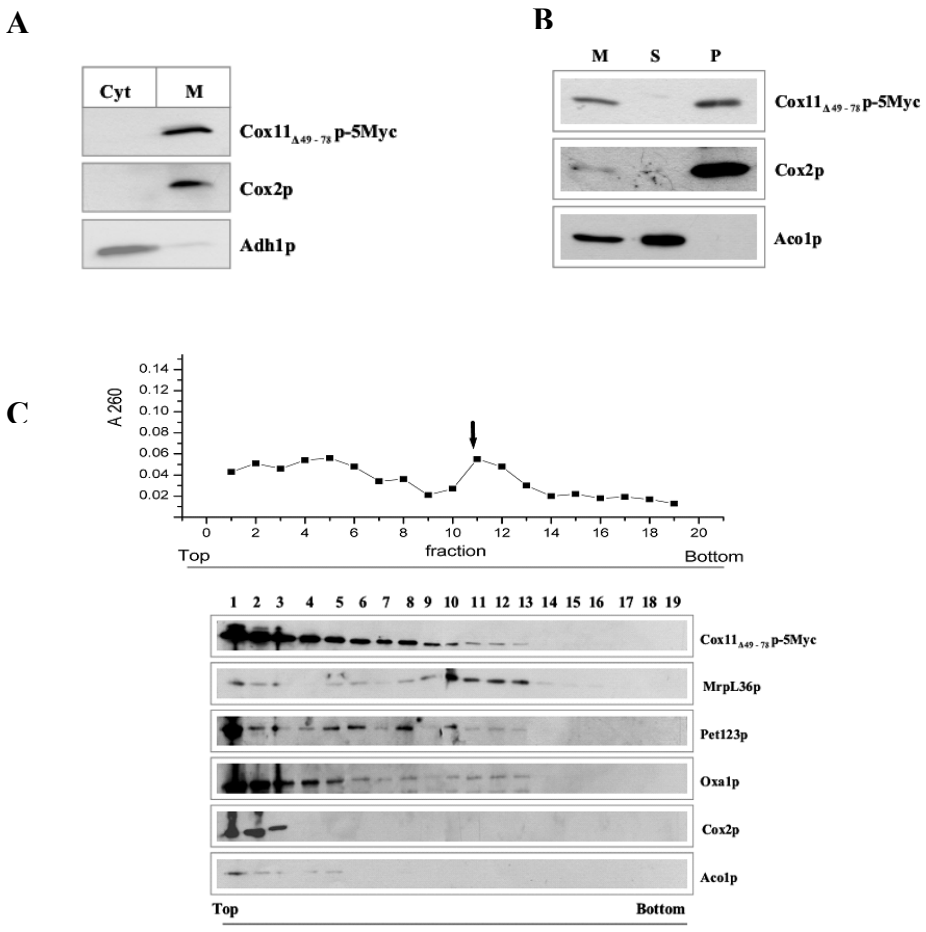



**Figure 25. Cox11$_{\Delta 49\text{-}78}$p-5Myc is localized to the mt membrane and co-sediments with mitoribosomes.** Mitochondrial (M) and cytosolic (Cyt) fractions were isolated from the *cox11*Δ strain Y06479 expressing Cox11$_{TMSco1}$p-5Myc, and analysed as described in the legend to Fig.24.

### 3.4.3 Cox11p association with mitoribosomes is mediated by its TM domain

Recently it was reported that a modified version of Cox11p in which aa residues 85-103 replaced by the TM domain of Sco1p failed to rescue *cox11*Δ mutation (Carr et al., 2005). A similar construct (Cox11$_{TMSco1}$p-5Myc), was created and its ability to confer respiratory competence to a *cox11* null mutant tested. As shown in Fig. 23, the respective transformants were respiratory deficient confirming the observation of Carr et al. (2005). Crosses with a *rho*$^0$ tester strain resulted in respiratory competent diploid cells, thus excluding that the failure to grow on non-fermentable carbon sources results from the deletion of mtDNA (data not shown).

$Cox11_{TMSco1}$p-5Myc is exclusively present in the mt fraction (Fig. 26 A). Within mitochondria $Cox11_{TMSco1}$p-5Myc is anchored in the membrane as documented by alkaline treatment with sodium carbonate, which does not release the protein from the membrane (Fig. 26 B). Thus the inability of $Cox11_{TMSco1}$p-5Myc to complement the *cox11*Δ mutation cannot be due to intracellular or intramitochondrial mislocalization of the protein.

The distribution profile of $Cox11_{TMSco1}$p-5Myc in sucrose gradients differs from that observed for $Cox11_{\Delta 59\text{-}78}$p-5Myc and $Cox11_{\Delta 49\text{-}78}$p-5Myc (Fig. 26 C). While Aco1p, Cox2p, MrpL36p and Pet123p show the same distribution as in the gradients described above, $Cox11_{TMSco1}$p-5Myc no longer co-migrates with mitoribosomes and Oxa1p (Fig. 26 C).

This result indicates that the TM helix is the part of Cox11p that defines the association with mitoribosomes.

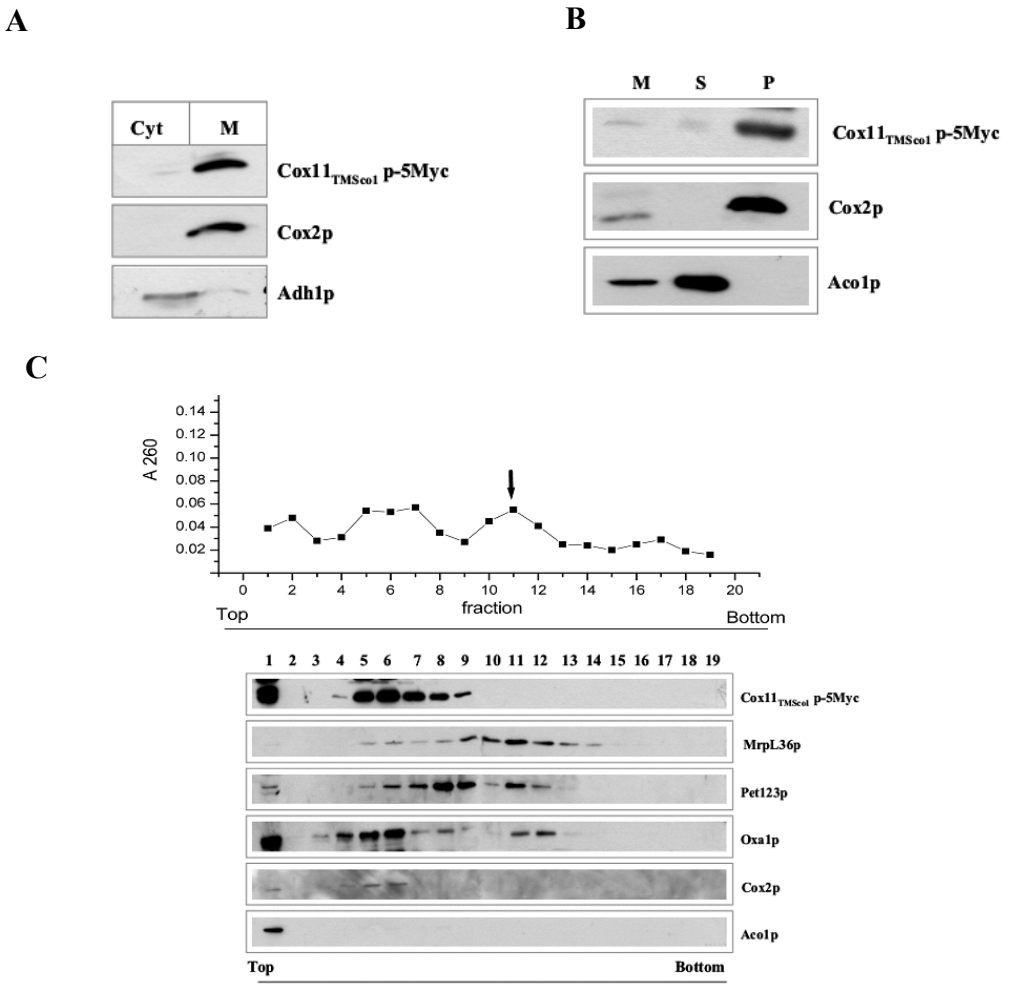



**Figure 26. Cox11$_{TMSco1}$p-5Myc is localized to the mt membrane and does not co-sediment with mitoribosomes.** Mitochondrial (M) and cytosolic (Cyt) fractions were isolated from the *cox11*Δ strain Y06479 expressing Cox11$_{TMSco1}$p-5Myc, and analysed as described in the legend to Fig. 24.

### 3.4.4 Site-directed mutagenesis of conserved amino acids in the TM of Cox11p

The segment of Cox11p TM domain that appears to be crucial for its interaction with mitoribosomes demonstrates a high degree of conservation between different organisms, in particular a conserved proline ($P_{105}$) and valine ($V_{104}$) residue (Fig. 27). In order to check whether these conserved residues are important for the association of Cox11p with mt ribosomes a C-terminally Myc-tagged Cox11p carrying a point mutation ($P_{105}$A or $V_{104}$A) was created. *cox11*Δ transformants expressing Cox11$_{P105A}$-5Myc or Cox11$_{V104A}$-

5Myc are respiratory competent when grow at 30°C or 37°C, however their growth on non-fermentable carbon sources is less efficient compared to that of transformants expressing Cox11p-5Myc (Fig. 28 A, C). Surprisingly, expression of $Cox11_{P105A}$-5Myc results in a very faint complementation of *cox11*-null mutant at 23°C (compared to that one at 30°C and 37°C). By contrast, transformation with $Cox11_{V104A}$-5Myc results in a slightly affected growth that shows no temperature dependence (Fig. 28 E). The growth characteristics correlate well with the measured COX activities. Enzymatic activity of mitochondria purified from strain BY4741 (wt) and *cox11*Δ strain Y06479 remains unchanged at all temperatures tested. COX activity in mt from strain Y06479 expressing

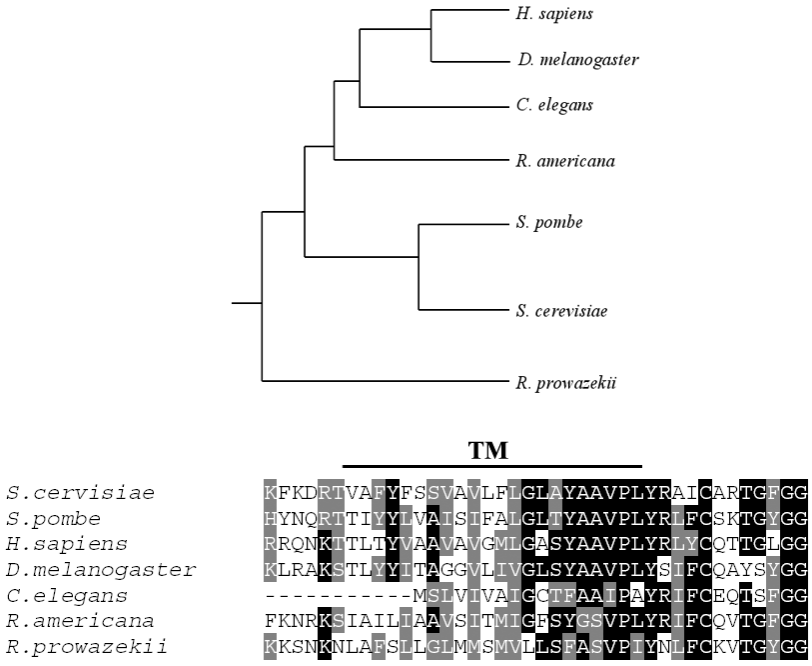



**Figure 27. Evolutionary conservation of the Cox11p TM domain.** In the upper part a schematic drawing of a phylogenetic tree is shown. Data are according to Hedges (2002). Lengths of the horizontal lines are not drawn to scale. In the lower part the TM sequence, including flanking aa of *S. cerevisiae* Cox11p is aligned with the respective aa stretches of the indicated organisms.

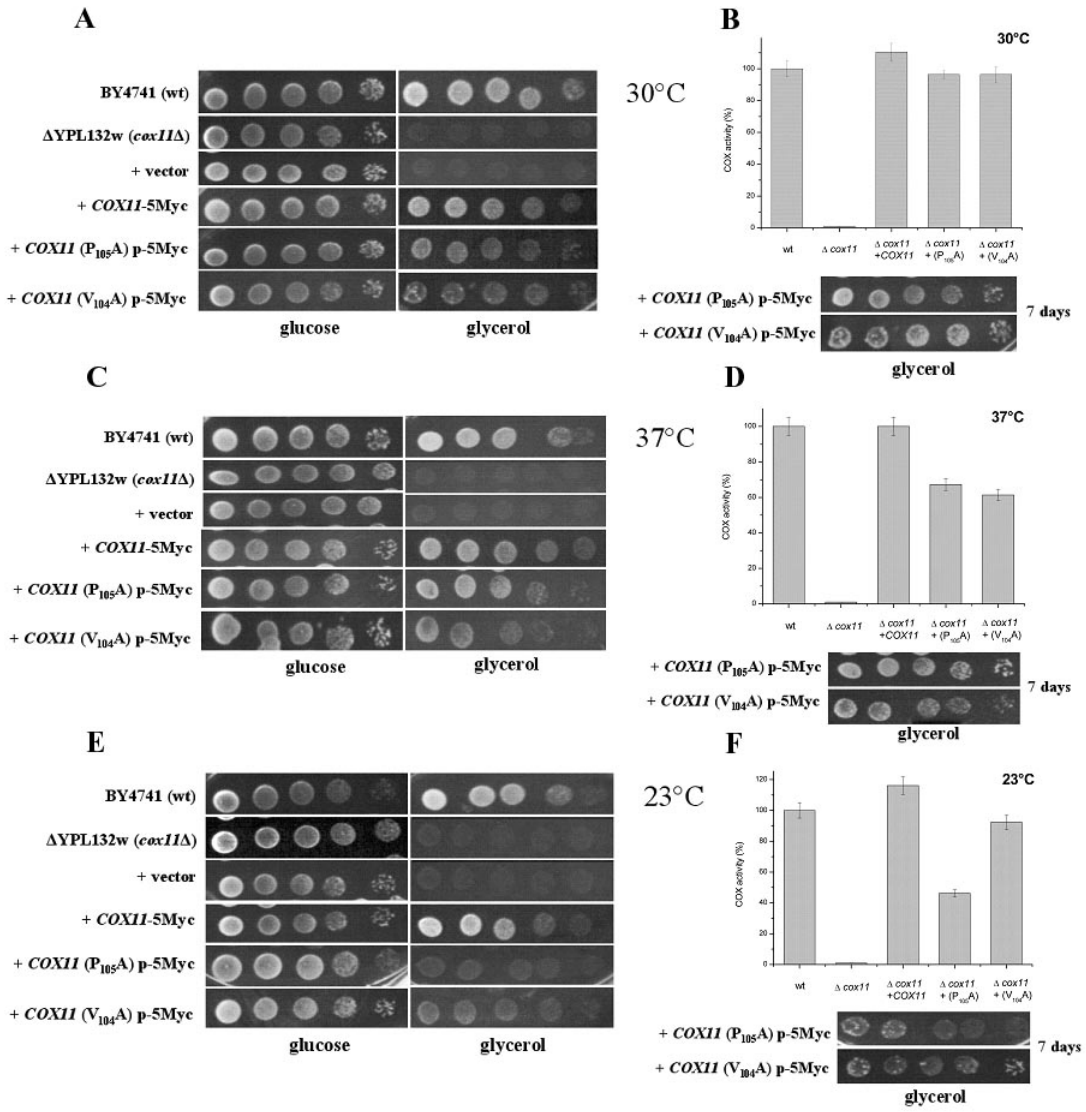



**Figure 28. Complementation analysis of *S. cerevisiae cox11*Δ mutant and COX activities.** Cells of the *cox11*Δ strain Y06479 bearing an empty vector or the indicated plasmids were serially diluted and growth was followed for 3 days (left panel) and for additional 7 days in the case of Cox11($P_{105}$A)p-5Myc and Cox11($V_{104}$A)p-5Myc (right lower panel) on selective media containing glucose or glycerol as the sole carbon source. Cells of wild-type BY4147 and of untransformed *cox11*Δ strain Y06479 were used as positive and negative controls, respectively. Growth characteristics and COX activites were tested at 30°C (A, B), 37°C (C, D) and 23°C (E, F).

Cox11p-5Myc is slightly higher than that of the wild-type at 30°C (Fig. 28 B) and 23°C (Fig. 28 F) and same as the wt activity at 37°C (Fig. 28 D). The faint increase of COX-activity may be explained by the slightly higher expression level due to the *ADH1* promoter compared to that provided by the native *COX11* promoter. Enzymatic activities of mt from strain Y06479 expressing Cox11($V_{104}$A)p-5Myc are about of 92% at 30°C (Fig. 28 B) and 23°C (Fig. 28 F). When mt were isolated from cells grown at 37°C, COX activity drops down to 61% of that of the wt (Fig. 28 D). Mitochondria isolated from strain Y06479 expressing Cox11($P_{105}$A)p-5Myc demonstrate COX activity of about of 96% at 30°C (Fig. 28 B). Activity is even lower at 37°C, near 67% of that of the wt (Fig. 28 D). Surprisingly, COX activity is extremely low in mt obtained from the Cox11($P_{105}$A)p-5Myc expressing cells grown at 23°C: they exhibit only 46% of the wt COX activity (Fig. 28 F). The results demonstrate that COX function is compromised when the conserved $V_{104}$ or $P_{105}$ is substituted by another unpolar residue. This effect appears to be more drastic in the case of $P_{105}$ replacement at 23°C.

Both mutated proteins are efficiently imported into the mt (Fig. 29). As both $V_{104}$ and $P_{105}$ reside at the end of TM segment, their substitution may result in disturbed membrane anchoring of Cox11p. To clarify this issue carbonate extractions of the respective mutant proteins were performed. As growth characteristics and COX activities at 30°C and 37°C are relatively similar, mt were isolated for that purpose from strain Y06479 expressing either Cox11($V_{104}$A)p-5Myc or Cox11($P_{105}$A)p-5Myc upon growth at 30°C and 23°C. Aco1p, that served as a soluble control protein, is exclusively present in the supernatant (Fig. 30, middle panel), whereas the integral membrane protein Tim50p is exclusively detected in the pellet fraction (Fig. 30, lower panel) in both mt preparations. Similarly, Cox11($V_{104}$A)p-5Myc or Cox11($P_{105}$A)p-5Myc expressed at either 23°C (Fig. 30 A, upper panel) or 30°C (Fig. 30 B, upper panel) are present in the pellet fraction. Thus, mutant Cox11p carrying $V_{104}$A or $P_{105}$A mutation in the C-terminal end of TM segment is still an integral mt membrane protein.

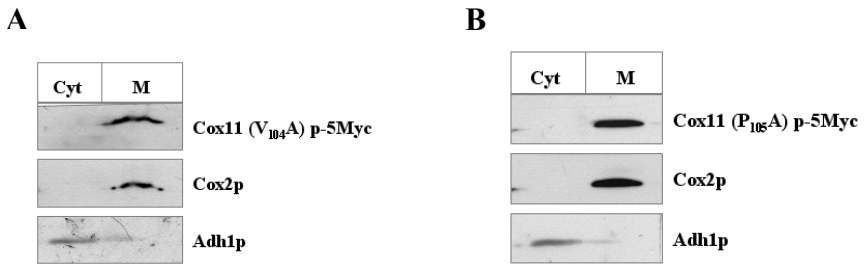



**Figure 29. Cox11($V_{104}$A)p-5Myc or Cox11($P_{105}$A)p-5Myc are imported into the mitochondria.** Mitochondria (M) and cytosolic (Cyt) fractions were isolated from the strain Y06479 expressing either Cox11($V_{104}$A)p-5Myc (A) or Cox11($P_{105}$A)p-5Myc (B), subjected to SDS-PAGE and analyzed by Western blot with antibodies raised against the HA-epitope, the mt protein Aco1p and the cytosolic protein Adh1p.

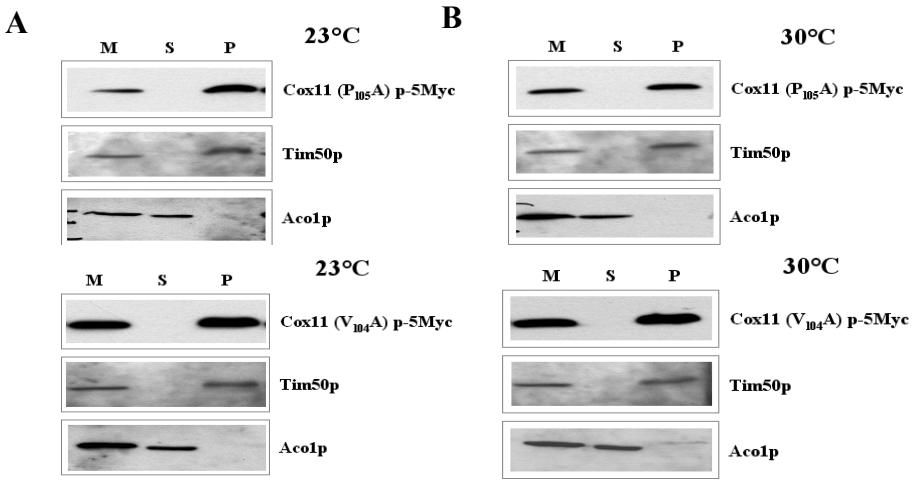



**Figure 30. Cox11($V_{104}$A)p-5Myc or Cox11($P_{105}$A)p-5Myc are mt membrane-anchored proteins.** Mitochondria (M) isolated from the strain Y06479 expressing either Cox11($V_{104}$A)p-5Myc or Cox11($P_{105}$A)p-5Myc at 23°C (A) or 30°C (B) were treated with 0.1 M $Na_2CO_3$. Pellet (P) and supernatant (S) fractions were collected, precipitated with TCA and resolved by SDS-PAGE. Western blot analysis was performed using antibodies directed against the Myc-epitope, the soluble matrix protein Aco1p and the integral membrane protein Tim50p.

### 3.4.5 Cox11p association with mitoribosomes does not depend on Oxa1p.

Oxa1p is known to play a crucial role in the insertion of the nascent mt polypeptides including Cox1p (Stuart, 2002). In order to clarify if Oxa1p may be the compound that mediates the interaction of Cox11p with mitoribosomes we compared co-sedimentation

of Cox11p with mitoribosomes in strains expressing either wild-type Oxa1p or Oxa1$_{\Delta}$Cp, a non-functional truncated version which is no longer able to associate with mitoribosomes (Szyrach et al., 2003). Sucrose gradients were run with mt lysates from the respiratory competent strain Y06479 expressing Cox11p-5Myc (Fig. 31 A), or from the respiratory deficient strain YPH499MO expressing Cox11p-5Myc (Fig. 31 B). Isolated mitochondria were lysed and lysate was applied onto the continuous sucrose gradient and subjected to the velocity centrifugation.

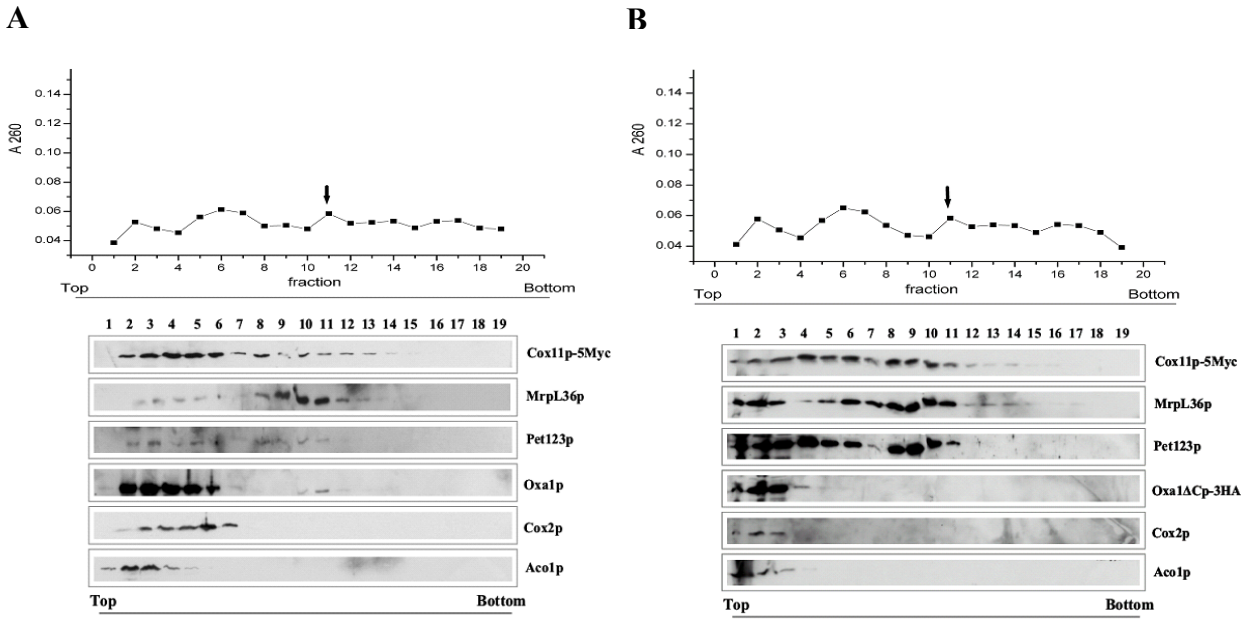



**Figure 31. The sedimentation profile of Cox11p-5Myc does not depend on Oxa1p.** Mitochondria isolated from strain Y06479 (A) or YPH499MO (B) expressing Cox11p-5Myc were solubilized and subjected to the high velocity centrifugation in a continuous 20-40% sucrose gradient. Fractions of 270 µl were collected, precipitated with TCA and subjected to SDS-PAGE. The distribution of Cox11p-5Myc, MrpL36, Pet123p, Oxa1p or Oxa1ΔCp-3HA, Aco1p and Cox2p was assessed by Western blot analysis. An aliquot of each fraction was taken to determine the A260. Peaks representing assembled mitoribosomes are marked by arrows.

The distribution of Cox11p-5Myc, mitoribosomal proteins (MrpL36p, Pet123p), Oxa1p or Oxa1$_{\Delta}$Cp-3HA, Cox2p and Aco1p was assessed by immunoblotting of the fractions from the sucrose gradient (Fig. 31). The profiles of Aco1p, Cox2p, MrpL36p and

Pet123p are like those observed in the gradients described above. The distribution of Cox11p-5Myc is similar to that of Cox11$_{\Delta 59\text{-}78}$p-5Myc and Cox11$_{\Delta 49\text{-}78}$p-5Myc (Fig. 31). Oxa1p migrates like MrpL36p and Pet123p, with the majority of the protein present in the top fractions and a significant portion in the bottom fractions (Fig. 31 A). However, co-sedimentation of Oxa1p with mitoribosomes is no longer detectable when its C-terminal part is deleted (Fig. 31 B). This observation is in agreement with the reported inability of Oxa1$_{\Delta}$Cp-3HA to associate with mitoribosomes (Szyrach et al., 2003). Nevertheless a portion of Cox11p is detected in the high-density fractions.

Next, BN-PAGE followed by Western blot analysis was performed as described above using the *oxa1* mutant strain YPH499MO expressing Cox11p-5Myc. Distribution of Cox2p, Atp2p, mitoribosomal proteins MrpL36p and Pet123p is similar to that observed in the previous experiments using other strains. Detection of Cox11p-5Myc also demonstrates the already described continuous tale-shaped signal with relatively high intensity in the range similar to that of the mitoribosomal subunits (Fig. 32). By contrast, distribution of Oxa1$_{\Delta}$Cp-3HA differs in that the mutant form of Oxa1p is not detected in high molecular weight complexes of the size of mitoribosomes.

These results suggest that Cox11p association with mitoribosomes appears not to be mediated by Oxa1p. Obviously factor(s) other than Oxa1p are involved in linking Cox11p to the mt translation machinery.

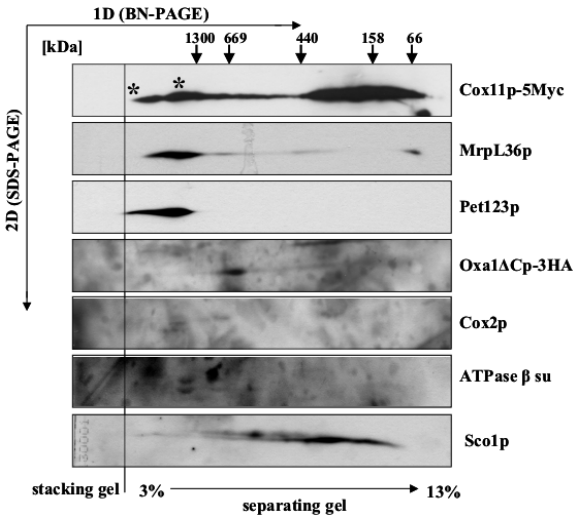



**Figure 32. Detection of Cox11p in high molecular weight complexes does not depend on Oxa1p.** Mitochondria isolated from strain YPH499MO expressing Cox11p-5Myc were lysed and analyzed by BN-PAGE at conditions maintaining the assembled forms of mitoribosomes. Western blot analysis was performed as described in Fig. 16.

## 3.5 Disruption of *S. pombe cox11*$^{+}$ genes

### 3.5.1 Disruption of either *S. pombe cox11*$^{+}$ or *cox11b*$^{+}$

A replacement cassette consisting of the *S. pombe ura4*$^{+}$ gene including its regulatory sequences (1411 bp), flanked on both sides by the 5'- (450 bp) and 3'-(226 bp) sequences of the *cox11*$^{+}$ reading frame was created to generate a *Spcox11*$^{+}$ knock-out mutant. The long flanking sequences were introduced to facilitate the recombination process which is less efficient than in *S. cerevisiae*. For the replacement a diploid strain was created by crossing *S. pombe* strains HE665 ($h^{+N}$, *ade6-M210*) and HE639 ($h^{-S}$, *ade6-M216*). To maintain the diploid state of the resulting *S. pombe* cells, the intragenic complementation of the two *ade6* alleles was used as described (Gutz, 1963). The resulting diploid strain was transformed with the replacement cassette. Uracil-positive clones (strains OK2n-1 and OK2n-2) were selected on supplemented minimal medium lacking uracil, sporulated on the sporulation medium, and spores obtained by tetrad dissection were screened for haploid *cox11* knock-out mutants. The presence of the replacement cassette was checked by PCR (Fig. 33 A). To figure out which of the two *cox11*$^{+}$ alleles was knocked-out a DNA stretch encompassing the divergent region was PCR-amplified and subjected to

direct sequence analysis. As already outlined the only difference between the two *cox11*$^+$ alleles are three nucleotides in positions 1659-1661, that result in polymorphic bands in the respective sequencing pattern.

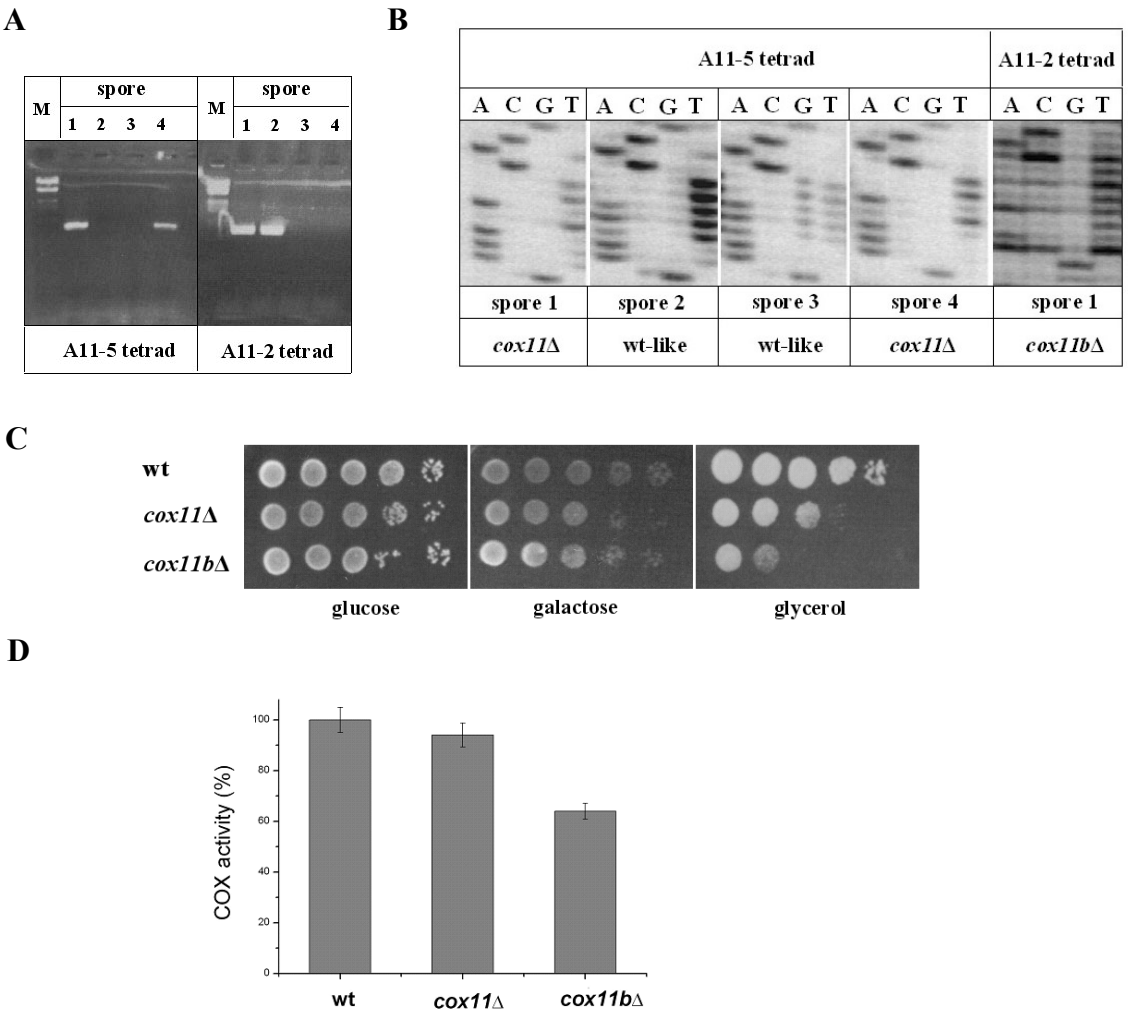



**Figure 33. Replacement of either *cox11*$^+$ or *cox11b*$^+$ in *S. pombe*.** The presence of *ura4*$^+$-replacement cassette in the spores of two tetrads was checked by PCR (A). Direct sequencing of the PCR products of the spores from tetrad A11-5 as well as spore from tetrad A11-2 was performed (B). Cells of the respective strains were serially diluted and growth on media with glucose, galactose or glycerol as the sole carbon source was followed for 6 days (C). The respective strains were grown in YPGG liquid medium and COX activities were determined (D).

Upon deletion of one of the two *cox11*$^+$ genes the polymorphic bands are replaced by an unambiguous sequencing ladder of the remaining *cox11* allele.

Sequence analysis showed that in 2 of the spores from tetrad A11-5 *cox11*$^+$ was replaced, whereas in 2 spores from tetrad A11-2 the *cox11b*$^+$ gene was deleted (Fig. 33 B). Surprisingly, the mutants were respiratory competent irrespective of which of the two *cox11*$^+$ alleles was deleted. However, growth on YPGG medium is slightly impaired in *cox11*Δ and more severely affected in the *cox11b* knock-out mutant (Fig. 33 C). This finding is consistent with the COX activities, which are 94% and 64% of the wt enzyme activity for *cox11* Δ and *cox11b*Δ, respectively (Fig. 33 C).

### 3.5.2 Generation of the double deletion (*cox11::ura4*$^+$, *cox11b::Kan*MX4) in *S. pombe*

A second replacement cassette carrying the *Kan*MX4 gene (1522 bp) flanked on both sides with the 5'- (450 bp) and 3'-(226 bp) sequences of the *cox11*$^+$ was introduced into the diploid strain OK2n-1 in which *cox11* gene is replaced by the *ura4*$^+$ replacement cassette. This diploid strain was used because of the possibility of a lethal effect of the deletion of both *cox11*$^+$ genes. Clones bearing both markers (uracil-prototroph and G418-resistant) were selected.

Four of the obtained transformants were initially analysed by tetrad dissection (36 tetrads), and a clone exhibiting two single spore colonies per tetrad was further analysed. 40 tetrads of this clone were dissected. If the deletion of both genes on the same chromosome is lethal only two of the four spores of a tetrad will form colonies.

Interestingly, a 2:0 segregation on YEA was observed for 9 tetrads and 1:0 segregation for 17 tetrads, respectively (Fig. 34 A). 14 tetrads did not give rise to visible colonies. Taken together these results suggest that the deletion of both *cox11* alleles is either lethal or affects spore germination. The presence of the replacement cassette was checked by PCR using both the diploid strain OK2n-1 and spores obtained in the tetrad dissection (Fig. 34 A). PCR of OK2n-1 cells detected the presence of *ura4*$^+$- and *Kan*MX4-replacement cassettes, both are integrated into the correct chromosomal locus, as well as wt *cox11*$^+$ Fig. 34 B, left panel). At the same time PCR of the haploid spores yields only a positive signal for *cox11*$^+$, but not for the replacement cassettes (Fig. 34 B, right panel). Unexpectedly, one of the spores of a 1:0 segregating tetrads showed a weak *Kan*MX4

signal (data not shown). Either a secondary integration or recombination events in the diploid cell may account for this observation.

In *S. cerevisiae rsm22* null mutant spore viability or germination is affected (Deutschbauer et al., 2002). Because a 1:0 segregation was observed in addition to the expected 2:0 segregation in *S. pombe* (see above), the *cox11* knock-out may severely affect spore viability in *S. pombe*. Therefore, spore viability was tested as described in Material and Methods. In the parental diploid strain (obtained by crossing of

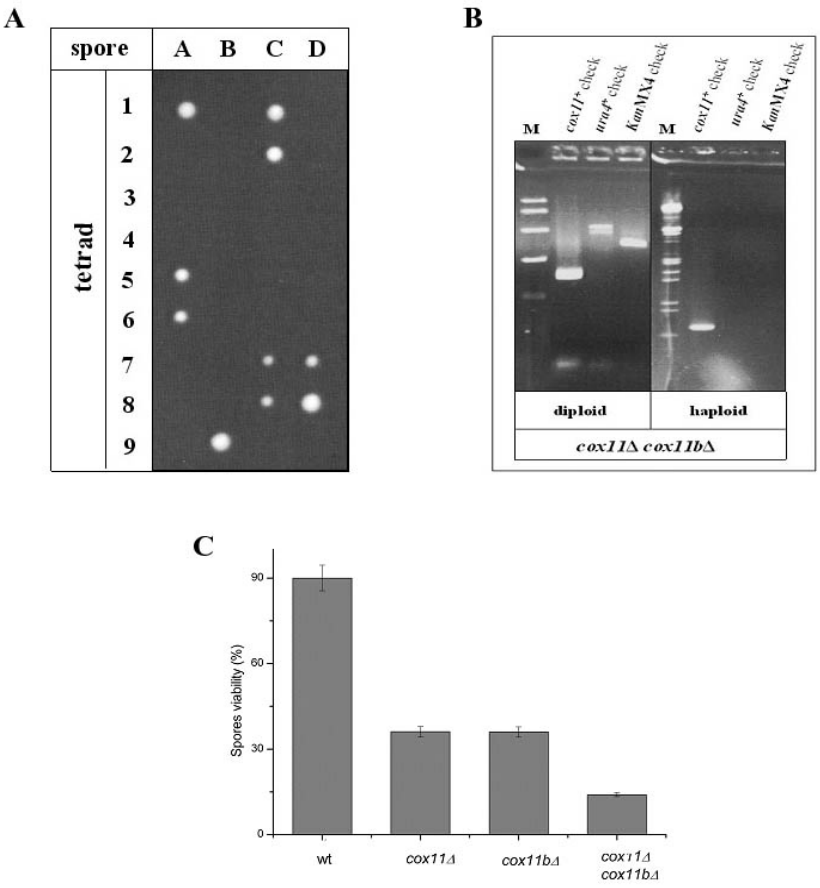



**Figure 34. Generation of the double deletion (*cox11::ura4*$^+$, *cox11b::Kan*MX4) in *S. pombe.*** *S. pombe* strain OK2n-2 was sporulated on a minimal sporulation medium (MMA) for 4 days, and the obtained tetrads were dissected. Spores were grown on complete medium (YEA) for 3 days (A). The presence of *ura4*$^+$-replacement cassette in the spores of two picked tetrads was checked by PCR (B). Spore viability of the single mutants as well as double *cox11*Δ *cox11b*Δ mutant was assessed (C).

HE665 and HE639 strains), which served as a positive control, about of 90% of the spores form colonies. The spore viability of OK2n-1 and OK2n-2 is significantly decreased to about 36%, and even lower in OK2n-3 (14%) (Fig. 34 C). These results indicate that the disruption of *S. pombe cox11* gene(s) affects spore viability.

## 3.6 Biochemical characterization of SpCox11p

### 3.6.1 The SpCox11p precursor is a fusion protein that is cleaved during its import into two mature protein species

As single *cox11*-null mutants of *S. pombe* are respiratory competent and double *cox11* knock-outs appear to be inviable, a C-terminally 3 x HA-tagged version of either SpCox11p or SpCox11bp under control of the strong thiamine-repressible *nmt1*$^+$ promoter was transformed into the respiratory competent *S. pombe* strain HE620 to check whether the respective protein will be processed. A similar experiment has been reported by Bureik et al. (2002) in the study of the *S. pombe* Yah1p-Cox15p fusion protein. Transformants were grown on selective minimal medium and cell lysates and mitochondria were prepared for Western blot analysis with HA-antibodies. Detection of a product with a molecular weight of about 25 kDa (Fig. 35) hints at processing of the putative SpCox11p precursor (around 87 kDa) that could not be detected in numerous

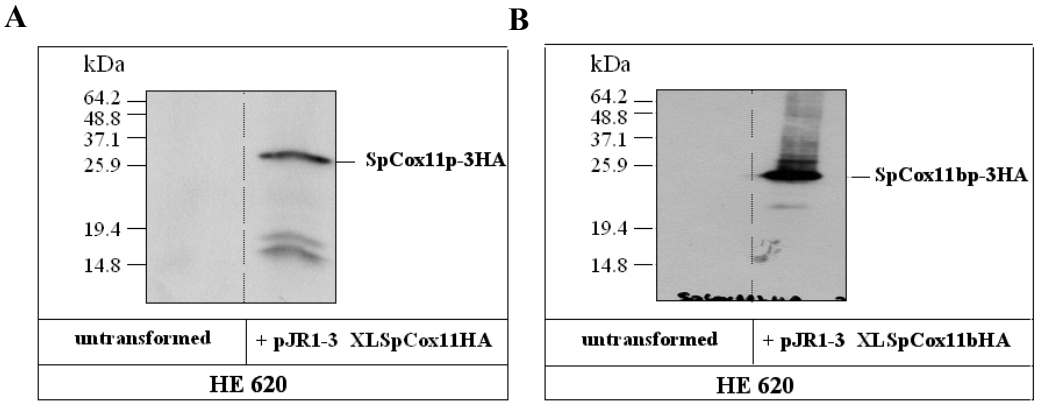



**Figure 35. *In vivo* processing of *S. pombe* Cox11p.** Mitochondria were prepared from cells of *S. pombe* strain HE620 (A and B, left lanes) and strain HE620 expressing either SpCox11p-3HA (A, right lane) or SpCox11bp-3HA (B, right lane). Total mt protein was separated by SDS-PAGE and subjected to Western blot analysis using HA-antibodies.

experiments. Less intense bands of lower molecular weight presumably reflect degradation products. As the bands were not detected in the untransformed strain, the observed signals are specific for the HA-tagged protein (Fig. 35A). The same result was obtained when SpCox11bp-3HA was expressed (Fig. 35B). A possible time point at which processing of the SpCox11p precursor might occur, is the mt import. To address this issue radiolabeled SpCox11p was produced by *in vitro* translation of the RNA-transcript obtained from *in vitro* transcription of plasmid pGEM3 or pGEM4 carrying *cox11*$^{+}$ ORF as an insert. Interestingly, radiolabeled SpCox11p precursor has an unusual migration behaviour in the SDS-PAGE. Instead of the expected 87 kDa band, a band of about 67 kDa is detected (Fig. 36A). This unusual migration behaviour may be due to the limited retention of the structure of the precursor protein, that decreases the sieving effects of the gel. SpCox11p radiolabeled precursor was used for *in vitro* import into isolated mitochondria of *S. pombe* strain L972 as described in Materials and Methods. The samples were taken at different time points of the import assay. Part of the samples was treated with PK to remove unbound and non-imported protein, or coverted to mitoplasts and then PK-treated. SpCox11p precursor protein is efficiently imported and cleaved in two sequential processing steps to give rise to three polypeptides that are resistant to PK treatment: a small fragment of around 14 kDa possibly reflecting the cleaved off N-terminal presequence, a mature fragment that likely corresponds to the Rsm22p segment (around 46 kDa), and a second mature fragment possibly corresponding to Cox11p protein (around 24 kDa). In addition a number of labeled protein bands of intermediate size (between 28 and 35 kDa) that persist PK treatment can be detected. It remains open whether these proteins reflect true processing intermediates or degradation products. Notably, the band assigned to processed Cox11p disappears when mitoplasts are PK-treated, while the protein assigned to Rsm22p remains protected, i.e. reaches the matrix (Fig. 36 A). These results are in agreement with the intraorganellar localization of both Cox11p and Rsm22p known from *S. cerevisiae*.

*In vitro* imported SpCox11p precursor protein was subjected to alkaline extraction and analysed by Western blot. As expected, the processed Rsm22p part is found in the supernatant, while the protein assigned to the Cox11p part is detected in the pellet (Fig. 36 B). Similar results were obtained upon detection of the processed parts of the Cox11p

isoforms, corresponding to SpCox11p and SpCox11bp, after alkaline extraction (data not shown). The results show that the tandem organisation of the SpCox11p precursor is not maintained *in vivo*, but distinct Rsm22p- and Cox11p-like moieties are present in *S. pombe* as in other species, in which these proteins are encoded by two different genes.

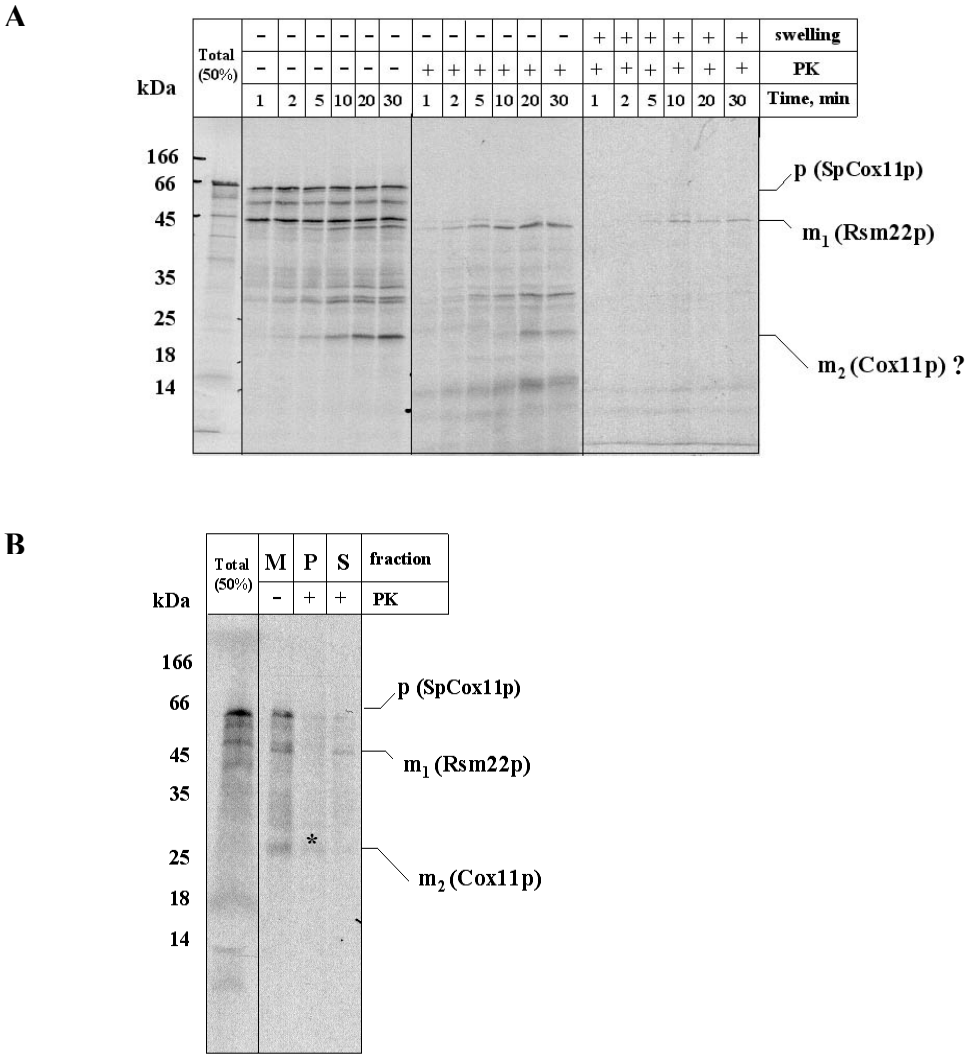



**Figure 36. Processing of SpCox11p during its *in vitro* import into *S. pombe* mitochondria.** Radiolabeled lysate (total 50%) containing SpCox11p precursor protein was incubated with the isolated mitochondria from *S. pombe* strain L972 to assess either the import kinetics (A) or topological properties of the mature proteins (B). Samples were taken after the depicted time points and treated as described. After incubation samples were resolved by SDS-PAGE and analysed by autoradiography.

### 3.6.2 Prediction of the cleavage site between the Rsm22p-Cox11p moieties in the SpCox11p precursor protein.

So far no mt protease cleaving fusion proteins in *S. pombe* has been identified. Because proteolytical cleavage of SpCox11p precursor occurs concomitantly or upon import, a possible candidate to mediate or to perform cleavage is the *S. pombe* homologue of MPP. In that case the cleavage site should show the characteristics of a classical mt presequence (von Heijne et al., 1989; Chaumont et al., 1990).

In order to define the cleavage site between the Rsm22p and Cox11p moieties of SpCox11p or SpCox11bp fusion, radiolabeled truncated versions of the protein were created by means of *in vitro* translation. For that purpose, DNA encoding the *cox11*$^+$ ORF was cut by the restriction enzymes that recognise unique sequences in the region between the two protein parts that demonstrate a charge distribution similar to presequence cleavage sites (Fig. 37 B). Plasmid pGEM4SpCox11 was cut with *Sca*I, *Pst*I and *Kpn*I restriction enzymes, respectively, and the resulting mixtures were transcribed *in vitro*. The RNAs were translated and the resulting proteins were used for *in vitro* import into the mt prepared from *S. pombe* strain L972. Following import mt were converted to mitoplasts, treated with PK and analysed by autoradiography. Clearly, a band of lower molecular weight, compared to the normally processed Rsm22p-mature part is observed in the case of translation product obtained from *Sca*I-treated DNA (Fig. 37 A). The minor band comparable in size with the control presumably arises from the incompletely digested DNA. In the case when *Pst*I-digested *cox11*$^+$ ORF was used as a template for *in vitro* transcription/translation, a band of slightly lower molecular weight, compared to the control, is observed (Fig. 37 A). However, in the case of the product obtained upon import of the precursor resulting from transcription/translation of the *Kpn*I-digested template, no difference in size compared to the control mature protein is observed (Fig. 37 A). These results suggest that the proteolytic cleavage must occur in the immediate vicinity of the sequence defined by the *Kpn*I site. The aa stretch (residues 541-568) encompassing the *Kpn*I site on DNA level was scanned using Helix Draw v1.00 program that allows to define the presequence-like motifs. A segment of 9 aa (positions 549-560) shows a charge distribution that is typical for mt presequences (Fig. 37 C). Interestingly, this region also harbours the aa residues that differ in SpCox11p and SpCox11bp (Fig. 37

C, lower panels). The significance of this, however, is not clear and will be discussed later.

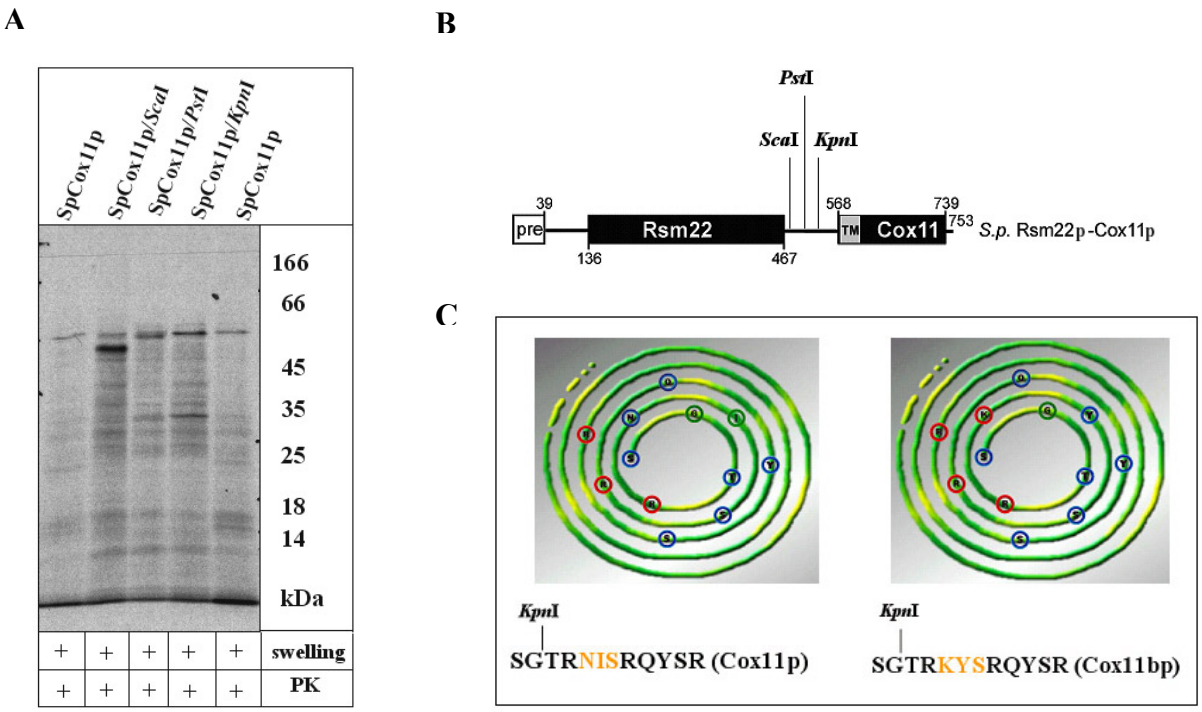



**Figure 37. Prediction of the cleavage site between the Rsm22p and Cox11p moieties of SpCox11p precursor protein.** (A) Radiolabeled lysates containing either SpCox11p precursor protein or truncated forms were incubated with isolated mitochondria from *S. pombe* strain L972. After incubation samples were resolved by SDS-PAGE and analysed by autoradiography. (B) Schematic view of the SpCox11p fusion protein. DNA-cleavage sites that were used to create the truncated versions of the precursor protein, are indicated. (C) The charge distribution in the sequence that may represent the cleavage site between the Rsm22p and Cox11p moieties of SpCox11p or SpCox11bp precursor protein were predicted using Helix Draw v1.00 program (http://www.bioinf.man.ac.uk/~gibson/HelixDraw/helixdraw.html).

# Chapter 4

# Discussion

## 4.1 Complementation analysis in *S. cerevisiae*

### *4.1.1 Complementation analysis of S. cerevisiae cox11Δ mutant*

The ability of Cox11p homologues from different organisms, namely *S. pombe* and human, as well as *S. cerevisiae* Rsm22p, a possibly functionally related protein, to complement the respiratory deficient phenotype of *S. cerevisiae cox11*Δ strain Y06479 was tested. The results suggest that despite of the high degree of similarity, these proteins can not substitute for ScCox11p. This also holds true if the respective proteins are overexpressed. Overexpression of either SpCox11p or SpCox11bp in the wt strain BY4741 causes no dominant-negative effect.

In the case of SpCox11p and SpCox11bp, two almost identical proteins, the presence of a long N-terminal extension with a significant degree of similarity to Rsm22p might be the reason for non-complementation of *S. cerevisiae cox11* null mutant. Such fusion protein may be less stable or characterized by improper topogenesis in *S. cerevisiae* mt. Preliminary results suggest that radiolabeled SpCox11p precursor protein is cleaved incorrectly upon *in vitro* import into *S. cerevisiae* mt (data not shown). In order to exclude this possibility, complementation analysis with truncated versions of SpCox11p containing only the C- or N-terminal part, respectively, were performed. As expected, the N-terminal Rsm22p-like part was not able to substitute for ScCox11p. The C-terminal part, however, also was not able to complement *S. cerevisiae cox11*Δ mutant. As inability of the mentioned constructs to complement *S. cerevisiae cox11* null mutant may be due to the differences in the recognition of the imported protein by the mt import machinery, a set of chimeras (Fig. 8) carrying N-terminal part of ScCox11p was created. These chimeras were also not able to restore the respiratory competence of strain Y06479. When ScCox11p is compared with SpCox11p or SpCox11bp, it is apparent that some aa residues within the 100 aa stretch that otherwise shows a high degree of conservation, significantly differ in their physical-chemical properties. It should be pointed that both SpCox11p, in contrast to most Cox11p homologues, contain a leucine residue instead of a conserved methionine ($M_{224}$, *S. cerevisiae* numbering). A similar situation is observed in

the *Caenorhabditis elegans* homologue. This residue is also believed to be implicated in copper binding (Carr et al., 2002). Interestingly, such a change in ScCox11p causes a decrease in copper binding by the modified protein when it is purified in form of a truncated version. However, this change does not impair the respiratory competence of the *S. cerevisiae cox11*Δ transformants (Carr et al., 2002). These residues may affect the function of chimeric protein in *S. cerevisae* cells. To exclude that possibility the respective modifications were created (Fig. 9). All tested chimeras also failed to complement strain Y06479.

These data imply that ScCox11p - despite of its high degree of conservation - possesses some specific features that can not be covered by the other homologues. The reason for non-complementation may be the presence of specific topogenic signals (Stuart and Neupert, 1996), e.g. short, mostly hydrophobic segments that are important not only for the protein sorting but also define the proper orientation and insertion of the protein into the IMM. Indeed, the fact that SpCox11p are synthesized as fusion proteins may suggest a different processing/insertion mechanism for the respective proteins. If such topogenic signals would be located in the SpCox11p-derived C-terminal part, this also might be the reason for non-functionality of the created chimeras. It is rather unlikely that topogenic signals may reside at the short C-terminal extention of about 37 aa, present in ScCox11p, as Carr et al. (2005) have shown that this segment is not important for the protein function. Another reason for non-functionality of the created chimeras may be the presence of the short specific region between the TM segment and the Cu-binding CFC motif that is crucial for the protein function, as it is described for Sco proteins (Lode, 2001; Paret, 2001).

The failure of HsCox11p to substitute for ScCox11p may be due to the same reasons as described above. Similar results were recently obtained by Carr et al. (2005). Complementation of *S. cerevisiae cox11*Δ mutant by the chimera consisting of the N-terminal part of ScCox11p including its TM domain and the C-terminal part derived from HsCox11p failed (Carr et al., 2005). Non-complementation has been already demonstrated for another protein involved in COX assembly, HsCox10p that substitutes its *S. cerevisiae* counterpart extremely poorly (Glerum and Tzagoloff, 1994).

In the case of functional complementation of yeast mutants by the respective human genes yeast can be successfully used as a model to study the human mutations, e.g. the pathogenic nature of $P_{174}L$ mutation in Sco1p (Paret et al., 2000). Due to the non-complementation of *HsCOX11*, the yeast system can not be efficiently used to study the mutations in the respective gene, although no mutations in *HsCOX11* were reported so far (Sacconi et al., 2003; Horwath et al., 2005).

*4.1.2 Complementation analysis of S. cerevisiae rsm22Δ mutant*

The ability of Cox11p homologues to complement *S. cerevisiae rsm22*Δ mutation was also tested. As expected, ScCox11p can not substitute for ScRsm22p. However, SpCox11p proteins both representing fusions of Rsm22p-Cox11p-like proteins also failed to confer respiratory competence to the *rsm22*Δ strain Y15005, even upon overexpression. However it is impossible to decide if the inability is due to the non-complementation of the proteins or whether it reflects extensive *rho*$^-$ formation due to the *rsm22*Δ background. This aspect became evident when ScRsm22p also failed to confer the respiratory competence of strain Y15005. Further studies with the diploid strains Y35005 and OK-R22 transformed with the plasmid that allows overexpression of ScRsm22p have shown that the haploid cells obtained by sporulation from the respective diploid transformants become very rapidly *rho*$^-$, even in the presence of plasmid-borne ScRsm22p. ScRsm22p is a protein of the small subunit of mt ribosomes (Saveanu et al., 2001; Gan et al., 2002). Deletion of *RSM22* causes respiratory deficiency. On the other hand it is known that that deletion of most of the genes whose products are part of the mt translation apparatus, in particular of mitoribosomes results in the loss of mtDNA (Graack and Wittmann-Liebold, 1998). These results are in line with the recently published data of Carr et al. (2005). These authors, by using a different approach, succeeded to obtain a haploid *rsm22*Δ mutant with intact mtDNA: they introduced a plasmid expressing ScRsm22p into a wt strain, that subsequently was used to disrupt the chromosomal copy of *RSM22*. In addition they demonstrated that the introduction of an artificial fusion protein consisting of ScRsm22p and ScCox11p, thus mimicking SpCox11p, can substitute for ScRsm22p. However, it is not clear if this holds true for native SpCox11p as its ability to complement *rsm22*Δ mutant was not checked.

### 4.2 The Cu-binding C-terminal domain of ScCox11p is exposed to IMS

Results of PK digestion of mitoplasts demonstrate that Cox11p has an $N_{in}$-$C_{out}$ topology in the IMM. This result confirms the prediction of Carr et al. (2002) that the copper-binding site in the C-terminal part of Cox11p protrudes into the IMS. Recent data by Horng et al. (2004), which were obtained by *in vitro* experiments and a yeast cytosolic expression system, strongly suggest that the primary copper donator of Cox11p is Cox17p. This copper-binding protein is partly present in the IMS and has been shown to be functional even when anchored in the IMM (Maxfield et al., 2004). Therefore it seems likely that a direct interaction between Cox17p and Cox11p in the IMS mediates the copper transfer. However, Horng et al. (2004) were not able to detect a stable interaction of these proteins. Possibly these interactions exist only transiently and require cross-linking in order to be detected.

Cox11p exhibits a number of similarities with Sco1p, which is engaged in the formation of the $Cu_A$ site. Like Sco1p, Cox11p exhibits an $N_{in}$-$C_{out}$ topology. Both proteins are anchored by a single TM domain in the inner mt membrane and form homodimers (Nittis et al., 2001; Carr et al., 2002). Both Cox11p and Sco1p possess a copper-binding site in the IMS-exposed C-terminal part and receive the copper ions from Cox17p. However, the inability of crosswise complementation and the observation that both proteins seem not to interact with each other led to the conclusion that formation of $Cu_A$ and $Cu_B$ centres are mechanistically independent (Leary et al., 2004).

### 4.3 Processing of *S. cerevisiae* Cox11p precursor protein

Originally Cox11p has been described by Tzagoloff et al. (1990) as 28 kDa protein, however, the molecular weight calculated from the DNA sequence is 34 kDa. This difference may indicate that Cox11p undergoes a proteolytical processing step during import into mitochondria as it described for most of the mt proteins of nuclear origin (Neupert and Brunner, 2002; Gakh et al., 2002). However inspection of the primary sequence of Cox11p does not reveal a typical mt targeting presequence, i.e. an aa stretch rich in hydroxylated and positively charged aa residues that forms an amphipathic α-helix. In order to clarify this issue, processing was tested by *in vitro* incubation of Cox11p with purified recombinant *S. cerevisiae* MPP, as well as by *in vitro* import of

radiolabelled Cox11p into isolated wild type mt. As expected, *in vitro* translated Cox11p has a molecular weight of about 34 kDa. Results of both *in vitro* processing and mt import show that the 34 kDa-form of Cox11p disappears, concomitantly with the appearance of a 28 kDa band that probably reflects the processed form of protein. Cox11p import depends on the mt membrane potential as no band corresponding to the processed form is observed when mt are treated with valinomycine, a de-energizing agent. A band of about 5 kDa that is observed upon MPP tretament is likely to correspond to the cleaved-off presequence. The exact cleavage site of the presequence in the Cox11p precursor is not yet defined, but Carr et al. (2005) have demonstrated that removal of the N-terminal 60 codons abolishes mt localization of Cox11p. The results obtained by *in vitro* MPP-processing and import studies suggest that the mt-targeting signal encompasses about 45 aa residues. Thus, the length of predicted presequence is in the range of typical mt-targeting presequences (10-80 aa residues) (von Heijne et al., 1989).

### 4.4 Association of ScCox11p with mitoribosomes

#### *4.4.1 ScCox11p is associated with mitoribosomes*

How does Cox11p mediate insertion of Cu(I) into the $Cu_B$ site of COX, which is deeply buried in the IM? One possibility is that the incorporation of copper into the $Cu_B$ site may occur during synthesis of Cox1p. The observation that the *S. pombe* homologues contain N-terminal extensions with significant homology to the *S. cerevisiae* mitoribosomal protein Rsm22p may hint at a link between mt translation and formation of the $Cu_B$ site. Fusion of two genes to yield a fusion protein with the activities of both single proteins is not without precedent in *S. pombe* and can hint at a cooperative action of the two proteins: for example, Cox15p, which is involved in the biosynthesis of heme a, is fused to Yah1p, an enzyme engaged in electron transfer in mitochondria (Barros et al., 2001; Bureik et al., 2002). The functional link between both proteins became evident by the finding that Yah1p acts as the electron acceptor for Cox15p in the course of heme O oxidation (Barros et al., 2002; Carr and Winge, 2003).

The obtained results hint at an association of Cox11p with mitoribosomes. It was observed that part of Cox11p co-fractionates with the fraction of assembled

mitoribosomes in sucrose gradients. It seems unlikely that Cox11p is part of a distinct high-molecular weight complex of similar size as mitoribosomes. The detection of Cox11p but not Cox2p in the bottom fractions of the gradient clearly demonstrates that the presence of Cox11p is not due to membrane fragments. The different fractionation profiles of Cox2p and Cox11p indicate that Cox11p is not associated with the assembled COX complex.

A second line of evidence for an association of Cox11p with mitoribosomes comes from our finding of co-immunoprecipitation of Cox11p and MrpL36p, a constituent of the large mitoribosomal subunit. It remains to be clarified whether the large subunit alone or the entire ribosome is required for this interaction. A recent proteomic approach to identify components of the mitoribosomes by mass-spectrometry failed to identify Cox11p (Gan et al., 2002). Interestingly, other ribosome-associated proteins like Oxa1p (Jia et al., 2003; Szyrach et al., 2003) or translational activator proteins (Krause-Buchholz et al., 2004; 2005) were also not identified in this study, presumably due to the stringent purification conditions. Another observation in favour of an association of Cox11p with mt ribosomes is the finding that the expression profiles of Cox11p upon different conditions correlate well with those of some mitoribosomal proteins, Mrp10p (Sudarsanam et al., 2000), Mrp7p (Travers et al., 2000), MrpL15p (Roberts et al., 2000), Mrp49p, MrpL44p (Huang et al., 2004), MrpL38p, Mrp13p, Mrp17p (Yoshimoto et al., 2002) and Mrp4p and YPL183w-A (Haugen et al., 2004). Interestingly, most of these proteins are constituents of the large mt ribosomal subunit.

Obviously the conditions for the isolation and purification of mitoribosomes used in co-sedimentation analysis were too stringent to maintain the association with peripherally associated proteins. It seems likely that the interaction of Cox11p with mitoribosomes is weak and possibly indirect and mediated by additional components. Cox11p possesses only a short N-terminal part protruding into the mt matrix. The site of potential interactions with the mt ribosome or "linker" proteins is therefore limited to this region, or – in case of a membrane protein as a "linker" – to the TM segment.

The association of Cox11p with mitoribosomes could allow the formation of the $Cu_B$ site in close proximity to the process of translation and membrane insertion of Cox1p. Integration of nascent Cox1p into the IMM is dependent on the Oxa1p complex (Stuart,

2002). This mt protein translocation machinery plays a pivotal role in the integration of both nascent mt polypeptides and of some imported proteins into the IMM (Stuart and Neupert, 1996; Stuart, 2002; Jia et al., 2003; Szyrach et al., 2003). The C-terminal part of Oxa1p has been shown to be associated with mitoribosomes. A cross-linking approach revealed that Oxa1p interacts with Mrp20p, a protein of the large mitoribosomal subunit (Jia et al., 2003). Interestingly, Mrp20p has been shown by tandem affinity purification to interact with MrpL36p, the mitoribosomal protein which we used in our study (Gavin et al., 2002). Therefore, the association of Cox11p with mt ribosomes could be indirectly mediated by Oxa1p. Co-operation of Cox11p and Oxa1p might allow the insertion of the copper ion to the nascent Cox1p during translocation. However, as discussed later, this is not the case.

Association of Cox11p with mitoribosomes was also documented by its purification with mitoribosomal proteins in the TAP procedure. Interestingly, active translation seems to be required for this interaction, as part of Cox11p is lost in the presence of puromycin that prematurely terminates translation and leads to a disassembly of RNA, nascent protein and mitoribosomal subunits. This may hint that Cox11p is not permanently and directly associates with mitoribosomes. These data are compatible with the model proposed below. Another line of evidence for association of Cox11p with the mt translation apparatus is the finding that Cox11p can be detected in high molecular weight complexes when separated by the BN-PAGE. Interestingly, its profile is similar to that of Oxa1p, whose mitoribosomal association has clearly been demonstrated (Jia et al., 2003; Szyrach et al., 2003). Notably, the association of Oxa1p with the ribosomes was reported to be independent on the presence of nascent polypeptide chains (Szyrach et al., 2003).

Based on the finding that ScCox11p is directly or indirectly associated with mt ribosomes, a model of the $Cu_B$ site formation is proposed (Fig. 38). According to it, the $Cu_B$ site is formed by a transient interaction of the C-terminal part of Cox11p with an IMS-exposed domain of Cox1p in the course of Oxa1p-mediated translocation process. Upon transfer of a Cu ion the nascent Cox1p is pushed further into the IMM and the $Cu_B$ site moves into the lipid bilayer of the IMM. The dimeric state of Cox11p might be disrupted during the interaction accompanied by the formation of a Cox11p-nascent Cox1p heterodimer. As functional COX acts as a dimer (Tsukihara et al., 1996), the

second $Cu^{+}$ ion of the Cox11p dimer could concomitantly be inserted into another nascent Cox1p. It has to be stressed, however, that it is still not clear whether Cox11p is directly involved in the formation of the $Cu_B$ site.

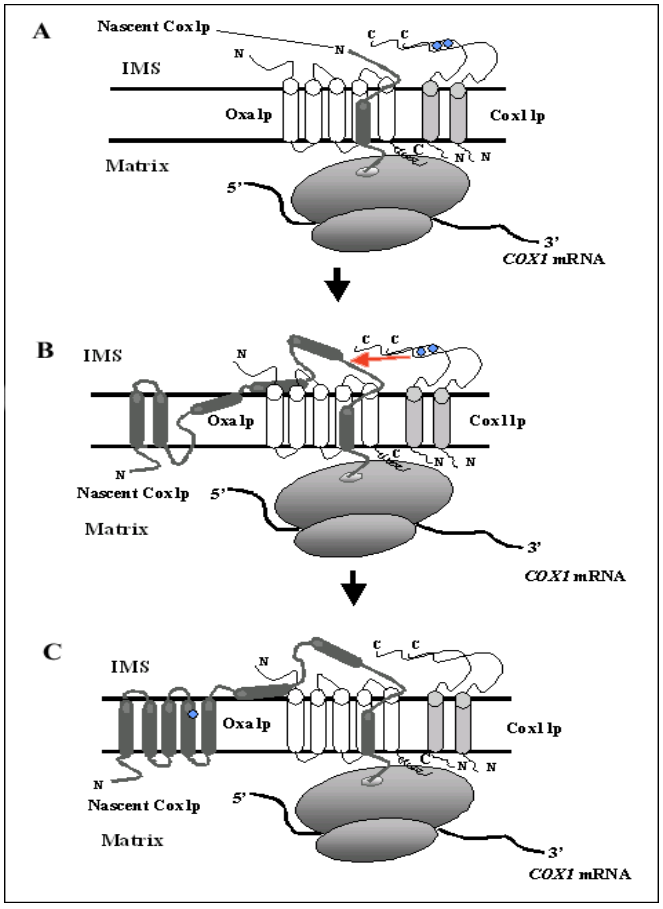



**Figure 38. Model of co-translational insertion of Cu ions into Cox1p.** (A) Cox1p is translated on a ribosome. The nascent polypeptide chain is held in proximity to the IM by Oxa1p (and eventually also by Cox11p) and inserted by the Oxa1p complex into the lipid bilayer. (B) While a stretch of the partially inserted Cox1p is exposed to the IMS, the $Cu_B$ site is formed. Upon transient interaction of the C-terminal part of the Cox11p dimer Cu(I) is loaded onto Cox1p. (C) While membrane insertion of Cox1p continues the $Cu_B$-site moves into the lipid bilayer.

### *4.4.2 Identification of amino acid stretches of ScCox11p that define its association with mitoribosomes*

Obviously the interaction of Cox11p with mitoribosomes should be mediated by its matrix-protruding part. In order to test this, the entire N-terminal matrix-exposed stretch

including the TM domain was replaced by the Sco1p-derived counterpart. As both Sco1p and Cox11p possess $N_{in}$-$C_{out}$ topology the resulting chimeric protein (Sco1N-Cox11Cp-3HA) should be imported into mt and anchored in the mt membrane. Although Sco1N-Cox11Cp-3HA fulfills both criteria, it is not able to substitute for the endogeneous Cox11p. At the same time it is no longer associated with mitoribosomes as shown by co-sedimentation analysis. Also Sco1N-Cox11Cp-3HA can no longer be co-purified with mt ribosomes in TAP procedure. These results suggest that either the N-terminal part of Cox11p and /or its TM domain mediates the association to mt ribosomes. However, BN-PAGE analysis demonstrates that a significant portion of the chimeric protein can still be found in high molecular weight complexes, similar to those observed for unmodified Cox11p. A possible explanation for this unexpected finding may be provided by the calculated pI values of the matrix-protruding moieties of Cox11p and Sco1p, respectively. In both cases the pI values are high and almost identical. The highly charged N-terminal stretches of both proteins may play a docking function to facilitate the various interactions. In line with this is the observation of Carr et al. (2005) that the replacement of part of the Cox11p matrix domain (aa 55-75) by the respective Sco1p-derived stretch yields a functional protein. An alternative explanation for the presence of Sco1N-Cox11Cp-3HA in a high molecular mass complex may come from the misassembly of COX due to the copper depleted Cox1p in *cox11* null mutants. Such a sub-complex, possibly associated with the chimeric protein, may be bound by prohibitins (Phb1p and Phb2p) and stay stablilized for a certain time. These proteins are known to prevent the components of COX assembly process from degradation by forming a high molecular weight complexes (Nijtmans et al., 2000).

Which part of Cox11p is responsible for the observed interaction with mitoribosomes?

Inspection of the primary sequence revealed a stretch of charged aa conserved in a probable translation elongation factor of *S. pombe* and in two SRPs of *A. thaliana*, where it is involved in protein-protein interactions (Funke et al., 2005). The previously reported finding of Carr et al. (2005) that replacement of the Cox11p matrix-exposed N-terminal stretch by its Sco1p counterpart yields a functional, albeit poorly effective protein, is not in contradiction with the assumption that the charged stretch could be responsible for Cox11p association with mitoribosomes. However, inspection of the respective stretch of

Sco1p reveals very similar physico-chemical properties which could account for the observed functional substitution. Both stretches in Cox11p and Sco1p exhibit a high concentration of charged amino acid residues and an almost identical pI value. Therefore the significance of this stretch for Cox11p function was directly tested by creating two deletion mutations in the N-terminal part of Cox11p, both removing the charged stretch. Interestingly, both deletions did not impair the respiratory growth of mutants. Thus it can be concluded that this charged stretch plays no crucial role for the protein's function. However, it cannot be excluded that it may be important for docking of the interacting partner protein(s) as it is described for number of charged stretches (Pool, 2005). For further studies it would be interesting to check the behavior of the respective modifications in the gradient gel under non-denaturing conditions.

Another candidate region for conferring interaction with mitoribosomes is the TM domain of Cox11p. The essential role of the TM helix has recently been shown by Carr et al. (2005), who reported that a chimeric Cox11 protein whose authentic TM was replaced by the Sco1p TM is non-functional. Sco1p and Cox11p have a similar topology in the IMM. A similar chimeric protein ($Cox11_{TMSco1}$p-5Myc), in which the Cox11p TM domain was replaced by the TM segment of Sco1p was created in this work. Its failure to confer respiratory competence to the *cox11* null mutant confirms the data of Carr et al. (2005).

In order to test whether non-functionality of this chimeric protein is accompanied by a disturbed interaction with mitoribosomes, its distibution in a sucrose gradient upon high velocity centrifugation was determined. The results clearly show that the mutant protein can no longer be detected in the fraction that contain the assembled mitoribosomes. Thus the TM domain seems to be crucial for the association of Cox11p with mt translational machinery. Interestingly, the BLAST search reveals that the seven C-terminal aa of the Cox11p TM domain are highly conserved between pro- and eukaryotic Cox11p homologues.

The high conservation of the TM domain is rather unusual as is the presence of a highly conserved proline ($P_{105}$) residue on its end. In order to test the significance of this conservation, point mutations were introduced, that change two of the conserved residues ($V_{104}$ or $P_{105}$) to alanine residues. This hydophobic aa should not affect the function of the

TM domain as a mere membrane anchor. Indeed, the results of carbonate extraction show that both mutant proteins are anchored in the mt membrane. Expression of Cox11$_{V104A}$-5Myc or Cox11$_{P105A}$-5Myc in a *cox11* null mutant strain (Y06479) confers respiratory growth, although it is less efficient and temperature-sensitive compared to the wt strain or strain Y06479 expressing Cox11p-5Myc. The respiratory growth is in line with determined COX activities of mt prepared from the transformants at the respective temperatures. Expression of *cox11*$_{P105A}$ affects aerobic growth at 37°C and - more severely - at 23°C, while *cox11*$_{V104A}$ transformants show moderate impairment at both temperatures.

Interestingly, the COX activity of mt from strain Y06479 that express Cox11p-5Myc is slightly higher at 30°C and 23°C than that one of the wt-mitochondria, presumably due to the higher level of expression provided by an *ADH1* promoter (Mumberg et al., 1995). This may hint at Cox11p as a limiting factor in COX formation at these temperatures.

Proline is known to bend the protein structure. Its high conservation and its position at the end of TM segment suggests that it may be either involved in a specific protein folding and/or in protein-protein interactions. As the effect of the $P_{105}A$ mutation is most deleterious at lower temperature it may be speculated that the introduction of the hydrophobic alanine residue at the IMM/IMS interface that is covered by polar head groups of phospholipids, leads to a local rearrangement of the lipid bilayer. This in turn may lead to a perturbed folding of Cox11p and its dysfunction. Alternatively, $P_{105}$ may be one of the crucial residues that define Cox11p interaction with the compound(s) mediating association with mt ribosomes and such interaction can no longer be conferred by the other residues of conserved stretch at 23°C.

Taken together, the obtained results could suggest that association of Cox11p with mitoribosomes is neither mediated unspecifically by lipid compounds nor by proteins that are exclusively present in fungi. Instead, they could hint at an evolutionary conserved protein as the interacting partner of Cox11p which links copper insertion to translation. As already mentioned above, Oxa1p is a candidate for such a conserved protein. This important component of the mt translocation machinery has been discussed as being involved in tethering the mitoribosomes to the IMM (Szyrach et al., 2003). To clarify if Oxa1p might be responsible for Cox11p association with mitoribosomes the distribution

of Cox11p in a mutant strain bearing a C-terminal deletion of *OXA1* was analyzed. This mutation affects the association of Oxa1p with mitoribosomes (Szyrach et al., 2003). Nevertheless, the distribution profile of Cox11p remains unchanged. In addition the distribution of Cox11p and of the mutant Oxa1p was analyzed by BN-PAGE. The results clearly demonstrate that the interaction of Cox11p with mt ribosomes is not mediated by Oxa1p.

Which other proteins engaged in COX biogenesis could function in mediating Cox11p/mitoribosome association? A component engaged in Cox1p formation that has been discussed as a putative interacting partner of Cox11p is Pet309p (Carr et al., 2005). This *COX1* translational activator with multiple TM domains has been shown to be associated with the translational activators of *COX2*- and *COX3*-mRNA (Naithani et al., 2003). Of these, only Pet122p, one of the Cox3p translational activators so far has been reported to directly interact with mitoribosomes (McMullin et al., 1990).

Another putative candidate to cooperate with Cox11p and mediating its binding to the ribosomes is Cox14p, a membrane-anchored chaperone required for Cox1p expression (Glerum et al., 1995). Both nascent Cox1p and Mss51p, a protein required for Cox1p expression and insertion (Perez-Martinez et al., 2003; Siep et al., 2000), have been reported to interact with Cox14p. The Cox1p/Mss51p/Cox14p complex is important for Cox1p assembly (Barrientos et al., 2004; Herrmann and Funes, 2005). The authors proposed that Shy1p, which is required for COX assembly and presumably involved in heme *a* insertion (Mashkevich et al., 1997; Smith et al., 2005), may cooperate with the Cox1p/Mss51p/Cox14p complex during Cox1p assembly, but it seems not to interact with the latter (Barrientos et al., 2004). Since Shy1p is a membrane-anchored protein it is also a candidate to mediate Cox11p/ribosome interaction. It has to be noted that orthologues of Pet309p, Mss51p and Cox14p have so far only been reported in fungi (Barrientos et al., 2004; Carr and Winge, 2003), whereas Shy1p shows a high degree of conservation in eukaryotes (Zhu et al., 1998; Poyau et al., 1999). If the evolutionary conservation of the TMD part is indeed indicative for a conserved interaction partner protein, Shy1p appears to be a prime candidate. It can be speculated that the insertion of the heme(s) into Cox1p also occurs in a co-translational manner, and that the $Cu_B$ site, a complex dimetallic motif formed by heme $a_3$ and copper moieties, is formed

simultaneously by the cooperative action of Cox11p and Shy1p. The findings that *cox11* mutation can be partially rescued by the addition of exogenous heme *a* (Church et al., 1996) and that the expression levels of Cox11p and Shy1p are similar when cells are treated with arsenic (Haugen et al., 2004) are compatible with the idea of a cooperate insertion of heme and Cu ions. Alternatively, Shy1p may simply facilitate insertion of heme, as it has been shown that heme *a* can be self-inserted in a *de novo* designed four helix bundle protein, mimicking the hydrophobic pockets of Cox1p (Gibney et al., 2000). Another possible candidate to mediate the association of Cox11p with mt ribosomes is the product of *YGR150c*, a protein with unknown function that exhibits weak similarity to the mitoribosomal protein Mrps5p. Ygr150cp is predicted to possess a TM segment and might be involved in tethering of the ribosome to the IMM and in mediating Cox11p/mitoribosomal association.

### 4.5. Replacement of *cox11*$^+$/*cox11b*$^+$ genes in *S. pombe*

Despite of the fact that the genome of *S. pombe* is less redundant than that of *S. cerevisiae* (Wixon, 2002) the overall number of duplicated genes is higher in the fission yeast (Wood et al., 2002). The presence of two copies of *cox11*$^+$ in the *S. pombe* genome raises the question if both versions are functional. In the case of the two identified human *COX11* alleles it was shown that one of them is a non-functional pseudogene (Petruzella et al., 1998). Expression profiles obtained for *cox11*$^+$ and *cox11b*$^+$ at different stress conditions appear to be quite similar (Fig. 39; Chen et al., 2003), however, it is almost impossible to attribute a signal to one or two genes. To clarify if both *cox11*$^+$ genes are functionally important *ura4*$^+$- and *KanMX4*-based disruption cassettes carrying flanking sequences with homology to 5'- and 3'-regions of *cox11*$^+$ (as pointed above, both *cox11*$^+$ genes are part of large 5.7 kb identical duplicated region) were created. Based on the results of tetrad analysis and direct sequencing, single *cox11*Δ and single *cox11b*Δ haploid mutants were obtained in the first round by using the *ura4*$^+$-based replacement cassette. Both mutants were viable and respiratory competent at all temperatures tested. These results show that the disruption of either *cox11*$^+$ or *cox11b*$^+$ does not affect essential cell functions. However, when the respiratory growth on glycerol medium was tested the deletion mutants showed differences with *cox11b*Δ having a more severe

defect. This effect may be due to the differences in steady-state levels of the remaining SpCox11p isoform as discussed below. Interestingly, further phenotypic analysis of diploids carrying *cox11*Δ or *cox11b*Δ allele revealed that both are characterized by reduced (up to 36%) spore viability compared to the parental strain bearing intact versions of *cox11*$^{+}$.

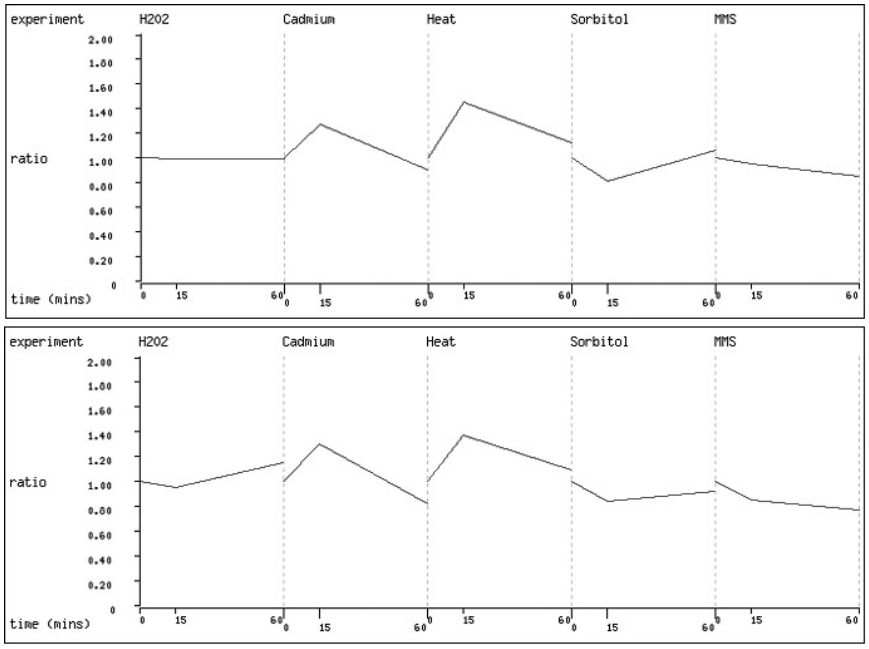



**Figure 39. Expression profiles of *S. pombe cox11*$^{+}$/*cox11b*$^{+}$ under different stress conditions.** Changes in expression of *cox11*$^{+}$ and *cox11b*$^{+}$ in *S. pombe* cells in response to the indicated stress conditions (modified from Chen et al., 2003).

To obtain haploid cells with deletion of both *cox11* genes, the *KanMX4*-based replacement cassette was applied in diploid strains that bear deletion of a single *cox11* allele. Results of tetrad analysis of the respective transformants suggest that haploid double *cox11*Δ *cox11b*Δ mutants are non-viable. Moreover, analysis of diploids carrying heterozygous *cox11*Δ and *cox11b*Δ mutations indicates a dramatic decrease in spore viability compared to the wt or the single deletion mutants. As *S. pombe* is a "*petite*-negative" yeast (Wolf et al., 1976; Seitz-Mayr and Wolf, 1982; Schäfer, 2003), depletion of the gene(s) important for mtDNA maintenance normally will result in lethality. As

both versions of SpCox11p with their ScRsm22p-like moieties appear to be the only Rsm22 homlogues in *S. pombe,* and Rsm22p is important for proper functionnig of mtDNA in *S. cerevisiae* (Saveanu et al., 2000; Carr et al., 2005), the lethal phenotype of *cox11* double deletion mutants is expected. However, dysfunction of the ScCox11p-like part may also contribute to the lethal effect as it was reported for deletion of the *S. pombe* *oxa1*$^+$ alleles (Bonnefoy et al., 2000). Taken together, the results show that both *cox11*$^+$ and *cox11b*$^+$ can fulfill the function of SpCox11p in respiration. Interestingly, *S. pombe* also possesses two distinct *oxa1*$^+$ orthologues and double inactivation is also lethal (Bonnefoy et al., 2000). However, in this case only one gene has been shown to be essential for respiration. Similar results were obtained, for instance, when the *msp1*$^+$ gene, whose product is important for maintenance of mt dynamics and mtDNA integrity, was deleted (Guillou et al., 2005). Decreased spore viability can be a rather unspecific effect and was described for a number of knock-outs in *S. pombe* (Muris et al., 1997; Grishchuk and Kohli, 2003). On the other hand, as it was pointed above, *S. cerevisiae* *rsm22*Δ mutants are also characterized by decreased spore viability (Deutschbauer et al., 2002).

### 4.6 Processing of *S. pombe* Cox11p

The sequencing of *S. pombe* genome revealed the presence of several genes encoding fusion proteins, several of which are predicted to be mt components (Fig. 40). These gene products contain classical mt targeting signals at their N-termini followed by a sequence which represents the homologues of two mt proteins arranged in tandem. It is unclear whether these tandem proteins are proteolytically processed or remain as fusion proteins in mitochondria of *S. pombe*. Both variants are possible as in diatom *Phaeodactylum tricornutum* triosephosphat isomerase (TPI)-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fusion protein is imported without proteolytical separation of the tandem parts (Liaud et al., 2000), on the other hand for Cox15p-Yah1p fusion in *S. pombe* (Bureik et al., 2002) or rice succinate dehydrogenase subunit B-mt ribosomal protein S14 fusion (Oshima et al., 2005) it was reported that the tandem precursor protein undergoes a processing step during the import.

As ScRsm22p is a constituent of mt ribosome in *S. cerevisiae* (Saveanu et al., 2001), it was speculated that the expression of Cox11p as a tandem protein with a ribosomal subunit in *S. pombe* might covalently tie Cox11p to the mitochondrial ribosome (Carr and Winge, 2003). In favour of this idea Carr et al. (2005), using the unprocessed fusion of ScRsm22p-ScCox11p that mimics SpRsm22p-Cox11p tandem, have demonstated that such a construct can rescue both *S. cerevisiae rsm22* and *cox11*-null mutants.

Results of import of radiolabeled Rsm22p-Cox11p precursor into *S. pombe* mt indicate that this fusion protein is efficiently imported and cleaved in two sequential processing steps to give rise to three polypeptides: The N-terminal presequence, a mature fragment

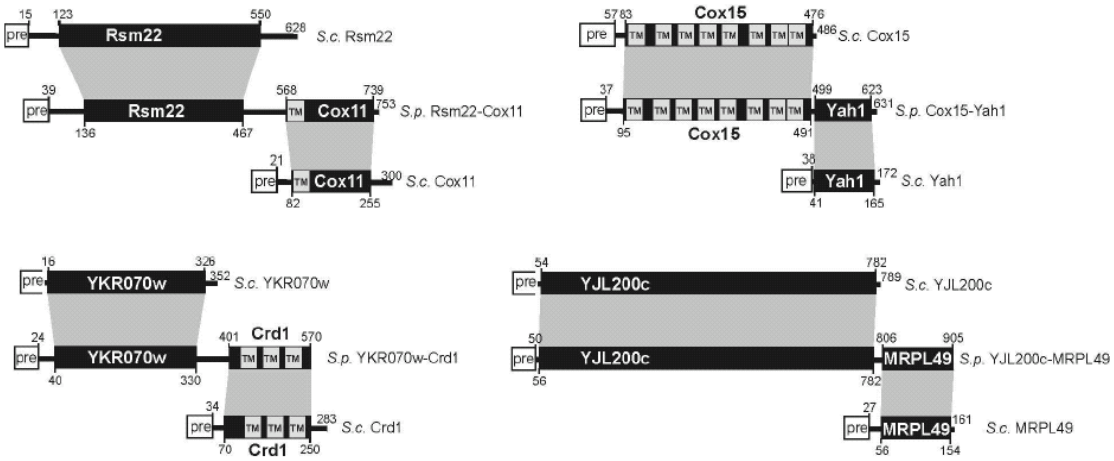



**Figure 40. Tandem mitochondrially-localized proteins in *S. pombe*.** Schematic view of the proteins predicted to localize to *S. pombe* mitochondria. The respetive *S. cerevisiae* counterparts as well as presequences and TM domains are indicated.

presumably corresponding to the matrix-located soluble Rsm22p segment and the membrane-embedded part assigned to Cox11p protein. In line with that are results obtained in *in vivo* studies, however in that case only HA-tagged Cox11p-corresponding part has been detected. Numerous attempts aiming to detect the precursor form were not successful. Obviously *in vivo* uptake into mt and processing of the precursor protein seems to be a fast process that does not allow to detect the precursor form. In order to prove that MPP is responsible for that cleavage it would be helpful to have a *ts*-allele of MPP subunit or to apply the specific inhibitors during *in vitro* import. However there is

an indication that standard procedures using *o*-phenantroline are inefficient in *S. pombe* mt (preliminary results, data not shown).

Results of import experiments using different radiolabeled truncates containing only the Rsm22p-like part plus putative linker part demonstrate that the cleavage between both mature proteins occurs in a region that shows the characteristics of a classical mt presequence (von Heijne et al., 1989; Chaumont et al., 1990). Interestingly, the divergent region between SpCox11p and SpCox11bp is located inside the stretch predicted to be the cleavage site between the two parts of tandem. The significance of that is not clear, as both versions of protein appear to be functional and processed. As both SpCox11p precursor proteins are cleaved in a similar way, this divergent amino acids obviously has no impact on the cleavage. On the other hand if the cleavage occurs within the divergent region, the resulting processed forms of SpCox11 and SpCox11b will differ in the N-terminal aa residue that may result in difference of the protein stability, similarly as it is described for cytosolic proteins.

Thus, the tandem organisation of this protein is not maintained in the endogenous protein, but Rsm22p and Cox11p are present in *S. pombe* as in other species as distinct polypeptides. The tandem organisation of mt proteins in *S. pombe* might be used to coordinate expression levels of proteins and/or to improve the efficiency of their mt import.

The importance of Rsm22p-Cox11p fusion in *S. pombe* is still puzzling and less clear as, for example, in the case of the Cox15p-Yah1p tandem: in this case the Cox15p homologue is directly involved heme *a* biosynthesis, while Yah1p acts as electron supplier for the Cox15 partner protein (Glerum et al., 1997; Barros et al., 2001; 2002; Carr and Winge, 2003). Why these fusion proteins appeared and persisted in evolution remains unclear. It is possible that the genomic rearrangements which resulted in the expression of these fusion proteins were physiologically neutral since the processing sites in the tandem proteins allowed their proteolytic separation in the matrix. However the effects of the tandem organisation might have several reasons: (1) The processing of one fusion protein warrants equal expression levels two proteins. (2) The separation of the tandem protein in the matrix ensures that both proteins emerge at the same entry site in the mitochondrion which might help the partnering of cooperating proteins. (3)

Noteworthy, in some of the tandem proteins in the *S. pombe* genome, one part represents a hydrophobic membrane protein. It is conceivable that the tethering to a hydrophilic polypeptide helps to increase the solubility of mitochondrial membrane proteins thereby improving the efficiency of their post-translational import into the organelle. Whatever the molecular basis for the benefit is, it most likely is not generally advantageous as only few examples of such tandem proteins are present in the genomes that were so far sequenced.

It should be also noted that if tandem organization should warrant a similar concentration of both parts, this is rather not the case for Rsm22p-Cox11p fusion, where the Rsm22p moiety should be required in higher concentrations than the Cox11p part. However it may also be possible that the final *in vivo* stoichiometry of both protein parts may differ due to different proteolytic sensitivities of the two moieties.

Another curious thing is that Rsm22p homologues were found so far only in fungi (Saveanu et al., 2001). Moreover, the aforementioned results indicate that Cox11p interaction with mt ribosomes and thus Rsm22p is rather indirect. It is not clear if expression of ScCox11p and ScRsm22p coincides. However, a possible hint for the significance of Cox11p/Rsm22p relation may come from the finding that in *S. cerevisiae* expression levels of Cox11p and Mrp4p, a ribosomal protein that directly interacts with Rsm22p (Gavin et al., 2002) are very similar (Haugen et al., 2004).

# Chapter 5

# Summary

Cytochrome *c* oxidase (COX) is so far the only known Cu-containing enzyme in mitochondria. Delivery and insertion of copper into the enzyme are very complex processes that require multiple steps and involve a large number of assembly factors. Many of these factors are involved in the mt copper shuttling and homeostasis. One of the involved components is Cox11p, a copper binding protein, that is conserved from prokaryotes to eukaryotes. Cox11p is essential for respiratory growth and implicated in the assembly of the $Cu_B$ site located in subunit Cox1p of COX. Interestingly, in *Schizosaccharomyces (S.) pombe* Cox11p is fused to a protein homologous to ScRsm22p, a constituent of small subunit of mt ribosome, and this finding may link Cox11p function to the translation of the mt encoded subunit Cox1p. The role of Cox11p in COX assembly was studied in this work using two model organisms: the budding yeast *Saccharomyces (S.) cerevisiae* and the fission yeast *S. pombe*.

The presence of two *COX11* versions (*Spcox11*$^+$ and *Spcox11b*$^+$) in the *S. pombe* genome was proven by direct sequencing of isolated DNA fragments. Neither of both versions nor their isolated N- and C-terminal parts are able to substitute for ScCox11p. Introduction of the *Spcox11*$^+$ under control of a high-effective promoter in a respiratory competent *S. cerevisiae* strain has no dominant-negative effect. Also the human homologue of Sc*COX11* (*hCOX11*), isolated from a liver cDNA, does not complement *S. cerevisiae cox11* null mutation. A set of chimeric genes composed of portions from the *S. cervisiae* and *S. pombe COX11* genes, was created. Surprisingly, none of them was able to complement the *S. cerevisiae cox11*Δ strain.

Overexpression of *ScRSM22*, the putative homologue of the gene fused to the *COX11* part in both versions of *Spcox11*$^+$ also does not complement the *S. cerevisiae cox11Δ* mutation. Notably, in the *S. cerevisiae rsm22*-null mutant strain stability of the mt genome is apparently affected already in the spore germination stage.

*S. pombe cox11*$^+$ and *cox11b*$^+$ knock-out mutants were constructed. Cells lacking only one of the *cox11*$^+$ copies remain respiratory competent. Replacement of both *S. pombe*

*cox11*$^{+}$ alleles appears to result in either spore lethality or in severe decrease of spores viability. Thus, both versions of SpCox11p are functional and important.

The topology of ScCox11p was determined by biochemical assays: it was demonstrated that it is an integral membrane protein with $N_{in}$-$C_{out}$ topology. ScCox11p is proteolytically processed during its import into mitochondria. Co-immunoprecipitation data showed that ScCox11p interacts with MrpL36p, a constituent of the large mitoribosomal subunit. Results of sucrose gradient centrifugations, TAP-co-purification and BN-PAGE provide clear evidence that ScCox11p is associated with mitoribosomes. Taken together, these data hint at a link between the formation of $Cu_B$ site of COX and the mt translation machinery. A model for the formation of $Cu_B$ center of COX is proposed, according to that the insertion of copper ion(s) may occur co-translationally while the nascent Cox1p is inserted into the lipid bilayer.

Replacement of the N-terminal part of ScCox11p including the single TM segment by its ScSco1p counterpart results in respiratory deficiency and inability to interact with mitoribosomes. Mutational studies demonstrate that the highly charged N-terminal part of ScCox11p adjacent to the TM helix is dispensable for the function of protein and its association with mitoribosomes. Instead, the evolutionary conserved ScCox11p TM segment was shown to be crucial for the association of ScCox11p with mitoribosomes. Point mutations in the conserved stretch of the TM segment affect respiratory growth: Replacement of the highly conserved $P_{105}$ residue that might be involved in protein-protein interactions, results in an almost complete abolishment of respiratory growth at 23°C. On the basis of these data it is proposed that ScCox11p interaction with mt ribosomes is indirect and is mediated by another conserved membrane protein(s). Clearly the mt translocase ScOxa1p can be excluded as a mediator protein. Possibly factors involved in the heme $a_3$ insertion into ScCox1p are involved in this process and allow concomitant insertion of the Cu ion and heme $a_3$.

Results of both *in vitro* and *in vivo* studies demonstrate that *S. pombe* Cox11p tandem precursor protein is cleaved during its import into two mature protein species corresponding to Rsm22p- and Cox11p-like moieties. Possibly the tandem organisation of some nuclear genes encoding mitochondrial proteins in *S. pombe* is used to coordinate expression levels of proteins and to improve the efficiency of their mitochondrial import.

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# Appendix

```
S.cerevisiae    1 ------------------------------------------------------------
S.pombe         1 MPILTCRYKILFLYNLRNCFTFQNQRCLIPYGTTTTIRWYNANFQAVQNNFSDYKNELIS
H.sapiens       1 ------------------------------------------------------------
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------

S.cerevisiae    1 ------------------------------------------------------------
S.pombe        61 SHRPEASSLLDFLVKDQKKSGDISLHTKFNLYVDDLLKKSEKGQIKKFINDIKKDLATES
H.sapiens       1 ------------------------------------------------------------
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------

S.cerevisiae    1 ------------------------------------------------------------
S.pombe       121 QLPLSAPFKDESTRTMTDPQVLAYIHQSMPYQYASLYSVLTDLKIVNSDVSCKSQHILDC
H.sapiens       1 ------------------------------------------------------------
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------

S.cerevisiae    1 ------------------------------------------------------------
S.pombe       181 GKGPGIGALASYSVFPTPNSVSIVEENPFLKKIIYDIHHNIYPSTSPNPTSPVTLNRLPL
H.sapiens       1 ------------------------------------------------------------
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------

S.cerevisiae    1 ------------------------------------------------------------
S.pombe       241 GKKDSYTLVIASNKLLEMKSEKELFDYLRSLWSLVSNDGGLLVLCERGTKRGFSLIQRAR
H.sapiens       1 ------------------------------------------------------------
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------

S.cerevisiae    1 ------------------------------------------------------------
S.pombe       301 TFLLQKSKNTSDKQFNAHIVAPCPHDGRCPIDIENGVRANICSFKQHFFLSPFSRLYVPR
H.sapiens       1 ------------------------------------------------------------
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------

S.cerevisiae    1 ------------------------------------------------------------
S.pombe       361 SHRRSSDRSHYSYVVIQKGITRPLNNTTQRFKNDEDLLENVNVTSPTLKNWPRIIRPPLK
H.sapiens       1 ------------------------------------------------------------
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------

S.cerevisiae    1 ------------------------------------------------------------M
S.pombe       421 RDGHVIIDVCDSDARLRRNIVPKSQGKLAYRLARKSAWGDLFPLEGKVQSTSPSSKITKH
H.sapiens       1 ----------------------------------------------MGGLWRPGWRCVPF
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------

S.cerevisiae    2 IRICPIVRSKVPLLGTFLRSDSWLAPHALALRRAICKNVALRSYSVNSEQPKHTFDI---
S.pombe       481 LKDASSTYSINPPSYNKPKVERNTTADPIFVGKRFYSTNRHKAFSRFADFNSHRFPCIFT
H.sapiens      15 CGWRWI--HPGSPTRAAERVEPFLRPEWSGTGGAERGLRWLGTWKRCSLRARHPALQPPR
C.elegans       1 ------------------------------------------------------------
R.americana     1 ------------------------------------------------------------
R.prowazekii    1 ------------------------------------------------------------
```

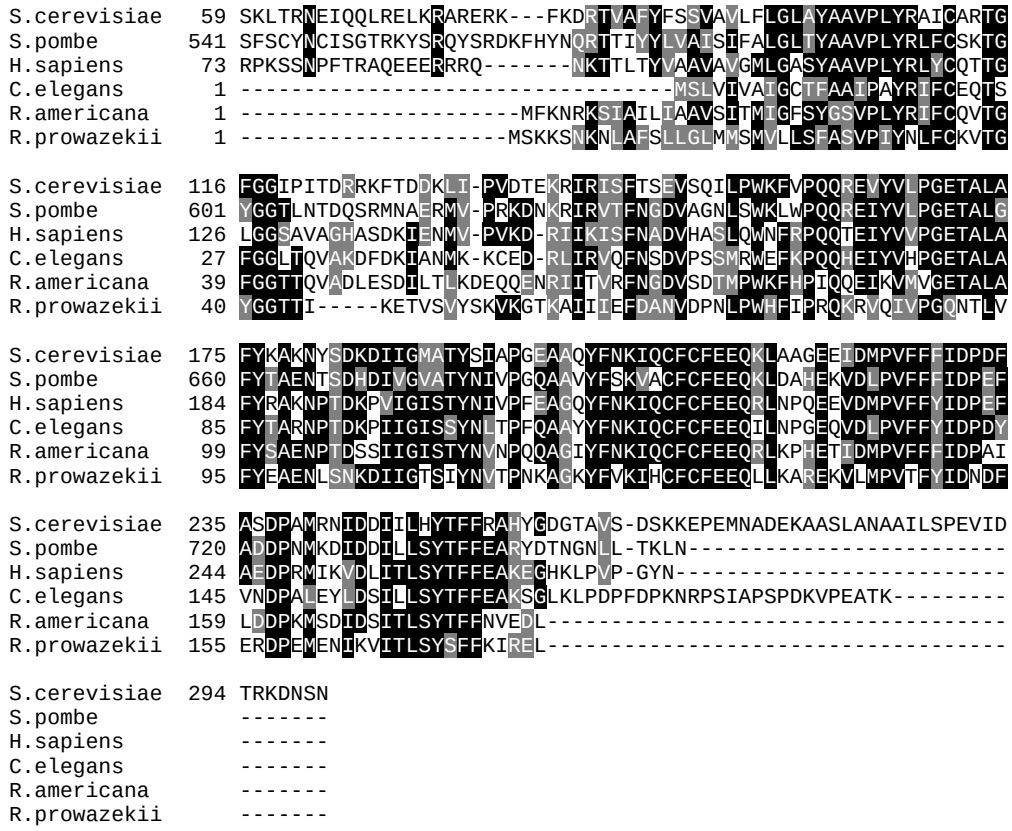


**Figure 41. Alignment of Cox11p from different organisms.** *S. cerevisiae* Cox11p is aligned with the respective amino acid sequences of the indicated organisms.

```
SpCox11p      1 MPILTCRYKILFLYNLRNCFTFQNQRCLIPYGTTTTIRWYNANFQAVQNNFSDYKNELIS
SpCox11bp     1 MPILTCRYKILFLYNLRNCFTFQNQRCLIPYGTTTTIRWYNANFQAVQNNFSDYKNELIS

SpCox11p     61 SHRPEASSLLDFLVKDQKKSGDISLHTKFNLYVDDLLKKSEKGQIKKFINDIKKDLATES
SpCox11bp    61 SHRPEASSLLDFLVKDQKKSGDISLHTKFNLYVDDLLKKSEKGQIKKFINDIKKDLATES

SpCox11p    121 QLPLSAPFKDESTRTMTDPQVLAYIHQSMPYQYASLYSVLTDLKIVNSDVSCKSQHILDC
SpCox11bp   121 QLPLSAPFKDESTRTMTDPQVLAYIHQSMPYQYASLYSVLTDLKIVNSDVSCKSQHILDC

SpCox11p    181 GKGPGIGALASYSVFPTPNSVSIVEENPFLKKIIYDIHHNIYPSTSPNPTSPVTLNRLPL
SpCox11bp   181 GKGPGIGALASYSVFPTPNSVSIVEENPFLKKIIYDIHHNIYPSTSPNPTSPVTLNRLPL

SpCox11p    241 GKKDSYTLVIASNKLLEMKSEKELFDYLRSLWSLVSNDGGLLVLCERGTKRGFSLIQRAR
SpCox11bp   241 GKKDSYTLVIASNKLLEMKSEKELFDYLRSLWSLVSNDGGLLVLCERGTKRGFSLIQRAR

SpCox11p    301 TFLLQKSKNTSDKQFNAHIVAPCPHDGRCPIDIENGVRANICSFKQHFFLSPFSRLYVPR
SpCox11bp   301 TFLLQKSKNTSDKQFNAHIVAPCPHDGRCPIDIENGVRANICSFKQHFFLSPFSRLYVPR

SpCox11p    361 SHRRSSDRSHYSYVVIQKGITRPLNNTTQRFKNDEDLLENVNVTSPTLKNWPRIIRPPLK
SpCox11bp   361 SHRRSSDRSHYSYVVIQKGITRPLNNTTQRFKNDEDLLENVNVTSPTLKNWPRIIRPPLK

SpCox11p    421 RDGHVIIDVCDSDARLRRNIVPKSQGKLAYRLARKSAWGDLFPLEGKVQSTSPSSKITKH
SpCox11bp   421 RDGHVIIDVCDSDARLRRNIVPKSQGKLAYRLARKSAWGDLFPLEGKVQSTSPSSKITKH

SpCox11p    481 LKDASSTYSINPPSYNKPKVERNTTADPIFVGKRFYSTNRHKAFSRFADFNSHRFPCIFT
SpCox11bp   481 LKDASSTYSINPPSYNKPKVERNTTADPIFVGKRFYSTNRHKAFSRFADFNSHRFPCIFT

SpCox11p    541 SFSCYNCISGTRNISRQYSRDKFHYNQRTTIYYLVAISIFALGLTYAAVPLYRLFCSKTG
SpCox11bp   541 SFSCYNCISGTRKYSRQYSRDKFHYNQRTTIYYLVAISIFALGLTYAAVPLYRLFCSKTG

SpCox11p    601 YGGTLNTDQSRMNAERMVPRKDNKRIRVTFNGDVAGNLSWKLWPQQREIYVLPGETALGF
SpCox11bp   601 YGGTLNTDQSRMNAERMVPRKDNKRIRVTFNGDVAGNLSWKLWPQQREIYVLPGETALGF

SpCox11p    661 YTAENTSDHDIVGVATYNIVPGQAAVYFSKVACFCFEEQKLDAHEKVDLPVFFFIDPEFA
SpCox11bp   661 YTAENTSDHDIVGVATYNIVPGQAAVYFSKVACFCFEEQKLDAHEKVDLPVFFFIDPEFA

SpCox11p    721 DDPNMKDIDDILLSYTFFEARYDTNGNLLTKLN
SpCox11bp   721 DDPNMKDIDDILLSYTFFEARYDTNGNLLTKLN
```

**Figure 42. Alignment of *S. pombe* Cox11p and Cox11bp.** SpCox11p is aligned with the SpCox11bp, a second version of the respective protein in *S. pombe*.

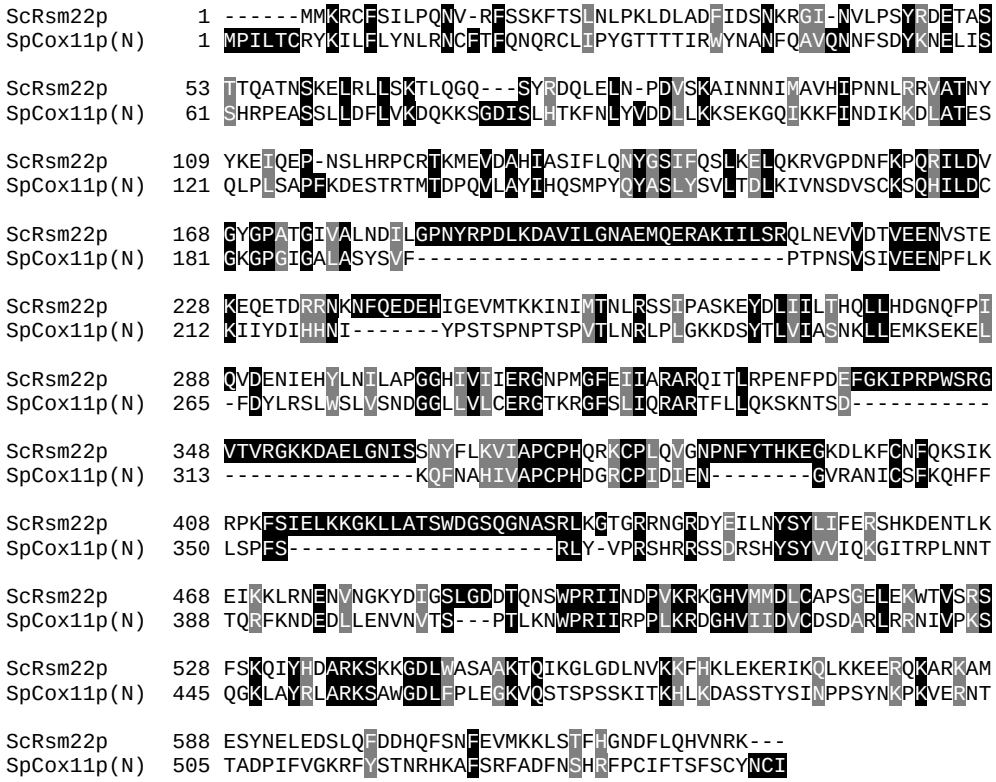


**Figure 43. Alignment of *S. cerevisae* Rsm22p and the N-terminal part of *S. pombe* Cox11p.** *S. cerevisiae* Rsm22p is aligned with the amino acid sequence of the N-terminal part of *S. pombe* Cox11p.

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# Curriculum vitae

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Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Die vorliegende Arbeit wurde am Institut für Genetik der Technische Universität Dresden unter der wissenschaftlichen Betreuung von Herrn Prof. Dr. rer. nat. habil. G. Rödel angefertigt.

Die Promotionsordnung wird anerkannt

Dresden, den 8. 12. 2005

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