Biogenesis of proteins of the mitochondrial intermembrane space: Identification and characterization of Mia40 in *Saccharomyces cerevisiae*

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To my father

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1. INTRODUCTION

1.1. Origin, structure and function of mitochondria

Mitochondria are ubiquitous organelles of eukaryotic cells that are involved in many cellular processes from energy production to apoptosis. This is quite astonishing, considering that mitochondria are believed to have evolved from a bacterial endosymbiont approximately two billion years ago (Margulis 1970). The recent sequence information of mitochondrial genomes and phylogenetic studies supported the idea of an α-proteobacterium inhabiting the cytosol of the first eukaryotes as the ancestor of mitochondria (Gray, Burger *et al.* 1999; Kurland and Andersson 2000; Herrmann 2003). Following the change from the endosymbiont to an organelle, a large fraction of the genetic information was transferred to the host's nucleus. In addition, the mitochondrial proteome has been complemented by hundreds of nuclear-encoded proteins of eukaryotic origin. Hence, about 99% of all mitochondrial proteins are synthesized as precursors in the cytosol and has to be imported into the organelle.

The translocation and integration of mitochondrial precursors is a challenging task as the organelle is bounded by two membranes of distinct lipid and protein composition. These two membranes separate two aqueous compartments: the intermembrane space (IMS) and the innermost matrix space. The outer membrane is strongly enriched in porins, which form channels in the lipid bilayer and allow the passage of water, many small molecules and ions. It contains also many proteins regulating the mitochondrial morphology and mediating apoptosis. The inner membrane is impermeable to polar molecules, thereby sustaining the electrochemical proton gradient, created by the activity of the respiratory chain. Compared to the outer membrane, the inner membrane has a considerably larger surface with two distinct subcompartments. These are: the inner boundary membrane, which is closely positioned to the outer membrane, and the cristae, which represent folds of the inner membrane into the matrix (Frey and Mannella 2000). The inner membrane is populated with the components of the respiratory chain, the ATP synthase complex, the protein import and insertion machineries and many metabolite transporters. The intermembrane space of mitochondria has a width of only a few nanometers but it harbours many proteins significant to the cell. Among these are components of the electron-transport chain, protein translocation factors, transporters for metal ions and redox equivalents, enzymes for metabolic processes and several apoptotic proteins. The IMS is connected to the cytosol by channels formed by porins in the outer membrane, therefore it is considered to have physicochemical features similar to those of the cytosol. The mitochondrial matrix is a space of a high density, enclosed by the inner membrane. It contains the mitochondrial DNA, specific mitochondrial ribosomes and a large number of enzymes. The size and coding capacity of the mitochondrial genome varies in different organisms and encodes rRNAs, tRNAs and essential mitochondrial proteins. In the matrix many metabolic processes occur, for example the ATP production, the citric acid cycle and fatty-acid oxidation. The diversity and complexity of the mitochondrial subcompartments reflect their highly specialized functions.

Mitochondria fulfill numerous and diverse tasks in eukaryotic cells. Their most well-known biochemical function is the generation of adenosine triphosphate (ATP) by oxidative phosphorylation. Mitochondria play essential roles in the iron-sulfur cluster biogenesis and synthesis of lipids, amino acids and heme (Scheffler 2001; Lill, Dutkiewicz *et al.* 2006). Furthermore, mitochondria are key players in cell stress response, apoptosis, calcium homeostasis and the generation/detoxification of reactive oxygen species (Newmeyer and Ferguson-Miller 2003). Since mitochondria perform so many essential roles in the life and death of the cell, it is not surprising that mitochondrial dysfunction has been associated with a

range of severe human disorders, e.g. Leber's hereditary optic neuropathy (LHON) or Parkinson's disease, and even the process of aging (Simon, Pulst *et al.* 1999; Chan 2006).

Mitochondria are dynamic organelles. There is a direct correlation between energy demand in the cell and mitochondrial abundance. Mitochondria move actively along the cytoskeletal elements and undergo constant fusion and fission events (Nunnari, Marshall *et al.* 1997; Reichert and Neupert 2002). The balance of these processes determines the mitochondrial morphology and position in the cell. The formation of reticular networks is an essential process in the normal function of mitochondria, and thus, the morphology of mitochondria is associated with the functions of cells. Mitochondria cannot be generated *de novo* but form from pre-existing organelles. This is achieved in a process that recruits new proteins, which are added to pre-existing subcompartments to a point where mitochondria divide in a fission event (Yoon and McNiven 2001).

1.2. Protein translocation into mitochondria

The importance of mitochondrial functions for eukaryotic cells and the involvement of mitochondrial dysfunctions in many human diseases have drawn much attention to the analysis of the mitochondrial proteome. Recently, detailed analyses on both the yeast *Saccharomyces cerevisiae* and human heart mitochondria led to the estimation that these mitochondria contain about 800 and 1.500 different proteins, respectively (Sickmann, Reinders *et al.* 2003; Taylor, Fahy *et al.* 2003). Notably, about a quarter of the identified proteins in either study were of unknown function, highlighting a number of mitochondrial constituents to be described in the future. Only 8 proteins in yeast and 13 in humans are encoded by mitochondrial DNA, while the great majority of mitochondrial proteins are nuclear-encoded. Therefore, the cell needs to deliver these proteins to the organelle to ensure the efficient function of mitochondria.

The mitochondrial precursor proteins are preferentially imported in a post-translational manner. However, some recent reports proposed that co-translational import also plays a role in the biogenesis of the organelle (Beddoe and Lithgow 2002; Marc, Margeot *et al.* 2002). Newly synthesized mitochondrial precursors are usually bound to molecular chaperones of the Hsp70 and Hsp90 families, as well as to some specific factors like mitochondrial import stimulation factor (MSF) in order to maintain an import-competent conformation in the cytosol (Deshaies, Koch *et al.* 1988; Murakami, Pain *et al.* 1988; Hachiya, Komiya *et al.* 1994; Young, Hoogenraad *et al.* 2003). Specific signals within the precursor proteins direct them to mitochondria and subsequently to their distinct subcompartment in the organelle. Sophisticated molecular machineries, named translocases, have evolved to mediate protein import and sorting and subsequent assembly into multi-subunit complexes (Neupert and Herrmann 2007). The present knowledge of mitochondrial protein translocation and sorting is mainly based on studies in fungi, but the mechanisms and components appear to be well conserved in animals and plants. An overview on the translocation and sorting routes in mitochondria is presented in Figure 1.

The initial entry of the mitochondrial preproteins is mediated by a high molecular weight machinery, termed the translocase of the outer mitochondrial membrane (TOM). Four different pathways downstream of the TOM complex perform the further translocation and assembly of proteins into a specific subcompartment. The TOB complex (topogenesis of mitochondrial outer membrane β-barrel proteins) mediates the insertion of the β-barrel precursors into the outer membrane. Precursor proteins, destined for the inner membrane or the matrix, are directed to one of the translocases of the inner membrane (TIM) after crossing the outer membrane. The TIM23 complex is responsible for the translocation of all presequence-containing precursors. The function of the TIM23 translocase requires the membrane potential across the inner membrane and energy in form of ATP. Hydrophobic

inner membrane proteins are directed with the help of the complexes of "small Tims" in the intermembrane space to the TIM22 translocase which inserts them into the inner membrane. Another translocase, the OXA1 complex, enables the export of proteins from the matrix site into the inner membrane and is used by proteins encoded in the mitochondrial genome, as well as by some nuclear encoded proteins.

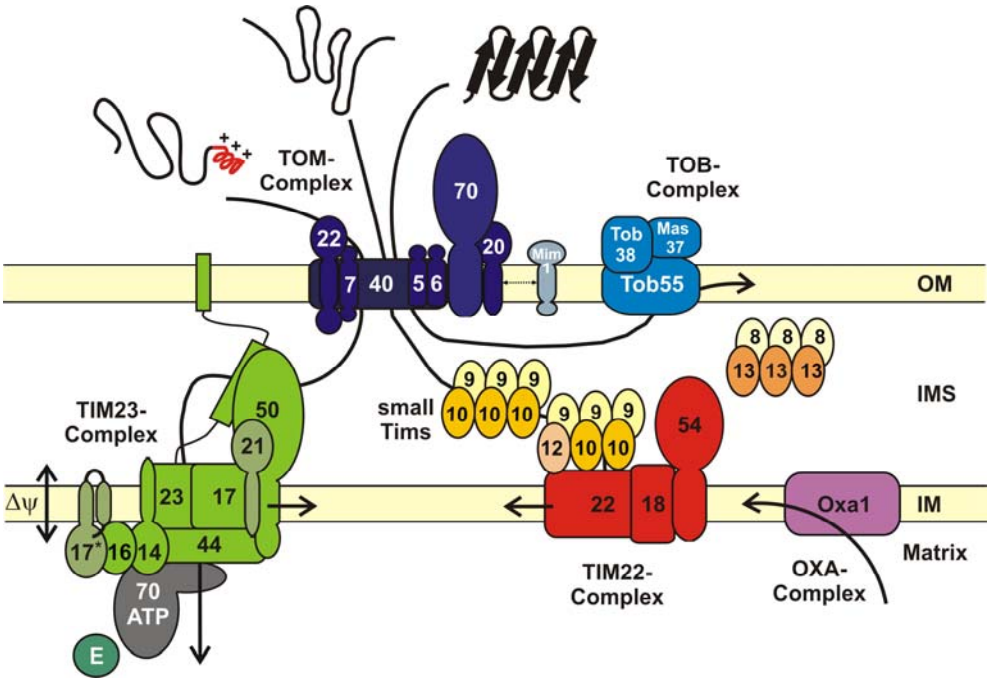

Figure 1. General pathways for import and sorting of mitochondrial preproteins. Preproteins first cross the outer membrane via the TOM complex. β-barrel proteins are transferred from the TOM complex to the TOB complex which mediates their insertion into the outer membrane. Matrix-destined preproteins are translocated further through the inner membrane via the TIM23 machinery. This process requires the membrane potential (ΔΨ) and the ATP-dependent action of the mtHsp70. The multispanning proteins of the inner membrane are guided by the "small Tims" across the intermembrane space to the TIM22 complex which inserts them into the inner membrane. The Oxa1 complex inserts proteins into the inner membrane from the matrix side.

1.2.1. Mitochondrial targeting signals

Precursors of mitochondrial proteins harbour sufficient information in order to direct them to the mitochondria. In most cases, proteins contain a cleavable N-terminal targeting signal sequence, also called presequence. However, many precursors, especially hydrophobic proteins of the outer and inner membranes, lack such presequences and instead possess internal targeting signals.

The amino-terminal targeting sequences are also named matrix targeting sequences (MTSs), as they direct proteins into the matrix in the absence of further sorting information. The N-terminal presequences usually consist of about 10-80 amino acids that form amphipathic α-helices enriched in positively charged, hydroxylated and hydrophobic residues (Von Heijne 1986; Roise and Schatz 1988). Specific primary sequence motifs have not been found. The physical interactions, which occur between the presequences and the translocation machinery, are not well understood, but data indicate that the basic and hydrophobic residues are vital for this process (Abe, Shodai *et al.* 2000). In the matrix the majority of the presequences are cleaved off by the mitochondrial processing peptidase MPP (Gakh, Cavadini *et al.* 2002) with a few exceptions, like the chaperonin 10 (Rospert, Junne *et al.* 1993).

Outer and inner membrane proteins often contain internal targeting sequences that are not removed after import and are not so well characterized. Outer membrane proteins with

single transmembrane domains harbour mitochondrial targeting signals in their hydrophobic anchors and the flanking positively charged residues. The β-barrel proteins of the outer membrane have their targeting information encoded in structural elements that engage different regions of the protein (Rapaport 2003). Some inner membrane proteins possess the classical presequences that are followed by a hydrophobic stretch which eventually arrest the translocation of these proteins at the level of the TIM23 complex. This results in lateral insertion into the inner membrane (Gärtner, Bomer *et al.* 1995; Rojo, Guiard *et al.* 1998). Interestingly, in some inner membrane proteins the presequence signals are located at the C-terminus of the hydrophobic regions and are proposed to form hairpin-loop structures during import (Foelsch, Guiard *et al.* 1996). Proteins of the metabolite carrier family of the inner membrane contain multiple signals distributed within the whole preprotein (Endres, Neupert *et al.* 1999; Curran, Leuenberger *et al.* 2002).

The intermembrane space (IMS) proteins can be classified into three categories based on their import characteristics. Members of the first class possess a classical N-terminal presequence, followed by the hydrophobic sorting domain (so called bipartite presequence). These proteins employ the TIM23 complex, sorted to the inner membrane by the hydrophobic segment, and are released into the IMS after a proteolytic cleavage. Typical examples for this class of IMS proteins are the cytochrome b_2, the fission component Mgm1 and the apoptotic factor Smac/Diablo (Glick, Brandt *et al.* 1992; Herlan, Bornhovd *et al.* 2004; Burri, Strahm *et al.* 2005). The second class consists of proteins with a low molecular weight that are imported by a "folding-trap" mechanism. Main feature of the members of this class are conserved cysteine residues, present within characteristic patterns. Following translocation through the TOM complex these proteins become folded by the acquisition of cofactors or by formation of disulfide bonds, which locks the proteins permanently in the IMS. The best characterized representatives of this class are cytochrome *c*, the small Tim proteins and the copper/zinc superoxide dismutase Sod1 (Dumont, Ernst *et al.* 1988; Diekert, Kroon *et al.* 2001; Field, Furukawa *et al.* 2003; Koehler 2004). Proteins of the third class do not contain mitochondrial presequences and associate with binding sites at the surface of the outer or inner membrane. The best studied members of this group are the heme lyases. The sorting signal in heme lyases seems to be internal and to comprise a pattern of hydrophilic residues, like in cytochrome *c* heme lyase (CCHL) (Steiner, Zollner *et al.* 1995; Diekert, Kispal *et al.* 1999).

1.2.2. Translocases of the outer mitochondrial membrane

1.2.2.1. The TOM translocase

The translocase of the outer mitochondrial membrane (TOM) acts as a general entry gate for virtually all nuclear-encoded mitochondrial proteins into mitochondria. The TOM complex consists of seven integral membrane proteins.

Tom70 and Tom20 are the major receptor components that recognize substrate proteins. They are anchored with N-terminal transmembrane domains in the outer membrane and expose hydrophilic segments to the cytosol. Precursors with N-terminal presequences interact with the Tom20 receptor, while others with internal targeting signals interact with Tom70, often together with cytosolic molecular chaperones (Brix, Dietmeier *et al.* 1997). Both receptors transfer their substrates to Tom22, which functions as a central organizer and is important for the integrity of the TOM complex (Mayer, Neupert *et al.* 1995; van Wilpe, Ryan *et al.* 1999). The preprotein-conducting channel of the TOM complex consists of two to three cation-selective pores with a diameter of about 20Å (Kunkele, Heins *et al.* 1998).These import pores consist most likely of several molecules of Tom40, which is the only subunit essential for viability in yeast. Tom40 spans the outer membrane several times and forms probably a β-barrel structure (Hill, Model *et al.* 1998). Purified Tom40 forms a single channel

with similar dimensions as the TOM pores (Ahting, Thieffry *et al.* 2001). The diameter of the pore is sufficient for the passage of up to two α-helical segments, but not folded domains. The three small associated subunits of the TOM translocase, Tom5, Tom6 and Tom7, form tail-anchored, α-helical structures with just a few residues exposed to the IMS. The deletion of single small TOM proteins leads to only minor effects, but the simultaneous loss of all three proteins is lethal (Dietmeier, Honlinger *et al.* 1997; Dekker, Ryan *et al.* 1998). The small TOM subunits seem to regulate the stability and the interactions within the complex, but their individual functions are still not well understood.

The driving force for translocation across the outer membrane seems to be the sequential increase in the binding affinity of different parts of the TOM complex to the precursor protein (Komiya, Rospert *et al.* 1998). The so-called *cis*-binding sites are present on the cytosolic domains of Tom70, Tom20 and Tom22. Cross-linking experiments suggest that the presequence of the substrate protein at this early stage of import is already in the vicinity of Tom40 (Rapaport, Neupert *et al.* 1997). Further movement of the presequence to the inner, IMS-exposed surface of the TOM complex, also referred to as *trans*-binding site, completes the translocation of the precursor across the outer membrane. The features of the *trans* site are not fully clear, but Tom22, Tom7 and Tom40 appear to play a role in precursor binding in the IMS (Bolliger, Junne *et al.* 1995; Mayer, Neupert *et al.* 1995). Thus, different affinities of the binding sites for the precursor protein in the TOM complex drive the vectoral translocation across the outer membrane and can even enable unfolding of the preprotein and protection from aggregation (Rapaport, Kunkele *et al.* 1998; Esaki, Kanamori *et al.* 2003).

1.2.2.2. The TOB complex

The TOM complex mediates not only the translocation of precursors across, but also the insertion of proteins into the outer membrane. An interesting class represent the β-barrel membrane proteins which contain multiple membrane-spanning β-strands, each formed by 9–11 amino acid residues (Wimley 2003). Only the outer membranes of mitochondria and chloroplasts house β-barrel proteins in eukaryotic cells, displaying the evolutionary origin of these organelles from bacterial ancestors. In mitochondria, porin, Tom40, Tob55, Mdm10 and Mmm2 are β-barrel proteins. Their insertion into the membrane depends on the function of both the TOM complex and the recently identified translocase of outer membrane β-barrel proteins (TOB) (also called sorting and assembly machinery-SAM complex) (Paschen, Waizenegger et al. 2003; Wiedemann, Kozjak et al. 2003). Precursors of β-barrel proteins first interact with the receptors of the TOM complex, mainly Tom20, and then translocate through the TOM pore to insert into the outer membrane from the IMS site. It was reported that β-barrel precursors interact with small Tim proteins in the IMS on their route from the TOM to the TOB complex (Hoppins and Nargang 2004; Wiedemann, Frazier *et al.* 2004).

The main component of the TOB complex is the essential protein Tob55 (also named Sam50) (Paschen, Waizenegger *et al.* 2003; Wiedemann, Kozjak *et al.* 2003; Gentle, Gabriel *et al.* 2004). Tob55 is homologous to the bacterial Omp85/YaeT protein (Voulhoux, Bos *et al.* 2003). Homologues of Tob55 can be also found throughout the complete eukaryotic kingdom. Tob55 collaborates with two further components of the TOB complex, Tob38 (Sam35) and Mas37 (Sam37), which are peripheral membrane proteins located at the cytosolic surface of the translocase (Wiedemann, Kozjak *et al.* 2003; Ishikawa, Yamamoto *et al.* 2004; Milenkovic, Kozjak *et al.* 2004; Waizenegger, Habib *et al.* 2004). Depletion of Tob55 or Tob38 inhibits the import and assembly of newly imported β-barrel proteins. Mas37 is dispensable in yeast, but the deletion of *mas37* results in defects in the biogenesis of β-barrel proteins at high temperatures. Notably, the morphology protein Mdm10 has been identified as the fourth subunit of the TOB complex (Meisinger, Rissler *et al.* 2004). While Mdm10 is required for the integration of Tom40 into the outer membrane, it is not essential for the

biogenesis of other β-barrel proteins, like porin. A further protein found in the outer membrane, Mim1, appears to be needed for the assembly of a functional TOM complex (Waizenegger, Schmitt *et al.* 2005). The exact functions of all the proteins within the TOB complex are still not well understood. The high conservation of Tob55, however, indicates that it is the essential functional component of the TOB complex and that the other proteins play rather supplementary functions.

1.2.3. Translocases of the inner mitochondrial membrane

After crossing the outer membrane through the TOM complex, all preproteins destined for the inner membrane and the matrix and some destined for the intermembrane space are directed to one of the translocases of the inner membrane (TIMs).

1.2.3.1. The TIM 23 translocase

Preproteins with the classical mitochondrial N-terminal presequence are directed to the TIM23 translocase. Transport through the TIM23 translocase is energetically driven by the electrical membrane potential across the inner membrane ($\Delta\psi$) and the hydrolysis of ATP. The central part of the TIM23 translocase is the membrane-embedded core, consisting of the essential integral membrane proteins Tim23 and Tim17, together with Tim50 and the recently identified Tim21 (Chacinska, Lind *et al.* 2005). Tim50 is a receptor, which associates with presequence-containing proteins entering the IMS and directs them to the TIM23 translocase (Geissler, Chacinska *et al.* 2002; Yamamoto, Esaki *et al.* 2002; Mokranjac, Paschen *et al.* 2003). Tim17 and Tim23 have four predicted membrane-spanning domains and form a 90kDa core complex (Kubrich, Keil *et al.* 1994). The precise role of Tim17 is still not clear. Purified Tim23, reconstituted into liposomes, appears to form a voltage-sensitive high-conductance channel with a width of ~13-24 Å (Truscott, Kovermann *et al.* 2001). The N-terminal region of Tim23 was shown to be integrated into the outer membrane, thereby linking both mitochondrial membranes (Donzeau, Kaldi *et al.* 2000). Precursor proteins are translocated into the core TIM23 channel in a manner dependent on a membrane potential ($\Delta\psi$). According to recent findings, Tim21 interacts with Tom22 and Tim17 and might determine whether the bound preprotein should be integrated into the inner membrane or translocated through into the matrix (Chacinska, Lind *et al.* 2005; Mokranjac, Popov-Celeketic *et al.* 2005).

Precursors containing a hydrophobic segment after the presequence can be sorted directly into the inner membrane by the TIM23 complex. Precursors requiring complete transfer into the matrix employ additionally the action of the second part of the TIM23 translocase, the ATP-dependent import motor (also named presequence translocase-associated protein import motor-PAM). The central player in the import motor is the mitochondrial Hsp70 (mtHsp70) in the matrix. The peripherally associated Tim44 protein appears to facilitate binding of mtHsp70 to the coming precursor, while the ATPase activity of the chaperone is stimulated by the co-chaperone, Tim14 (Pam18) (Mokranjac, Sichting *et al.* 2003; Truscott, Voos *et al.* 2003). The association of Tim14 with the import motor is modulated by a further component, Tim16 (Pam16) (Frazier, Dudek *et al.* 2004; Kozany, Mokranjac *et al.* 2004). The nucleotide exchange is mediated by Mge1, enabling further round of cycling of mtHsp70. Two recently identified components, Pam17 and Mmp37/Tam41, are suggested to be involved in the assembly or function of the import motor (van der Laan, Chacinska *et al.* 2005). However, the specific molecular role of these proteins remains elusive. A final step for matrix proteins is, in most cases, the proteolytic removal of the presequence by the mitochondrial processing peptidase (MPP) (Gakh, Cavadini *et al.* 2002). Then, the proteins are folded to their native state with the help of different factors, such as mtHsp70, the chaperonin Hsp60 and the Peptidyl-Prolyl cis/trans isomerase.

1.2.3.2. The TIM22 translocase

The TIM22 complex binds only membrane proteins with many transmembrane segments, destined for integration into the inner membrane. Members of the metabolite carrier family and the membrane-embedded TIM subunits Tim17, Tim22, and Tim23 are the known substrates of this pathway. The TIM22 complex consists of the three membrane proteins Tim22, Tim54, and Tim18, to which small Tim proteins Tim9–Tim10 peripherally bind via the adaptor protein Tim12. Soluble small Tim proteins, forming hexameric complexes consisting of Tim9 and Tim10 or Tim8 and Tim13, respectively, mediate the passage of polytopic membrane proteins through the aqueous IMS to the mitochondrial inner membrane (Curran, Leuenberger *et al.* 2002; Vial, Lu *et al.* 2002). Tim22 is an essential inner membrane protein and structurally related to Tim23 and Tim17 (Sirrenberg, Bauer *et al.* 1996). Recombinant Tim22 was shown to form a voltage-activated channel that specifically responds to the addition of peptides resembling internal targeting signals (Kovermann, Truscott *et al.* 2002). The exact functions of Tim54 and Tim18 are not well-known. The translocation and insertion of inner membrane proteins by the TIM22 complex does not require ATP, but depends on the electrochemical potential across the inner membrane. The carrier precursors are inserted into the twin-pore channel of the TIM22 complex in loop conformations. The membrane potential across the inner membrane activates the channel protein Tim22 and drives the insertion process, followed by a final assembly into functional dimers (Rehling, Model *et al.* 2003). The import process of the TIM subunits Tim17, Tim22 and Tim23 is not as well characterized as the one of carrier proteins, but appears to use the same mechanisms (Leuenberger, Bally *et al.* 1999; Paschen, Rothbauer *et al.* 2000). Notably, Tim23 uses both the Tim9-Tim10 and the Tim8-Tim13 complexes in the IMS for its biogenesis.

1.2.3.3. The OXA1 translocase

A group of inner membrane proteins (both nuclear and mitochondrial encoded) are inserted into the inner membrane from the matrix. The protein translocase mediating this process is the OXA1 complex (Hell, Neupert *et al.* 2001). Oxa1p is a member of the highly conserved Oxa1p/YidC/Alb3 protein family whose members are present also in archaea, bacteria and thylakoid membranes of chloroplasts (Herrmann and Neupert 2003). Characteristically, proteins from this family contain five transmembrane segments of similar lengths which form a conserved core of the protein. In addition to the membrane-spanning regions, Oxa1 has a long α-helical C-terminal domain exposed in the matrix. This domain binds mitochondrial ribosomes and thereby it brings the precursors to the site of their integration into the lipid bilayer (Jia, Dienhart *et al.* 2003; Szyrach, Ott *et al.* 2003). Proteins that employ the OXA1 complex for their membrane insertion include the mitochondrial-encoded subunit 2 of the cytochrome oxidase complex (Cox2p) or the nuclear-encoded subunit 9 of the F_oF_1-ATPase of *N. crassa* and Oxa1 itself (Hell, Herrmann *et al.* 1997; Herrmann, Neupert *et al.* 1997).

1.3. Formation of disulfide bonds in living cells

The biologically active, three-dimensional structures of proteins are encoded by their amino-acid sequences and represent their energetically most favorable conformations. On the way to the native state, molecular chaperones help the newly synthesized proteins by protecting them from premature association or aggregation. Moreover, folding catalysts accelerate the rate-limiting steps in the folding pathway. One of the steps, which are rate-limiting in protein folding, is the formation of correct disulphide bonds between cysteine residues.

Introduction

Cysteines are one of the most rarely used amino acids in proteins of all organisms (Pe'er, Felder *et al.* 2004). If conserved, they play usually essential roles in the structure of proteins by forming disulfide bonds or by coordinating metal ions. Furthermore, cysteines often are present in the active sites or regulate the protein's activation and deactivation due to the unique biochemistry and high reactivity of the thiol group (Giles, Giles *et al.* 2003; Linke and Jakob 2003). Formation of a disulphide bond from the thiol groups of two cysteine residues generates two protons and two electrons. Therefore, there is a need of an oxidant which accepts the two electrons. Thiol-disulfide exchange reactions mediate the oxidation and the reduction of disulfide bonds between the active site cysteine residues of the enzyme and the cysteine residues in the target protein (Fig.2). The accessibility of the reactive groups and the difference of the redox potential between the redox partners influence the rate of the thiol-disulfide exchange reaction (Englander and Kallenbach 1983). For proteins with many disulfide bonds, the correct spatial arrangement of the disulfide bonds is absolutely crucial. Failure to form or to position the disulfide bonds correctly results in protein misfolding, leading to aggregation and degradation by proteases.

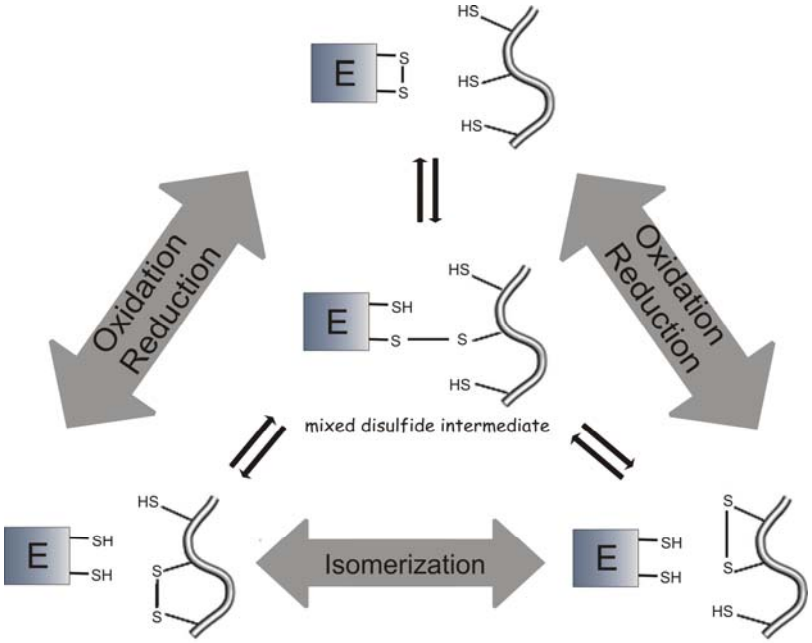

Figure 2. Thiol-redox reactions. The disulfide bond reduction, oxidation and isomerization between the enzyme (E) and a substrate (S) protein are presented. The complex in the middle shows one of the six possible mixed disulfide intermediates.

In vitro protein folding studies have shown that the presence of oxygen (or other strong oxidant like oxidized glutathione) is sufficient to promote disulfide bond formation in proteins (Anfinsen 1973). Nevertheless, air oxidation is a slow process and can take several hours or even days. In contrast, formation of disulfide bonds in the cell occurs much faster, within minutes or even seconds after synthesis. For example, the refolding of RNAse A, which contains four disulfide bonds, needs several hours *in vitro* but less than 2 min *in vivo*. The inconsistency between the oxidation rates *in vivo* and *in vitro* led to the discovery of the first catalyst of disulfide bond formation, the protein disulfide isomerase (PDI) about 40 years ago (Goldberger, Epstein *et al.* 1963). PDI is a eukaryotic protein in the endoplasmic reticulum and it is a member of a group of proteins involved in disulfide bond formation. The search for factors mediating disulfide bond formation in bacteria resulted in the identification of DsbA by two separate groups many years later (Bardwell, McGovern *et al.* 1991; Akiyama, Kamitani *et al.* 1992). Due to the accessibility of genetic tools and the simplicity of protein biochemistry in prokaryotes a fast progress was achieved in understanding the mechanisms of

disulphide bond formation in the bacterial periplasm. The enquiries in prokaryotes have, in turn, encouraged new studies on the disulfide bond formation in eukaryotes, which led to the discovery of redox systems in higher organisms similar to those present in bacteria.

A large number of enzymes catalyzing protein disulfide formation belong to a set of thiol-disulfide oxidoreductases found in all cells. Many of these proteins can be grouped into the so-called thioredoxin superfamily that is described by an active site containing a CXXC motif (cysteine residues separated by two amino acids) and by a thioredoxin fold (Holmgren, Soderberg *et al.* 1975). In extracytoplasmic compartments these proteins usually act as oxidants, whereas the enzymes found in the cytoplasm perform mainly reductive steps. Recently, an increasing number of enzymes are being identified that do not belong to the thioredoxin superfamily. Their active site cysteine pairs are separated by more than two amino acids and their three-dimensional structures are completely different from thioredoxin. Moreover, these enzymes often utilize small molecules, such as FAD, NADPH, NADH and quinones, as electron donors and acceptors. *In vivo*, the final electron acceptor for thiol oxidation is typically O_2, with the exception of anaerobic conditions when an appropriate anaerobic electron acceptor, e.g. fumarate, is used. The ultimate source of electrons for disulfide reduction is frequently NADPH.

Proteins with stable disulfide bonds are usually secreted into the extracellular space or widely found in the periplasm in bacteria and in the endoplasmatic reticulum in eukaryotic cells. In contrast, disulfide bonds are very rare in the cytosolic compartments of most organisms, as a result of the reductive properties of the cytosol (Derman and Beckwith 1991; Kadokura, Katzen *et al.* 2003). Occasionally disulfide bonds are formed transiently in the cytosol as part of the catalytic mechanism or are used to regulate enzymatic activity. One good example is the bacterial heat shock protein Hsp33, which acts as a molecular chaperone with a complex functional regulation. The transcription of the Hsp33 gene is under heat shock control, while on posttranslational level the Hsp33 protein is regulated by oxidative stress. The redox sensor of Hsp33 is found in the C-terminus of the protein and involves four conserved cysteine residues. These cysteines under non-stress conditions bind one zinc ion, while under oxidizing conditions they form two intramolecular disulfide bonds, resulting in the activation of the Hsp33 (Jakob, Eser *et al.* 2000). This redox-regulated chaperone activity of Hsp33 specifically protects proteins and cells from the damaging effects of the reactive oxygen species. Other exceptions are the recently discovered disulfide-bonded proteins in the cytoplasm of certain archaea, e.g. the protein disulfide oxidoreductases (PDOs) (Mallick, Boutz *et al.* 2002; Ladenstein and Ren 2006).

1.3.1. Bacterial periplasm

Important advances in understanding the most fundamental aspects of protein disulphide bond formation have come from studying the pathways found in the periplasm of bacterial cells (Kadokura, Katzen *et al.* 2003; Messens and Collet 2006). In Gram negative bacteria, such as *Escherichia coli*, periplasmic oxidoreductases, termed Dsb (for disulphide bond), are involved in two pathways of disulphide bond formation: (1) the oxidative pathway, which is responsible for the introduction of disulfides, and (2) the isomerization pathway, which shuffles incorrectly formed disulfides. (Fig.3). Most of the Dsb proteins are members of the thioredoxin superfamily and harbour in their active site the conserved CXXC motif.

The DsbA-DsbB pathway is responsible for the *de novo* disulfide-bond formation. DsbA is a small soluble protein, whose two active site cysteine residues are present in a CXXC motif. DsbA from *E. coli* was the first disulfide-catalyzing protein to be structurally characterized, revealing a thioredoxin fold with an inserted helical domain (Martin, Bardwell *et al.* 1993). The oxidized DsbA, containing a highly unstable disulfide bond, reacts fast with unfolded substrate proteins and donates to them a disulfide bond. The reduced DsbA is

reoxidized by the integral membrane protein DsbB. The recent crystal structure of *E. coli* DsbB–DsbA complex showed that the mobile periplasmic loop of DsbB interacts with DsbA and that DsbB consists of an anti-parallel four-helix bundle in the membrane (Inaba, Murakami *et al.* 2006). Notably, this core structure of DsbB has similar architecture as the soluble eukaryotic analogues, Ero1p and Erv2p. After donating the disulfide to DsbA, DsbB is reoxidized by an oxidized ubiquinone that is regenerated subsequently by the electron transport chain (Bader, Muse *et al.* 1999; Kobayashi and Ito 1999). Thus, DsbB links the disulfide bond formation to the electron transport chain. Under anaerobic conditions, menaquinone can replace ubiquinone and transports electrons to alternative electron acceptors such as fumarate or nitrate.

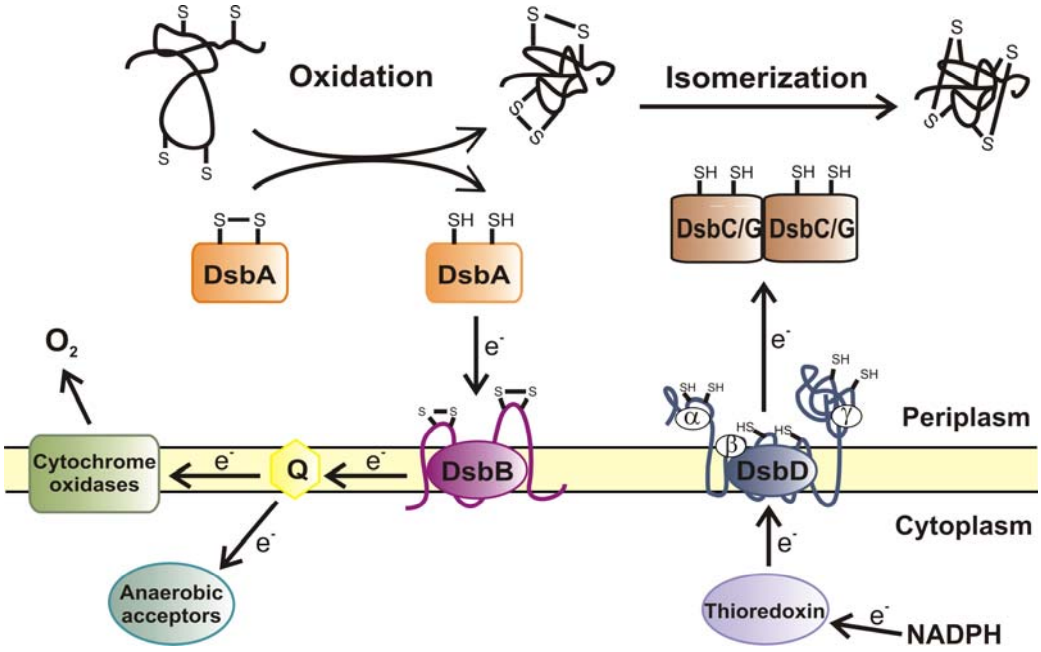

Figure 3. Protein oxidation and isomerization pathways in *E.coli* periplasm. The soluble thiol-disulphide oxidoreductase DsbA interacts with a newly translocated protein and introduces a disulfide bond into a target protein (left). The reduced DsbA is released and then reoxidized by the membrane protein DsbB. Next, the electrons flow from DsbB to a quinone cofactor (Q), further to cytochrome oxidases and ultimately they are transferred to oxygen under aerobic conditions. Under anaerobic conditions, DsbB passes the electrons to menaquinone and then to nitrate or fumarate. Another pathway in the periplasm catalyzes isomerization of incorrectly formed disulphide bonds (right). The thiol-disulphide oxidoreductases DsbC and DsbG mediate this process and they are kept in a reduced state by the membrane protein DsbD. To be active DsbD is reduced by the cytoplasmic thioredoxin, which gains electrons from the cytosolic NADPH.

Since DsbA has no proofreading ability, the formation of wrong disulfide bonds in proteins with many S–S bonds is corrected efficiently by the disulfide isomerases DsbC and DsbG (Missiakas, Georgopoulos *et al.* 1994). They share about 30% sequence identity and act as homodimers. Each monomer consists of a C-terminal catalytic thioredoxin domain, which is connected to the N-terminal dimerization domain by a α-helix. Two subunits form a V-shape, where the inner surface is extensively hydrophobic and represents most likely the substrate binding site (McCarthy, Haebel *et al.* 2000; Heras, Edeling *et al.* 2004). As DsbC and DsbG differ in their dimerization domains and in the putative protein-binding clefts, they most likely acquire different substrate specificities. To be active as disulphide isomerases, DsbC and DsbG need to remain reduced within the oxidizing periplasm. This is achieved by the inner membrane redox-active protein DsbD (Missiakas, Schwager *et al.* 1995). DsbD has a molecular mass of 59 kDa and is, thus, the largest protein in the Dsb protein family. DsbD has three diverse domains:1) a N-terminal immunoglobulin-like periplasmic domain (α-

domain), 2) a hydrophobic core with eight transmembrane stretches (β-domain), 3) a C-terminal thioredoxin-like periplasmic segment (γ-domain). Each fragment contains two conserved cysteine residues that are important for DsbD activity. To be able to reduce DsbC and DsbG, the DsbD itself needs to be in a reduced state. Genetic studies have shown that DsbD is maintained reduced by the cytosolic thioredoxin in an NADPH-dependent reaction (Rietsch, Belin *et al.* 1996; Rietsch, Bessette *et al.* 1997). It has been recently proposed that the transfer of reducing equivalents across the cytoplasmic membrane occurs by a cascade of thiol-disulfide exchange reactions within the DsbD protein (Katzen and Beckwith 2000; Collet, Riemer *et al.* 2002). The electrons flow from thioredoxin to the β-domain of DsbD, then to the γ- and the α-domains of DsbD and finally to DsbC or DsbG.

In the oxidative pathway, DsbA has to be kept oxidized to act as a disulphide donor. On the other hand, DsbC must be maintained reduced to function as a disulphide isomerase. Since these two pathways are present within the same compartment, the question of how they are kept separated appears. The barrier inhibiting any cross-talk between the two pathways is the dimerization of DsbC, which ensures protection of the DsbC active-site cysteines from oxidation by DsbB (Bader, Xie *et al.* 2000; Bader, Hiniker *et al.* 2001).

1.3.2. Endoplasmatic reticulum in eukaryotes

The endoplasmic reticulum (ER) provides an environment that is highly optimized for oxidative protein folding. A combination of genetic and biochemical studies using the yeast *Saccharomyces cerevisiae*, and more recently mammalian and plant systems, have revealed the proteins and mechanisms behind this fundamental protein folding process. Two pathways for the formation of disulfide bonds in proteins have been described in the ER of eukaryotes (Fig.4). The main pathway involves the membrane-associated flavoprotein Ero1 (ER oxidoreductin 1) that transfers oxidizing equivalents directly to protein disulfide isomerase (PDI), which then oxidizes the substrate proteins. In fungi, a second protein oxidation pathway exists, where another ER oxidase, known as Erv2, catalyzes disulfide bond formation also by interaction with PDI and its oxidation. A wealth of genomic sequencing data has revealed an abundance of enzymes sharing homology with Ero1, Erv2, or PDI. Currently, two Ero1 paralogues, three Erv2-like proteins and seventeen PDI homologues have been confirmed in human cells. In yeast, one Ero1 protein, two Erv2-like proteins and five PDI homologues have been identified (for review see (Sevier and Kaiser 2006).

Protein disulfide isomerase (PDI) is an essential protein in yeast and an astonishingly versatile enzyme. The formation, reduction or isomerization of disulphide bonds can be performed by the PDI, depending on the redox environment and the features of the substrate proteins (Wilkinson and Gilbert 2004). However, the specific contribution of PDI to the formation of new disulfides versus rearrangement of non-native disulfides is poorly understood. The PDI homologues compose a superfamily that is characterized by the presence of one or more domains with a predicted thioredoxin fold, a signal sequence and an ER retention signal. PDI itself contains four thioredoxin domains: two catalytic, a-type, domains with a CXXC motif and two non-catalytic, b-type, domains with a thioredoxin-like fold but no active site (Tian, Xiang *et al.* 2006). Within the PDI superfamily proteins with zero to four a-type domains, with only b-type domains or proteins containing non-traditional active site sequences (CXXS and SXXC) can be found. The characterized members of the PDI superfamily perform distinct functions and participate in specific ER folding pathways.

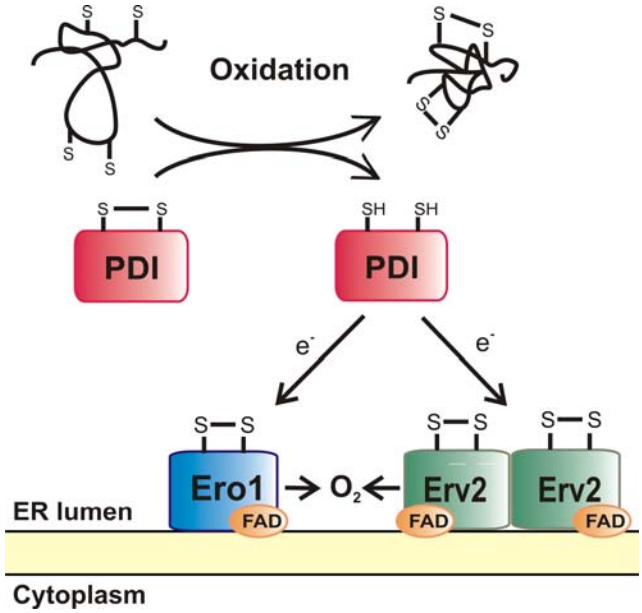

Figure 4. Protein oxidation pathways in the endoplasmic reticulum of *Saccharomyces cerevisiae*. Two parallel pathways provide oxidizing equivalents for formation of disulphide bonds in the endoplasmic reticulum (ER). The first pathway is the major one in the ER and key player here is the membrane-associated oxidase Ero1. Ero1 oxidizes the protein disulphide isomerase (PDI), which then introduces disulfide bonds into the substrate proteins. The second pathway, described so far only in fungi, involves the oxidase Erv2, which also oxidizes PDI. Both Ero1 and Erv2 receive oxidizing equivalents from a flavin adenine dinucleotide (FAD) cofactor and the final electron acceptor appears to be oxygen.

The oxidase Ero1, with the use of its cofactor flavin adenine dinucleotide (FAD), can recharge the reduced PDI and is indispensable for the introduction of oxidizing equivalents into the ER (Frand and Kaiser 1998; Pollard, Travers *et al.* 1998; Frand and Kaiser 1999; Tu, Ho-Schleyer *et al.* 2000). Ero1 is tightly associated with the ER membrane and contains two active-site cysteine pairs, forming CXXXXC and CXXC pattern, which contribute to the oxidative activity of this protein (Frand, Cuozzo *et al.* 2000; Frand and Kaiser 2000). Two mammalian homologues of the yeast Ero1 have been identified, Ero1α and Ero1β, which both can complement Ero1-deficient yeast strain and therefore seem to have role in oxidative folding analogous to Ero1 (Cabibbo, Pagani *et al.* 2000; Pagani, Fabbri *et al.* 2000).

Apart from the Ero1 homologues, another ER protein, Erv2, can restore viability to *S. cerevisiae* strain lacking Ero1, but only when overexpressed (Gerber, Muhlenhoff *et al.* 2001; Sevier, Cuozzo *et al.* 2001). Erv2p is also a FAD-binding protein and a member of a large family of thiol oxidases found in various eukaryotic organisms and in some viruses. The Erv2 activity is dependent on a pair of cysteines found in a CXXC motif in a highly conserved region, as well as on a second pair of cysteines in a CXC arrangement in the C-terminal fragment of the protein (Gross, Sevier *et al.* 2002). Erv2, like Ero1, can reoxidize reduced PDI. However, Erv2 is not essential and deletion of the *ERV2* has a moderate effect on protein oxidation, in contrast to the yeast *ERO1* deletion (Sevier, Cuozzo *et al.* 2001). Thus, the Erv2 pathway appears to be either a minor pathway for protein disulfide bond formation or is specialized for a group of not yet identified, non-essential proteins.

As the crystal structures of both Ero1 and Erv2 were solved, it became clear that these proteins are not only functionally but also structurally related, despite the lack of sequence similarity (Gross, Sevier *et al.* 2002; Gross, Kastner *et al.* 2004). The catalytic core of Ero1 and Erv2 is composed of a bundle of four anti-parallel α-helices that form the flavin-binding segment. The active site CXXC pair of Ero1 and Erv2 is positioned between two of the helices, near the stably bound FAD cofactor. The second cysteine pair in these proteins, the

CXXXXC motif in Ero1 and the CXC pattern in Erv2, is situated on a flexible segment in close proximity to the CXXC pair. The two oxidases function by a transfer of disulfide bonds from the rigid CXXC motif to the cysteine pair in the flexible loop, which then donates disulfide bonds to PDI. Notably, while the disulfide shuttle in Ero1 is intramolecular, the thiol-transfer mechanism of Erv2 requires its dimerisation (Gross, Sevier *et al.* 2002; Gross, Kastner *et al.* 2004). The CXC pair of one subunit of Erv2 then provides the disulfide bond for the CXXC pair of the other Erv2 subunit.

1.4. Proteins with disulfide bonds in the intermembrane space of mitochondria

According to most of the cell biology textbooks, formation and maintenance of disulfide bonds in proteins is limited to the secretory compartments of the eukaryotic cell. Recent discoveries of few disulfide-bonded proteins in the cytosol and mitochondria challenged that view and led to a growing interest in the role of cysteine residues in the reducing compartments of cells. The mitochondrial intermembrane space (IMS) is believed to be in equilibrium with the reducing cytosol, with free exchange of metabolites via porin channels. Interestingly, many proteins in the intermembrane space contain disulfide bonds (Table 1). In all of these proteins, the cysteine residues are highly conserved, often form specific motifs and play structural or catalytic roles. Although the IMS is reducing, this compartment seems to have developed mechanisms to form and maintain disulfide bonds in proteins.

Table 1. Proteins for which disulfide bonds in the IMS have been reported.

Protein	Motif	Function	Reference
Rieske	None	Subunit of complex III	Iwata, Saynovits *et al.* 1996
CCS	CX_2C, CXC	Copper chaperone for Sod1	Lamb, Torres *et al.* 2001
Sod1	None	Superoxide dismutase	Field, Furukawa *et al.* 2003
Tim8	Twin CX_3C	Protein import component	Curran, Leuenberger *et al.* 2002
Tim9	Twin CX_3C	Protein import component	Curran, Leuenberger *et al.* 2002
Tim10	Twin CX_3C	Protein import component	Curran, Leuenberger *et al.* 2002
Tim13	Twin CX_3C	Protein import component	Curran, Leuenberger *et al.* 2002
Cox12	CX_9C, $CX_{10}C$	Subunit of complex IV	Tsukihara, Aoyama *et al*. 1995
Cox17	Twin CX_9C	Copper chaperone	Arnesano, Balatri *et al.* 2005
Cox19	Twin CX_9C	Assembly factor for complex IV	Nobrega, Bandeira *et al.* 2002
Cox23	Twin CX_9C	Assembly factor for complex IV	Barros, Johnson *et al.* 2004
Sco1	CX_3C	Assembly factor for complex IV	Williams, Sue *et al.* 2005
Erv1	Two CX_2C	Sulfhydryl oxidase	Lee, Hofhaus *et al.* 2000

1.4.1. Cu/Zn superoxide dismutase Sod1 and the copper chaperone CCS

A first line of defence against reactive oxygen species includes the superoxide dismutase enzymes that catalyze the disproportionation of superoxide to hydrogen peroxide and water. The copper/zinc-superoxide dismutase Sod1 and its copper chaperone CCS are located mainly in the cytosol, but a fraction is also found in the intermembrane space of mitochondria, peroxisomes, lysosomes and the nucleus (Geller and Winge 1984; Sturtz, Diekert *et al.* 2001). Localization of Sod1 to the mitochondrial IMS is probably to protect against superoxide radicals produced by the respiratory chain. Structurally, all eukaryotic Sod1 proteins possess one copper ion, one zinc ion and one disulfide bond per monomer, that are required for activity (Culotta, Yang *et al.* 2006).

Insertion of the copper cofactor into Sod1 requires CCS (copper chaperone for superoxide dismutase) and occurs via a transient disulfide-bonded heterodimer (Lamb, Torres *et al.* 2001). The accumulation of Sod1 within mitochondria is greatly dependent on the mitochondrial form of CCS, because overexpression of CCS targeted to the intermembrane space results in elevated levels of Sod1 in the same compartment (Sturtz, Diekert *et al.* 2001). Notably, only a precursor form of Sod1, lacking copper and zinc and possessing a reduced disulfide, is competent for import into the mitochondrial IMS (Field, Furukawa *et al.* 2003). The two cysteine residues in Sod1 forming the intramolecular disulfide bond appear to contribute to the retention mechanism of Sod1. The CCS with the bound copper ion facilitates the oxidation and disulfide isomerization of the cysteines, which leads to the maturation of Sod1 (Furukawa, Torres *et al.* 2004). Thus, the correct disulfide bond formation in Sod1 is crucial for regulation of enzyme activity, for prevention of misfolding and for import into the intermembrane space of mitochondria.

1.4.2. Proteins with the twin CX_3C motif

The mitochondrial intermembrane space harbours the small Tim proteins that are marked by a unique twin CX_3C motif separated by 11 to 16 amino acids (Koehler 2004). They are conserved from yeast to mammals and plants, but absent in prokaryotes. Yeast cells contain five members: Tim8, Tim9, Tim10, Tim12 and Tim13, whereas other organisms contain a slightly different complement. The small Tim proteins assemble in 70 kDa complexes, which act as chaperones to lead the inner membrane proteins from the outer to the inner membrane (Curran, Leuenberger *et al.* 2002). In yeast, three subunits of Tim9 and three subunits of Tim10 form a complex; similarly Tim8 assembles with Tim13. Additionally, a portion of the Tim9 and Tim10, together with Tim12, associates with Tim22 at the inner membrane. Mutations in the human homolog of Tim8, DDP1 (deafness dystonia polypeptide 1), cause the inherited Mohr–Tranebjaerg syndrome, a progressive neurodegenerative disorder that is characterized by deafness, dystonia, blindness and mental retardation (Roesch, Curran *et al.* 2002).

The cysteine residues of the twin CX_3C are crucial for mitochondrial import and stable folding of the small Tim proteins. Two alternative structural arrangements of the cysteine residues have been proposed: coordination of zinc ions or formation of disulfide bridges. Some studies have shown that small Tim monomers bind zinc in a 1:1 ratio and that coordination of zinc is required to trap the small Tim protein in the intermembrane space (Sirrenberg, Endres *et al.* 1998; Lutz, Neupert *et al.* 2003). A different set of studies has shown that recombinant and native Tim9–Tim10 and Tim8–Tim13 complexes do not coordinate zinc ions, but forms disulfide bridges (Curran, Leuenberger *et al.* 2002). In addition, both the purified and the endogenous complexes showed similar binding properties for the transmembrane domains of the substrates, indicating that even if during purification oxidation steps took place, the complex was still functional. Another study by Allen *et al.*

characterized the assembly of Tim9 and Tim10 and showed that Tim10 folds into a structure resembling the one predicted from zinc coordination, but the cysteine residues form juxtaposed disulfide bonds (Allen, Lu *et al.* 2003). Moreover, Allen *et al.* suggested that binding of zinc ions to the fully reduced Tim proteins is possible, but it does not support complex formation. Nonetheless, all studies suggest that folding of the small Tim proteins after import is required to maintain their localization in the intermembrane space. Both states, zinc-bound or containing disulfide bonds, might represent physiological forms of the small Tim proteins. Recently, it was proposed that the exchange between oxidized and reduced states might depend on the redox conditions in the IMS (Curran, Leuenberger *et al.* 2004). Additional factors in the IMS might be required for incorporation of zinc ions or formation of disulfide bridges.

1.4.3. Proteins with the twin CX_9C motif

Another group of intermembrane space proteins possess the twin CX_9C motif in which two cysteine residues are separated by nine amino acids. Members of this group are Cox17, Cox19 and Cox23, proteins that are involved in cytochrome oxidase assembly (Nobrega, Bandeira *et al.* 2002; Barros, Johnson *et al.* 2004; Arnesano, Balatri *et al.* 2005). The best characterized representative is Cox17, a copper-binding protein located both in the mitochondrial IMS and the cytosol. Cox17 was identified in a genetic screen for components required for cytochrome oxidase maturation (Glerum, Shtanko *et al.* 1996). Yeast lacking COX17 are respiratory deficient, however the respiration can be restored by the addition of copper to cells, suggesting that Cox17 functions as a copper chaperone. Cox17 acts as a donor of copper to both Sco1 and Cox11 in yeast (Horng, Cobine *et al.* 2004).

Cox17 contains six conserved cysteine residues. The last four constitute to the twin CX_9C motif and form two disulfide bonds that connect two anti-parallel α-helices. This form represents the apoCox17 protein. Structural studies have shown that the oxidation state of all cysteine residues influence the copper binding and Cox17 can exist in two distinct conformers (Palumaa, Kangur *et al.* 2004; Arnesano, Balatri *et al.* 2005). The first conformer consists of a single copper ion bound to a monomer with two stabilizing disulfide bonds. Coordination of the copper ion requires an isomerization of the disulfide bonds prior to copper binding. The second Cox17 conformer is an oligomeric protein complex coordinating many copper ions via its fully reduced cysteine thiols. It appears that the binding and release of copper ions is driven by redox-regulated cycling through these different conformations. Notably, only the first three cysteines are essential for *in vivo* function (Heaton, Nittis *et al.* 2000). A mutant form of Cox17 lacking the remaining three conserved cysteines is functional, indicating that the two disulfides in the twin CX_9C motif are not essential for physiological activity of Cox17 and play rather a structural role.

The two α-helices of Cox17 are preceded by two coiled-coil regions. This pattern is termed as a coiled-coil–helix–coiled-coil–helix (CHCH) domain (Westerman, Poutsma *et al.* 2004). The CHCH domain is present also in the other proteins with the twin CX_9C motif. Cox19 was very recently reported to resemble Cox17 in its ability to coordinate copper ions and in the importance of copper coordination for the *in vivo* function (Rigby, Zhang *et al.* 2007). Interestingly, the subunit VIa of the cytochrome oxidase, Cox12, contains a CX_9C-$CX_{10}C$ pattern comparable with the twin CX_9C motif and forms similar helical disulfide-bonded structures as Cox17 (Tsukihara, Aoyama *et al.* 1995). Coiled-coil domain proteins have a propensity to oligomerize and may be important for heterodimerization with another coiled-coil protein containing a twin CX_9C motif (Arnesano, Balatri *et al.* 2005). For example, Cox12 may act as a docking site for Cox17 to facilitate copper transfer to the cytochrome oxidase.

1.4.4. Erv1, a sulfhydryl oxidase of the mitochondrial intermembrane space

Yeast Erv1 (essential for respiration and vegetative growth) was identified as the first mitochondrial sulfhydryl oxidase (Lee, Hofhaus *et al.* 2000). Erv1 is conserved in plants, fungi, and animals, but not found in prokaryotes. The human Erv1 protein was proposed to function as a growth factor for hepatocytes and therefore was named ALR (augmenter of liver regeneration) or hepatopoietin (Pawlowski and Jura 2006). In yeast, Erv1 is essential for viability and related to Erv2 of the endoplasmic reticulum. As FAD-dependent sulfhydryl oxidase, Erv1 use molecular oxygen to form disulfide bonds coupled with the generation of hydrogen peroxide.

Erv1 consists of two structural domains. The N-terminal fragment is not conserved but harbours an invariant CX_2C motif. This region contains many glycine and proline residues and represents a flexible part which functions in the transfer of disulfides from the redox-active CX_2C centre in the C-terminus onto the specific substrates (Hofhaus, Lee *et al.* 2003). The N-terminal cysteine pair mediates also the dimerization of yeast Erv1. Dimer formation seems critical for function because the plant Erv1 has no N-terminal cysteine residues, but dimerization is mediated by cysteine residues in the C-terminus (Levitan, Danon *et al.* 2004). The C-terminal fragment of Erv1 forms an FAD-binding domain of about 100 amino acid residues and contains a redox-active CX_2C motif and an additional cysteine pair forming a structural disulfide bridge. This domain of Erv1 is the hallmark of the recently established Erv1/ALR protein family (Coppock and Thorpe 2006). The first structural data for yeast Erv2 and human ALR displayed a unique four-helix bundle conformation for the FAD binding (Gross, Sevier *et al.* 2002; Wu, Dailey *et al.* 2003). A close proximity of the FAD cofactor to the redox-active CX_2C motif was revealed, suggesting that this CX_2C is oxidized by transfer of its electrons to the FAD moiety. It appears that interdomain disulfide shuffling between the catalytical redox-active CX_2C motif and the additional cysteine pair either in the N-terminus (ALR) or in the C-terminus (Erv2) is a characteristic feature of the FAD-binding sulfhydryl oxidases.

In yeast, mutations in the Erv1 protein lead to different defects such as respiratory deficiency and an altered mitochondrial morphology (Lisowsky 1994; Becher, Kricke *et al.* 1999). The first mitochondrial function for Erv1 was described by Lill and colleagues, demonstrating that Erv1 play a role in the export of FeS clusters from the mitochondrion (Lange, Lisowsky *et al.* 2001). The fact that the defects in Erv1 mutants are so diverse suggests a variety of Erv1 substrate proteins or role of Erv1 in oxidation of essential components in mitochondria. Both the molecular function and the physiological substrates of Erv1 are yet to be identified.

1.5. Aims of the present study

The main objective of this study was to investigate the biogenesis of the intermembrane space proteins containing conserved cysteine motifs. Particular interest was put on the identification of factors required for the import and stable folding of these cysteine-rich proteins in the mitochondrial IMS. The putative factors were expected to be essential mitochondrial proteins, since some of the small IMS proteins are essential for viability of the cells. The protein Mia40 was selected as a potential factor, because it is an essential protein predicted to locate in mitochondria and it harbours, similarly to the small IMS proteins, conserved cysteine residues. In this work, Mia40 was characterized in terms of its molecular function and structure.

2. MATERIALS AND METHODS

2.1. Molecular biology methods

(Sambrook, Fritsch *et al.* 1989)

2.1.1. Isolation of plasmid DNA from *E. coli*

Small scale preparation of plasmid DNA was performed according to the alkaline lysis method (Birnboim and Doly, 1979). LB-medium (2 ml) containing the appropriate antibiotic was inoculated with a single bacterial colony and incubated (ON, 37°C) under vigorous agitation conditions. Cells from 1.5 ml culture were harvested by centrifugation (7,500xg, 30 sec). The resulting cell pellet was resuspended in 250 µl buffer E1 (50 mM Tris-HCl, 10 mM EDTA-Na_2 x H_2O, 37% HCl, pH 8.0) containing 100 mg/ml RNase, and cell lysis was performed by adding 250 µl buffer E2 (0.2 M NaOH, 1% SDS). The samples were mixed by inverting the tubes 5 times and left for 5 min at RT. For neutralization, 250 µl buffer E3 (3.1 M KOAc, pH 5.5) was added, and the samples were mixed immediately by inverting the tubes 5 times. After centrifugation (35,000xg, 10 min), 700 µl of the supernatant (containing the plasmid DNA) was transferred to a new tube and the DNA was precipitated by adding 500 µl isopropanol (96%). The samples were pelleted via centrifugation, washed with 70% cold ethanol and, after drying at RT, resuspended in 30 µl H_20 and used for further analysis.

For large scale preparation of plasmid DNA a PureYield™ Plasmid Midiprep System (Promega) was used. Bacterial strain carrying the plasmid of interest was inoculated in 50 ml LB medium supplemented with the appropriate antibiotic and incubated overnight at 37ºC with vigorous shaking. The bacteria were harvested by centrifugation (10000 x g, 10 min, RT) and resuspended in 6 ml of Cell Resuspension Solution (50 mM Tris-HCl pH7.5, 10 mM EDTA pH 8.0, 100 µg/ml RNase A). Cell lysis was performed by adding 6 ml of Cell Lysis Solution (0.2 M NaOH, 1% SDS), invertion 5 times and incubation for 3 min at RT. After neutralization with 10 ml of Neutralization Solution (4.09 M guanidine hydrochloride pH4.8, 759 mM KOAc, 2.12 mM glacial acetic acid), tubes were inverted again 5 times and incubated for 3 min at RT to ensure thorough clearing. Samples were centrifuged (10000 x g, 10 min, 4ºC), and the supernatants immediately applied onto a blue Pure Yield™ Clearing Column standing on top of a white Pure Yield™ Binding Column placed onto a vacuum manifold. After the passage of the sample through column stack under applied vacuum, the Pure Yield™ Clearing Column was removed and the Pure Yield™ Binding Column was washed first with 5 ml of Endotoxin Removal Wash and then with 20 ml of the Column Wash Solution (60% ethanol, 60 mM KOAc, 8.3 mM Tris-HCl pH 7.5, 0.04 mM EDTA pH8.0). The column was left to dry for 30 sec under vacuum. Plasmid DNA was eluted with 600 µl of Nuclease-Free Water and stored at –20ºC.

2.1.2. Amplification of DNA fragments by polymerase chain reaction (PCR)

DNA sequences were amplified by PCR as described previously (Sambrook *et al.*, 1989). The DNA templates for PCR were: (i) DNAs isolated from yeast or bacteria (the PCR product was used for further cloning) and (ii) whole cell extracts from yeast or bacteria (to test the success of cloning). Two thermostable DNA polymerases were used: *Taq* (isolated from *Thermus aquaticus*) and *Pfu* (isolated from *Pyrococcus furiosus*). Since *Taq* DNA polymerase has no proofreading ability, *Pfu* DNA polymerase was added in the PCR mix when the PCR product was used for subsequent cloning.

Typical 50 µl PCR reaction mix contained: 1U DNA polymerase (*Taq* DNA polymerase and/or *Pfu* DNA polymerase), 5 µl 10 x PCR-buffer (1% Triton X-100, 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.8), 2 µl dNTPs (10 mM stock), 1 µl of each primer (100 pmol/µl) and 20 ng plasmid DNA or 200 ng genomic DNA as templates. When cloning success was checked by PCR, single *E.coli* colonies were resuspended in 15 µl sterile H_2O and 1 µl was used as a template for test PCR.

The following PCR program, with small changes depending on the DNA sequence, was used:

1) 95°C, 4min	Nuclease inactivation and complete DNA denaturation;	
2) 30-35 cycles	DNA amplification:	
	95°C, 30 s	DNA denaturation
	52- 60°C, 1 min	Primer annealing
	72°C, 1 min per 1 kb	DNA synthesis (extension of primers)
3) 72°C, 10 min	Final extension reaction	
4) 4°C	Cooling	

The PCR products were subsequently analyzed by agarose gel electrophoresis.

2.1.3. *QuickChange® Site-Directed Mutagenesis* (Stratagene)

QuickChange® Site-Directed Mutagenesis method enables a site-specific mutation in almost any double-stranded plasmid and requires no specialized vectors. The procedure is quite simple and highly efficient. The method uses *PfuTurbo* ® DNA polymerase, which replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure is based on a double-stranded DNA (dsDNA) vector with the insert of interest and two oligonucleotide primers containing the desired mutation. The primers, each complementary to opposite strands of the vector, are extended and incorporated during amplification, thus generating a mutated plasmid. Next, the product is treated with *DpnI*, an endonuclease specific for methylated and hemimethylated DNA, leading to digestion of the parental DNA template. Finally, the new vector harbouring the desired mutation is transformed into *E.coli* competent cells.

The *QuickChange® Site-Directed Mutagenesis* method was used for generation of some single and double cysteine-to-serine mutants of Mia40. PCR mix (total volume of 50 µl) contained: 2.5U *PfuTurbo* ® DNA polymerase, 5 µl 10 x reaction buffer (1% Triton X-100, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM$MgSO_4$, 100 mM Tris-HCl, pH 8.8), 1 µl dNTPs mix, 1 µl of each primer (100 pmol/µl) and 20 ng plasmid DNA template. The used cycling parameters were:

1) 95°C, 30s
2) 16-18 cycles:
   95°C, 30 s
   55°C, 1 min
   68°C, 1 min/ 1 kb of plasmid length
3) 68°C, 10 min
4) cooling to 30°C.

After 1hour-treatment with *DpnI*, the product was transformed into MH1 or XL1-Blue cells.

2.1.4. Purification and analysis of DNA

2.1.4.1. Agarose gel electrophoresis of DNA

Electrophoresis in a horizontal agarose gels (0.8- 2% w/v) was used to separate DNA fragments according to their sizes. The samples were mixed in 4:1 ratio with 5 x loading dye (30% (v/v) glycerol, 0.25% (w/v) bromphenol-blue, 0.25% (w/v) xylencyanol) and loaded on the agarose gel. Gels were run in TAE buffer (40 mM Tris-acetate, pH 7.5, 20 mM Na-acetate, 1 mM EDTA) at 80-140 V. The agarose solution contained 0.5 µg/ml ethidium bromide, to allow visualization of DNA in gel under UV light. Molecular weight markers (New England BioLabs) were used in each gel run.

2.1.4.2. Isolation of DNA from agarose gels

DNA fragments of interest were excised from the gel with a clean scalpel and DNA was extracted from the agarose using the "QIAquick Gel extraction kit" (Qiagen) or "E.Z.N.A.® Gel Extraction Kit" (peQLab). In principal, the agarose piece with the desired DNA was dissolved with addition of binding buffer specific for the kit and incubation in 55°C for 10min. Then the solution was loaded on a DNA-binding silica column (provided in the kit) by centrifugation in the top centrifuge for 1 min. After two washing steps with a kit-specific washing buffer (containing ethanol), the column was dried and the DNA was eluted with 30-50 µl sterile H_2O or elution buffer delivered in the kit. 1-2 µl of the eluted DNA were loaded on an agarose gel to verify the efficiency of purification. The extracted DNA samples were stored at –20ºC.

2.1.4.3. Measurement of DNA concentration

DNA concentration was determined by measuring the absorption of the DNA solution at 260 nm. One optical unit (OD = 1.0) corresponds to a concentration of 50 µg/ml of double stranded DNA and 33 µg/ml of single stranded DNA.

2.1.5. Enzymatic manipulation of DNA

2.1.5.1. Digestion of DNA with restriction endonucleases

DNA to be digested for analytical or preparative purposes was diluted in a recommended by the manufacturer buffer, specific for the restriction enzyme. After addition of 2-5 U of the restriction endonuclease per 1 µg of DNA, the mixture was incubated for 3 h at 37ºC. The DNA fragments obtained were analyzed by agarose gel electrophoresis or directly purified using DNA-binding silica columns (from Qiagen or peQLab, see2.1.4.2.).

2.1.5.2. Ligation of DNA fragments

DNA ligase from bacteriophage T4 was used to ligate DNA fragments. Linearized cloning vector (50-200 ng) and 3-5 times molar excess of DNA fragment to be inserted, were incubated with 2 µl of 10x ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5% (w/v) PEG-8000, 1 mM DTT, 1 mM ATP), and 1 µl (2 U) T4-DNA ligase (Gibco-BRL) in a 20 µl reaction. The ligation reactions were performed for 1 h at 25°C or at 14°C for 16 h. The ligated DNA (0.5-1 µl) was used to transform electrocompetent *E. coli* cells.

2.1.6. Transformation of electrocompetent *E. coli* cells

2.1.6.1. Overview of E. coli strains used

Strain	Genotype	Reference
MH1	MC1061 derivative; araD139, lacX74, *galU*, *galK*, *hsr*, *hsm*+, strA	Casadaban and Cohen, 1980
XL1-Blue	*supE*44, *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1, *lac*$^-$, F'[*proAB*$^+$, *lacI*^{q} *lacZ*ΔM15, Tn*10*(tet^r)]	commercially available from Stratagene

2.1.6.2. Media for E. coli

LB medium: 1% Bacto-Tryptone, 0.5% Yeast extract, 1% NaCl (all w/v)

LB-Amp medium: LB-medium supplemented with 100 µg/ml ampicilline

10x M9 salts: 60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl, H_2O to 1l

Trace elements mix: 50 g ethylenediamine tetaacetic acid, 22 g $ZnSO_4$ x $7H_2O$,
5.54 g $MnCl_2$ x $4H_2O$, 4.99 g $FeSO_4$ x $7H_2O$,
1.1 g $(NH_4)_6Mo_7O$ x $24H_2O$, 1.57 g $CuSO_4$ x $5H_2O$,
1.61 g $CoCl_2$ x $6H_2O$, H_2O to 1 l

M9 low-sulphate medium: 10 ml 10x M9 salts, 2 ml 20 % glucose, 1 ml 100 mM $MgCl_2$,
1 ml 10 mM $CaCl_2$, 0.1 ml vitamine b1 (1 mg/ml), 50 µl trace elements mix, 30 µl ampiciline (100 mg/ml), 1 ml stock of 17 amino acids (2 mg/ml each, without Cys, Met and Tyr),
2 ml tyrosine (1 mg/ml), 5 ml 1mM $MgSO_4$, H_2O to 100 ml

These media were used for preparing liquid cultures. The M9 low-sulphate medium was used for radiolabeling with ^{35}S the proteins expressed in *E. coli*. For solid media (LB-Amp plates), 2% (w/v) bacto-agar was added to the liquid solutions. Bacto-agar, glucose and the liquid solution were autoclaved separately. The antibiotic was added after the media cooled to 50°C.

2.1.6.3. Preparation of electrocompetent cells (Dower, Miller *et al.* 1988)

For preparation of electrocompetent *E. coli* cells, a small culture, usually 50 ml, of LB-Amp medium was inoculated with a single colony of the corresponding strain and grown overnight at 37ºC under shaking at 140 rpm. The following day, preheated 1 l of LB-Amp medium was inoculated with the overnight culture and the cells were grown until they reached $OD_{578} \approx 0.5$. After keeping the culture on ice for 30 min, the cells were harvested by centrifugation (4,400 x g, 5 min, 4°C) and washed sequently with 400 ml, 200 ml and 50 ml of sterile 10% (v/v) glycerol. Finally, the competent cells were resuspended in 500 µl 10% (v/v) glycerol, aliquoted and stored at – 80°C.

2.1.6.4. Transformation of E. coli cells by electroporation

The ligation mixture or isolated plasmid DNA (0.5-1 µl) was added on ice to the aliquot of electrocompetent cells. Then, the suspension was transferred to a cold electroporation cuvette, which was introduced in an electroporation apparatus, Gene Pulser (BioRad) (settings: 2.5 kV, 400 ohm, 25µF; time constant 7-8 ms). High electric voltage pulse was applied shortly to the bacterial cells followed by dilution with 1ml of LB-medium and incubation for 30-60 min at 37ºC with shaking to allow cell recovery. After a brief centrifugation and removal of the majority of the medium, cell pellet was resuspended in the remaining medium and plated on LB-Amp plates. The plates were incubated at 37°C overnight.

2.1.7. Bacterial plasmids used

Plasmid	Reference
Plasmids for transcription/translation:	
pGEM4-Tim13	Lutz, Neupert *et al.* 2003
pGEM4-Tim13^{SSSS}	Lutz, Neupert *et al.* 2003
pGEM4-Tim10	Sirrenberg, Endres *et al.* 1998
pGEM4-CCHL	Steiner, Zollner *et al.* 1995
pGEM4-Porin	Mayer, Lill *et al.* 1993
pGEM4-Su9(1-69)DHFR	Pfanner, Tropschug *et al.* 1987
pGEM4-Cox19	Nikola Mesecke, unpublished
pGEM3-Erv1	Terziyska, Grumbt *et al.* 2007
Plasmids used for protein expression in bacteria:	
pMalcRI (MBP)	New England BioLabs
pMalcRI-Mia40ΔTM	Terziyska, Lutz *et al.* 2005
pMalcRI-Tim13	Lutz, Neupert *et al.* 2003
pETGEXCT-Cox17	Mesecke, Terziyska *et al.* 2005

2.1.8. Transformation of *S. cerevisiae* cells

2.1.8.1. Media for S.cerevisiae

YP-medium: 1% Yeast extract, 2% peptone, pH 5.0 (adjusted with HCl)

YPD-medium: YP-medium containing 2% glucose

YPGal-medium: YP-medium containing 2% galactose

Lactate medium: 3 g yeast extract, 1 g NH_4Cl, 1 g KH_2PO_4, 0.5 g $CaCl_2$ x $2H_2O$, 0.5 g NaCl, 1.06 g $MgSO_4$ x $6H_2O$, 0.3 ml 1% $FeCl_3$, 22 ml 90% lactic acid, H_2O to 1 l, pH 5.5 (adjusted with KOH);

supplemented with 0.1% glucose or 0.1% -0.5% galactose

SLac medium: 1.7 g yeast nitrogen base, 5 g ammonium sulfate, 22 ml 90% lactic acid, 1.5 g "Dropout mix" powder (mix containing equal weight of all amino acids 20mg/l; for selecting one auxothrophic marker, the corresponding amino acid was left out), H_2O to 1 l, pH 5.5 (adjusted with KOH);

supplemented with 0.1% glucose or 0.1% galactose

The described media were used for preparing liquid cultures. If corresponding plates were made, 2% (w/v) agar was added. Bacto-agar, glucose and media were autoclaved separately. For selective media, amino acids solutions (His, Leu, Lys, all 10 mg/ml) and uracil and adenine solutions (both 2 mg/ml) were separately autoclaved for 20 min at 120°C, except for tryptophan (10 mg/ml) which was filter sterilized. They were added to the medium before pouring the plates. The amino acid solutions were added to the selective media just before pouring the plates.

2.1.8.2. Transformation of S. cerevisiae cells (Lithium-acetate method) (Gietz, St Jean *et al.* 1992)

The yeast strain to be transformed was grown overnight at 30°C with shaking in YPD-medium and diluted the following morning to an OD_{600} ca. 0.2 in 50 ml medium. When the culture reached OD_{600} ca. 0.5, cells were collected by centrifugation (1500 x g, 3 min, RT), washed with 25 ml sterile H_2O and resuspended in 1 ml 100 mM Li-acetate. After transfer into a 1.5 ml Eppendorf cup, cells were harvested again (7500 x g, 15 sec, RT) and resuspended in 400 µl 100 mM Li-acetate. For each transformation 50 µl of this suspension was centrifuged (3000 x g, 5 min, RT) and the supernatant was removed. The pellet was overlayed in the following order: 240 µl PEG 3350 (50% v/v), 36 µl 1 M Li-acetate, 5 µl single stranded salmon sperm DNA (10 mg/ml; previously heat-denatured and quickly cooled on ice), 70 µl H2O containing 0.1-10 µg of plasmid DNA. The mixture was vigorously vortexed for 1 min and incubated at 30°C for 30 min, followed by heat-shock at 42°C for 20-25 min. Cells were pelleted by centrifugation (15 s, 6000 x g, RT), resusepended in 800 µl YPD-medium and further incubated for 2 h at 30ºC. After harvesting, the cells were resuspended in a small volume of sterile H_2O and plated on selective medium. The plates were incubated for 2-4 days at 30°C.

2.1.9. *S. cerevisiae* strains used and cloning strategies

2.1.9.1. Overview of yeast strains used

Yeast strain	**Reference**
Wild type strains	
YPH499 (Mat a)	(Sikorski and Hieter 1989)
W303-1A (Mat a) W303-1B (Mat α)	R. Rothstein, Department of Human Genetics, Columbia University, New York

Materials and methods

Strains generated by homologous recombination	
GAL-MIA40	Terziyska, Lutz *et al.* 2005
GAL-Erv1	Mesecke, Terziyska *et al.* 2005
Mia40-His_6	Mesecke, Terziyska *et al.* 2005
Δ*cox18*	Bihlmaier, Mesecke et al. 2007
Δ*rip1*	Bihlmaier, Mesecke et al. 2007
Δ*cyc1*/ Δ*cyc7*	Bihlmaier, Mesecke et al. 2007
Δ*mia40*	Dr. Kai Hell, unpublished
Strains generated by transformations with yeast vectors	
Δ*mia40* + pYX142	Dr. Christian Kozany, unpublished
Δ*mia40* + pYX142-Mia40His WT	Dr. Christian Kozany, unpublished
Δ*mia40* + pYX142-Mia40His C1S	Dr. Christian Kozany, unpublished
Δ*mia40* + pYX142-Mia40His C2S	Dr. Christian Kozany, unpublished
Δ*mia40* + pYX142-Mia40His C3S	Dr. Christian Kozany, unpublished
Δ*mia40* + pYX142-Mia40His C4S	Dr. Christian Kozany, unpublished
Δ*mia40* + pYX142-Mia40His C5S	Dr. Christian Kozany, unpublished
Δ*mia40* + pYX142-Mia40His C6S	Dr. Christian Kozany, unpublished
Δ*mia40* + pYX142-Mia40His C1,2S	This thesis
Δ*mia40* + pYX142-Mia40His C3,6S	This thesis
Δ*mia40* + pYX142-Mia40His C5,6S	This thesis
Δ*mia40* + pYX142-Mia40His C4,5S	This thesis
GAL-MIA40 + pYX142	Dr. Christian Kozany, unpublished
GAL-MIA40 + pYX142-Mia40His WT	Dr. Christian Kozany, unpublished
GAL-MIA40 + pYX142-Mia40His C1S	Dr. Christian Kozany, unpublished
GAL-MIA40 + pYX142-Mia40His C2S	Dr. Christian Kozany, unpublished
GAL-MIA40 + pYX142-Mia40His C3S	Dr. Christian Kozany, unpublished
GAL-MIA40 + pYX142-Mia40His C4S	Dr. Christian Kozany, unpublished

GAL-MIA40 + pYX142-Mia40His C5S	Dr. Christian Kozany, unpublished
GAL-MIA40 + pYX142-Mia40His C6S	Dr. Christian Kozany, unpublished
GAL-MIA40 + pYX142-Mia40His C1,2S	This thesis
GAL-MIA40 + pYX142-Mia40His C3,6S	This thesis
GAL-MIA40 + pYX142-Mia40His C5,6S	This thesis
GAL-MIA40 + pYX142-Mia40His C4,5S	This thesis

2.1.9.2. Cloning strategies for plasmids used for the transformation of yeast

a) pYX142-Mia40His C1,2S

C1,2S mutation in *MIA40* was obtained by amplification using pYX142-Mia40His C1S as a template and the primers KH # 96 and NT # 22 (introducing the C2S mutation). The PCR product and the pGEM4-Mia40His plasmid were cut with SpeI and NcoI, purified and ligated. Next, the sequence coding for the C1,2S mutant of Mia40 was cut out of the resulting plasmid and cloned into pYX142 vector using EcoRI and HindIII restriction sites. The obtained plasmid was transformed in Δ*mia40* and GAL-MIA40 yeast strains that were subsequently grown on selective medium lacking leucine and containing glucose (Δ*mia40)* or galactose (GAL-Mia40).

NT # 22 (YKL195Rev-C1,2S)	5'– GGG TCC ATG GGC CAT ACC TCC CAA GGA AGG AGA GTC CCA ATT TAT CTC ACC – 3'
KH # 96 (YKL195For571SpeI)	5'– GAA GAA AAT ACT AGT GAC AAA AC – 3'

b) pYX142-Mia40His C3,6S and pYX142-Mia40His C5,6S

C3,6S or C5,6S mutations in *MIA40* were obtained by *QuickChange® Site-Directed Mutagenesis* method using primers NT # 7 and # 8 (introducing the C6S mutation) and pGEM4-Mia40His C3S or pGEM4-Mia40His C5S as a template, respectively. The sequence coding for each mutant of Mia40 was cut out of the obtained plasmid and cloned into pYX142 vector using EcoRI and HindIII restriction sites. The resulting plasmids were transformed in Δ*mia40* and GAL-MIA40 yeast strains that were subsequently grown on selective medium lacking leucine and containing glucose (Δ*mia40)* or galactose (GAL-Mia40).

NT # 7 (YKL195wFor1006-1046; C340S)	5'– CAT ATG CAA GAC AGT TTC CGG AAA TAC CCC GAG CAT TAT GC – 3'
NT # 8 (YKL195wRev1006-1046; C340S)	5'– GCA TAA TGC TCG GGG TAT TTC CGG AAA CTG TCT TGC ATA TG – 3'

c) pYX142-Mia40His C4,5S

C4,5S mutation in *MIA40* was obtained by *QuickChange® Site-Directed Mutagenesis* method using primers NT # 5 and # 6 (introducing the C5S mutation) and pGEM4-Mia40His C4S as a template. The sequence coding for this mutant of Mia40 was cut out of the obtained plasmid and cloned into pYX142 vector using EcoRI and HindIII restriction sites. The resulting plasmid was transformed in Δ*mia40* and GAL-MIA40 yeast strains that were subsequently grown on selective medium lacking leucine and containing glucose (Δ*mia40)* or galactose (GAL-Mia40).

NT # 5 (YKL195wFor973-1003; C330S)	5'– CCA AAA GGT ATC GAT TCT GTT GAA AAG TTT C – 3'
NT # 6 (YKL195wRev973-1003; C330S)	5'– GAA ACT TTT CAA CAG AAT CGA TAC CTT TTG G– 3'

2.2. Protein biochemistry methods

2.2.1. Protein analysis

2.2.1.1. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) (Lämmli 1970)

The proteins were separated according to their molecular weights under denaturing conditions using vertical one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The concentrations of acrylamide and bis-acrylamide in the separating gel were selected according to the molecular sizes of proteins of interest. Glass plates of 160 x140 mm and spacers of 1 mm thickness were used. The probes were supplemented with the sample buffer with or without 5% (v/v) β-mercaptoethanol depending if the electrophoresis was performed under reducing or non-reducing conditions (total volume of 20-50 µl). The samples were incubated at 95ºC for 3 min before loading and the electrophoresis was performed at 35 mA for 100 min. Protein molecular weight markers (peQLab) were used.

Running gel*:* 8-16% (w/v) acrylamide, 0.16-0.33% (w/v) bis-acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED

Stacking gel: 5% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 60 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED

1 x sample (Laemmli) buffer: 60 mM Tris·HCl, pH 6.8, 2% (w/v) SDS, 10 % glycerol, 0.05% (w/v) bromphenol-blue

Electrophoresis buffer: 50 mM Tris base, 384 mM glycine, 0.1% (w/v) SDS, pH 8.3 without adjustment

2.2.1.2. Urea-gel electrophoresis

For better separation of small proteins (below 15 kDa) an urea-gel electrophoresis was used. The same system as for the SDS-PAGE was employed, just the gel mixtures and the running buffer were slightly different. The electrophoresis was performed at 30mA for about 170min.

Running gel*:* 18 % (w/v) acrylamide, 0.25 % (w/v) bis-acrylamide, 33% urea, 700 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.1 % (w/v) APS, 0.05% (v/v) TEMED

Stacking gel: 6 % (w/v) acrylamide, 0.09 % (w/v) bis-acrylamide, 48 % urea, 170 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1 % (w/v) APS, 0.05% (v/v) TEMED

Electrophoresis buffer: 100 mM Tris base, 384 mM glycine, 0.1% (w/v) SDS, pH 8.3 without adjustment

2.2.1.3. Transfer of proteins onto nitrocellulose membrane (Western-Blot)

Proteins separated on SDS or urea gels were transferred onto nitrocellulose membrane using the semi-dry blotting method. The gel, the membrane, and four sheets of Whatman filter paper (3MM) were incubated in transfer buffer (20 mM Tris base, 150 mM glycine, 20% (v/v) methanol, 0.08% (w/v) SDS). Two sheets of filter paper were placed on the anode electrode then the membrane and the gel followed. The stack was covered with another two filter papers and the cathode electrode. The transfer was performed at 2 mA/cm^2 for 1-1.5 h. To verify transfer efficiency and label bands of the marker proteins, the nitrocellulose membranes were reversibly stained with Ponceau S solution (0.2% (w/v) Ponceau S in 3% (w/v) TCA). The membrane was then immunodecorated or the radioactive signals were visualized by autoradiography.

2.2.1.4. Coomassie Brilliant Blue (CBB) staining of SDS-PAGE gels

The gel was incubated in CBB staining solution containing 30% (v/v) methanol, 10% (v/v) acetic acid, and 0.1 (w/v) Coomassie-Brilliant-blue G-250 at RT for 30 min. The gel was destained several times with aqueous solution containing 30% (v/v) methanol and 10% (v/v) acetic acid until the protein bands were clearly visible. The gel was dried overnight between two gel-drying films (Promega).

2.2.1.5. Detection and quantification of radiolabeled proteins by autoradiography and phosphorimaging

Dry membranes carrying radiolabelled proteins were exposed to X-ray films ((Kodak Bio Max MM) and, after a desired exposure time, the film was developed in a developing machine (Gevamatic 60, AGFAGevaert). The period of exposure depended on signal intensities. Signals were quantified by densitometry using Image Master 1D Elite software (Amersham). Alternatively, the nitrocellulose membranes were exposed to Phosphroimaging plates and the intensity of the bands was determined with the Phosphorimager (Fuji BAS 1500).

2.2.1.6. Determination of protein concentration

Protein concentration was determined according to Bradford assay (Bradford, 1976). Protein solutions (1-10 µl) were diluted with 1 ml of 1:5 diluted "Bio-Rad-Protein assay" Reagent (BioRad) and incubated for 10 min at RT. The absorbance was measured at 595 nm using a 1cm path-length microcuvette. Protein concentration was determined from a calibration curve obtained with the known amounts of bovine IgGs (BioRad) as a standard.

2.2.2. Protein preparation

2.2.2.1. In vitro synthesis of radiolabeled mitochondrial preproteins

Mitochondrial preproteins used for import experiments *in organello* were cloned into pGEM vectors under the control of *Sp6* promoter. To label them radioactively, the preproteins were synthesized either by separate transcription reaction followed by translation reaction in the presence of ^{35}S-methionine or by TNT system that combines transcription and translation in the same reaction mixture.

a) In vitro Transcription

The transcription mix (100µl) contained: 20 µl 5x transcription buffer (200 mM TRIS-HCl, pH 7.5, 50 mM NaCl, 30 mM $MgCl_2$, 10 mM spermidine), 10 µl 0.1 M DTT, 4 µl RNAsin (40 U/µl), 20 µl NTPs (10 mM each), 5.2 µl $m_7G(5')ppp(5')G$, 3 µl *Sp6* polymerase, 27 µl H_2O and 10 µl plasmid DNA. The reaction mix was incubated for 1 h at 37°C and then followed by addition of 10 µl 10 M LiCl and 300 µl ethanol. Reaction was cooled at - 20°C for 30 min and RNA was precipitated by centrifugation for 20 min at 36670 x g and 2ºC. RNA pellet was washed with ice-cold 70% (v/v) ethanol, dried and resuspended in H_2O supplemented with 1 µl RNasin (40 U/µl), aliquoted and kept at – 80°C till use.

b) In vitro Translation (Pelham and Jackson 1976)

The translation mix contained: 50 µl rabbit reticulocyte lysate (Promega), 20 U RNAsin, 1.75 µl amino acid mix (1mM each, without methionine), 6 µl ^{35}S-methionine, 3.5 µl 15 mM Mg-acetate and 12.5 µl RNA. After incubation for 1 h at 30°C, the reaction was stopped by addition of 6 µl 58 mM non-labeled methionine. Upon addition of 12 µl sucrose (1.5 M), ribosomes and aggregated proteins were removed by centrifugation (30 min, 45000 rpm, TLA 45 rotor, 4°C). The supernatant was aliquoted, frozen in liquid nitrogen and stored at –80ºC.

c) TNT Coupled reticulocyte lysate system

TNT reaction mixture (50µl) contained: 25µl TNT rabbit reticulocyte lysate (Promega), 2 µl TNT reaction buffer, 1 µl TNT *Sp6* RNA polymerase, 1 µl amino acid mix (without methionine), 4 µl ^{35}S methionine (10 mCi/ml), 1 µl RNasin (40 U/µl), 1 µl DNA template. The TNT reaction was incubated for 1h at 30°. After adding 4 µl of cold methionine (58 mM) and 8 µl sucrose (1.5 M), aggregated proteins were removed by centrifugation (30 min, 45000 rpm, TLA 45 rotor, 4°C). The supernatant was aliquoted, frozen in liquid nitrogen and stored at -80°C.

2.2.2.2. Trichloroacetic acid (TCA) precipitation of proteins

Proteins from solutions were precipitated by addition of 72% TCA to a final concentration of 12%. The samples were incubated for 30 min at –20ºC and centrifuged for 20 min at 30000 x g. The precipitated proteins were washed with acetone (kept at –20ºC) and centrifuged again (30,000xg, 10 min). Protein pellet was shortly dried at RT and dissolved in sample buffer.

2.2.2.3. Radiolabeling of MBP-Tim13 and GST-Cox17 with ^{35}S during expression in E. coli

Tim13 protein fused to the maltose binding protein (MBP) or Cox17 fused to the glutathione S-transferase (GST) were expressed in *E.coli* from pMalcRI or pETGEXCT vector, respectively. Five ml of an overnight *E.coli* preculture were diluted into 100 ml M9 low-sulphate medium and grown until OD_{600} of ca. 0.5-0.8 at 37°C with moderate shaking. Expression of the recombinant protein was induced by 0.5-1 mM isopropyl-β, D-tiogalactopyranoside (IPTG), 10 µM $MgSO_4$ and 5 mCi of ^{35}S-sulfate and the culture was grown for additional 2-3 h at 37°C. At the end, cells were harvested by centrifugation (5000 x g, 10 min, RT), washed once with M9 low-sulphate medium and the cell pellet was stored at -20°C before purification. Expression and purification were monitored by SDS-PAGE, transfer onto nitrocellulose membrane and autoradiography.

2.2.2.4. Purification of radiolabeled recombinant MBP-Tim13 from E. coli

The harvested *E.coli* cells containing the radiolabeled MBP-Tim13 protein were resuspended in 10 ml binding buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol, 10 mM EDTA) with addition of 3 mg DNase I and 3 mg lysosyme and incubated at 4°C for 45min. After centrifugation for 20 min at 16000 rpm in JA-20 rotor, the supernatant was loaded on a 1 ml amylose column (New England BioLabs) preequilibrated with binding buffer. Column was washed three times with 5 ml of binding buffer and once with 5 ml of binding buffer supplemented with 2 mM tris carboxyethyl phosphine hydrochloride (TCEP). The bound proteins were eluted in 8x 300 µl fractions with the binding buffer containing additionally 10 mM maltose and 2 mM TCEP. The fractions containing most of the radiolabeled MBP-Tim13 protein were supplemented with 18 U Factor Xa (Novagen) and incubated overnight at 4°C in order to cleave the Tim13 from the fusion protein. After cleavage, a dialysis against denaturing buffer (6 M guanidinium chloride, 20 mM potassium phosphate pH 7.4, 10 mM EDTA, 10 mM β-mercaptoethanol, 2 mM TCEP) was performed, followed by aliquoting and storage of the purified protein at -80°C.

2.2.2.5. Purification of radiolabeled recombinant GST-Cox17 from E. coli

The cell pellet was resuspended in 2 ml lysis buffer (1 M NaCl, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 1% Triton X-100, 50 mM Tris pH 8.0) with addition of 1 mg/ml lysosyme. After incubation for 10 min at 25°C, 20 mM K-phosphate, pH 7.2 and 275 mM NaCl were added and the sample was incubated for further 10 min. The resulting cell extract was cleared by centrifugation for 15 min at 16000 rpm and applied to 0.5 ml of glutathione sepharose 4 Fast Flow resin (Amersham Biosciences). After 1 h incubation, the affinity matrix was washed extensively with TCB buffer (50 mM Tris pH 8.0, 5 mM $CaCl_2$, 250 mM K-acetate, 15% glycerol). Cox17 was eluted by overnight incubation with 9 U of thrombin protease in 1ml of TCB buffer. Finally, a dialysis against denaturing buffer (6 M guanidinium chloride, 20 mM K-phosphate pH 7.4, 10 mM EDTA, 10 mM β-mercaptoethanol, 2 mM TCEP) was performed, followed by aliquoting and storage of the purified protein at -80°C.

2.2.2.6. Purification of recombinant MBP-Mia40ΔTM from E. coli

Mia40 protein lacking the first 67 amino acids fused to MBP was expressed in *E.coli* from pMalcRI vector (New England Biolabs). An overnight *E. coli* culture was diluted into 400 ml LB-Amp and grown until OD*600* of ca.0.5-0.8 at 37°C. Expression of the recombinant protein was induced by 0.5 mM IPTG and the culture was grown in the presence of 100 µM zinc acetate or copper sulphate for additional 2-3 h at 37°C. Cells were pelleted by centrifugation for 10 min at 5000 rpm in JA-10 rotor and resuspended in 20 ml binding buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol, 1mM PMSF) with addition of 4 mg lysosyme and 4 mg DNase I. Following incubation for 15 min at 4°C, cells were completely broken by sonication (10x12 s, Branson sonifier, setting 4, 80% duty cycle). After centrifugation for 20 min at 16000 rpm in JA-20 rotor, the supernatant was loaded on a 3 ml amylose column (New England Biolabs) preequilibrated with 35 ml binding buffer. The flow-through was collected and loaded again onto the beads. The column was washed twice with 15 ml of binding buffer lacking the 200 mM NaCl, followed by elution of the bound proteins in 1 ml fractions with binding buffer lacking 200 mM NaCl but supplemented with 10 mM maltose. The maltose binding protein (MBP) was used as a control in metal determination analysis. Therefore, MBP was purified in the same way as MBP-Mia40ΔTM protein. Expression and purification were monitored by SDS-PAGE and CBB staining. The metal content of the proteins was measured by ICP-AES (induction coupled plasma atomic emission spectroscopy) with a VARIAN-VISTA instrument (Varian Inc., Palo Alto, CA).

2.2.3. Protein modification

2.2.3.1. Modification with iodoacetamide or N-ethylmaleimide (NEM)

Analysis of the redox states of Mia40 was performed using reducing agents, like dithiothreitol (DTT), and reagents modifying cysteine residues, namely iodoacetamide and N-ethylmaleimide (NEM). For modification, 80 mM iodoacetamide or 50 mM NEM were dissolved in a buffer used in the particular experiment and incubated with isolated mitochondria for 20 min at 25°C. Then the samples were analysed further according to the experimental setup.

2.2.3.2. Modification with $mPEG_{5000}$-maleimide (PEG-mal)

$mPEG_{5000}$-maleimide (PEG-mal) is a thiol-specific agent, which attaches one PEG_{5000} moiety to each free cysteine residue of the target protein, slowing down this way the migration of the modified protein on a SDS-PAGE gel. PEG-mal and isolated mitochondria harbouring wild type and single cysteine mutants of Mia40 were used in order to study the number and accessibility of the disulfide bonds in Mia40 protein. First, mitoplasts were generated by resuspension of mitochondria in 100 µl 100mM Hepes pH 7.4 with 5 mM TCEP (Tris-(2-carboxyethyl) phopshine hydrochloride). Second, mitoplasts were incubated 3 min on ice or at 95^0C and after 10 min recovery on ice they were mock-treated or incubated with PEG-mal for 90 min on ice. Finally, the probes were loaded on a non-reducing SDS gel, transferred onto nitrocellulose membrane and Mia40 was visualised by immunodecoration.

2.3. Cell biology methods

2.3.1. Cultivation of *S.cerevisiae*

S. cerevisiae growth was performed as described in Sambrook *et al.* (Sambrook *et al.*, 1989). Yeast was normally grown on lactate medium or YP medium supplemented with glucose or galactose. In case of the strains expressing the single and double cysteine mutants of Mia40, SLac medium was used. Liquid cultures were inoculated with yeast strains from the glycerol stocks or from the agar plates and were grown in the appropriate liquid medium at 30°C while shaking at 140 rpm. Prior to the isolation of mitochondria, cells were passaged for approximately 3 days in the way that OD_{600} never exceeded 1.2. For depletion of Mia40, yeast strains harbouring *MIA40* gene under *GAL* promoter, were grown for ca. 60 h on galactose-containing media and then cells were collected, washed with water, resuspended in glucose-containing media and grown in the latter media for 12-27 h depending on the strain. For depletion of Erv1, the GAL-Erv1 strain was grown 3-4 days in glucose-containing lactate media. For isolation of mitochondria with increased levels of Mia40, the GAL-MIA40 strain was grown in lactate medium supplemented with 0.5% galactose.

2.3.2. Complementation of the *MIA40* gene disruption in *S. cerevisiae* (Plasmid shuffling)

The haploid strain (Δ*mia40*) harbouring a chromosomal *MIA40* gene disruption and the coding sequence of the wild type Mia40 in pVTU-102 plasmid (containing the URA3 marker) was transformed with the plasmids carrying the cysteine to serine mutants of Mia40 and containing the LEU2 marker. The resulting strains were tested for their ability to complement the *MIA40* gene disruption on 5-fluoroorotic acid plates, which allowed elimination of the pVTU-102-Mia40 WT plasmid.

5-FOA plates: 100 ml 5x S medium (1.7 g yeast nitrogen base, 5 g ammonium sulphate),
25 ml 40 % glucose, 500 mg 5-fluoroorotic acid (FOA), 2 % Agar,
12,5 ml uracil (2 mg/ml), 5 ml adenine (2 mg/ml), 1.5 ml lysin (10 mg/ml),
1 ml histidin and tryptophan (10 mg/ml), H_2O to 500 ml

2.2.3. Determination of the growth characteristics of yeast strains (Drop dilution test)

To determine the growth phenotype of a specific yeast strain, the growth assays were performed either on solid media, i.e. drop dilution test on agar plates, or in liquid media. For drop dilution tests, the strains were grown in liquid culture for 2 days, diluted to identical $OD_{578} \approx 0.5$ and then a series of 1:10 dilutions were made for each strain. From each dilution 3.5 µl were spotted on plates with the adequate medium under sterile conditions. Plates were incubated for 2-5 days at 30^0C. For determination of growth phenotype in liquid medium, the strains were grown in liquid culture for 18-24 h, diluted to identical OD and then grown at 30^0C for up to 60 h with regular passaging. Cell growth was measured spectrophotometrically every 1.5-2 h, whereas at time zero the cell number was calculated as one.

2.3.4. Subcellular fractionation of yeast (Caplan and Douglas 1991)

For the subfractionation the wild type yeast cells (W303-1B) grown in lactate medium were used. First, spheroplasts were generated from yeast cells as described in 2.3.8.and collected by centrifugation (5 min, 5000 rpm, 4°C). After resuspension in lysis buffer (0,6 M sorbitol, 1 mM DTT, 3 mM EDTA, 1 mM PMSF, 20 mM HEPES/KOH pH 7.4) the spheroplasts were broken in a cooled homogeniser on ice. Not open cells, nuclei and

membranes were removed by centrifugation (10 min, 10 000 x g, 4°C). The supernatant was divided into two halves. One half was loaded on 30 % (v/v) Percoll-gradient in lysis buffer and centrifuged for 50 min at 2°C and 76 000 x g. The upper of the two bands occurring in the gradient was taken and concentrated by centrifugation for 1 h at 150 000 x g and 4 °C. The microsomal fraction was obtained on top of the sedimented Percoll layer. The other half of the spheroplasts lysate was centrifuged (12 min, 17 500 x g, 4°C) to isolate mitochondria. The mitochondria in the pellet were resuspended in the lysis buffer. The supernatant was again centrifuged for 1 h at 15 000 x g and 4°C to obtain the soluble cytosolic fraction.

2.3.5. Submitochondrial localisation of proteins

To determine the submitochondrial location of proteins, the outer mitochondrial membrane was selectively disrupted by 10-dold dilution of 100 µg of mitochondria with 20 mM HEPES/KOH pH 7.4 buffer. Addition of 50 µg/ml proteinase K and incubation for 20 min on ice led to degradation of the accessible proteins in the intermembrane space. After block of the protease with 2 mM PMSF, the mitoplasts were reisolated, washed and resuspended in sample buffer. As control, 100 µg of mitochondria were solubilised completely in SH containing 0.5 % Triton X-100 in the presence of 100 µg/ml proteinase K. This way all proteins were accessible to the protease activity. The mitochondrial protein extract was TCA precipitated and resuspended in the sample buffer. Finally, all the probes were analysed by SDS-PAGE and immunoblotting with antibodies against proteins from different compartments of mitochondria.

2.3.6. Generation of mitoplasts

Mitochondria whose outer membrane has been disrupted are known as mitoplasts. The outer mitochondrial membrane is specifically opened by swelling of mitochondria in a hypotonic solution which keeps the inner membrane intact. Mitochondria in SH buffer were diluted 1:10 in 20 mM HEPES/KOH pH 7.3 and 1 mM ATP and incubated for 20 min on ice. As a control, mitochondria were diluted in isoosmotic SH buffer in the same manner. If necessary, mitoplasts were reisolated by centrifugation (10 min, 17 000 x g) and resuspended in a desired buffer.

2.3.7. Carbonate extraction

Carbonate extraction was used to analyse the association of a protein with the membrane. For this purpose, 50 µg of precipitated mitochondria were resuspended in 100 µl of 0.1 M Na_2CO_3 pH 11.5 and incubated for 30 min on ice. The probes were then centrifuged (183 000 x g, 20 min, 2ºC), soluble proteins in the supernatant were precipitated using TCA, while the pellet containing membrane proteins was resuspended directly in 2 x sample buffer. The samples were analyzed by SDS-PAGE and immunodecoration.

2.3.8. Isolation of mitochondria from *S. cerevisiae* (Herrmann, Foelsch *et al.* 1994)

Yeast cells, grown to OD_{578} of 0.8-1.5, were collected by centrifugation (5 min, 4000 rpm, JA-10, RT), washed with water and resuspended in a buffer containing 10 mM DTT, 100 mM Tris/SO_4, pH 9.4, to a concentration of 0.5 g/ml (2 ml of buffer/ g of cell wet weight). Cells were incubated for 10 min at 30ºC with shaking, followed by the repeated centrifugation step and washed in 200 ml of 1.2 M sorbitol solution. Cells were harvested by centrifugation and resuspended to a concentration of 0.15 g/ml in buffer containing 1.2 M sorbitol, 20 mM KH_2PO_4·KOH pH 7.4 and 4 mg zymolyase per 1 g cell wet weight. Addition of zymolyase

leads to digestion of the cell wall and formation of the spheroplasts. The cell suspension was incubated for 30-60 min at 30ºC with gentle shaking. To test the cell wall digestion, 25 µl cell suspension was diluted into 1 ml H_2O or 1 ml 1.2 M sorbitol. Formation of spheroplasts was complete, when the OD of the H_2O dilution was 10-20% of the OD of the sorbitol dilution. All the subsequent steps were performed at 4°C. The spheroplasts were isolated by centrifugation (3000 x g, 5 min), resuspended (0.15 g/ml) in homogenization buffer (0.6 M sorbitol, 10 mM Tris-HCl, 1 mM EDTA, 0.2% (w/v) fatty acid-free BSA, 1 mM PMSF, pH 7.4), and dounced 10 times in a cooled douncer (homogeniser) on ice. Cell debris, intact cells and nuclei were removed as pellets by centrifugation performed twice (2000 x g, 5 min). Mitochondria were pelleted from the supernatant (12 min, 17400 x g) and resuspended in 25 ml SH buffer (0.6 M sorbitol, 20 mM HEPES/KOH, pH 7.3). After two centrifugation steps (2000 x g, 5 min), mitochondria were sedimented again as above and finally resuspended in 0.5-1 ml SH (0.6 M sorbitol, 20 mM HEPES/KOH pH 7.3) buffer. The protein concentration was determined by Bradford assay and mitochondria were diluted to 10 mg/ml, aliquoted, frozen in liquid nitrogen and stored at -80°C.

2.3.9. Import of preprotein into isolated mitochondria

Import of radiolabelled precursor proteins into *S. cerevisiae* mitochondria was performed in SI buffer (3% BSA (w/v), 0.6 M sorbitol, 50 mM HEPES-KOH, 80 mM KCl, 10 mM Mg-acetate, 2 mM KH_2PO_4, 2.5 mM EDTA, 2.5 mM $MnCl_2$, pH 7.2). For the import of small Tim proteins and Cox17 and Cox19, EDTA was omitted in the import buffer. In some cases, 2 mM NADH, 1 mM ATP, 10 mM creatine phosphate and 100 µg/ml creatine kinase were added to the import mixture. For inhibition of the respiratory chain activity, 10 µM KCN or 100 µg/ml antimycin A were added and incubated with mitochondria prior to import. For import experiments under oxygen-deprived conditions, the used buffers were degassed for 10 min using a PC 2001 Vario diaphragm vacuum pump (Vacuubrand, Wertheim, Germany) and subsequently purged with nitrogen gas for 2 min. The import reactions were carried out under nitrogen atmosphere in air-tight glove bags. Import reaction was started by addition of radiolabeled precursor (for lysate 1-3% (v/v)) and continued for different time points at 25°C (or at 12°C). Import was usually stopped by dilution 1:10 in ice cold SH buffer with or without 50 µg/ml proteinase K. Protease treatment was stopped after 15 min of incubation on ice by addition of 2mM PMSF. In some cases, instead of proteinase K, trypsin was used for 15 min on ice, followed by addition of 20-fold trypsin inhibitor. Finally, mitochondria were reisolated by centrifugation (12,000 x g, 10 min, 4ºC), washed once with 0.6 M sorbitol, 20 mM HEPES/KOH pH 7.3, 80 mM KCl and analyzed by SDS-PAGE, transfer to nitrocellulose membrane and phosphorimaging or autoradiography.

2.3.10. Trypsin treatment of endogenous Mia40 protein

To detect folded protease-resistant domains in the endogenous Mia40, mitoplasts were incubated with different concentrations (5, 10, 25 µg/ml) of trypsin for 30 min on ice. To check what role metal ions play in the stability of Mia40, prior to the treatment with trypsin, mitoplasts were incubated for 10 min in the absence or presence of chelators (10 mM EDTA, 2 mM bathophenanthroline-disulfonic acid). Following centrifugation (10 min, 14000rpm, 4ºC) and TCA precipitation of the supernatants, the protease-resistant fragments of Mia40 were identified by SDS-PAGE and immunoblotting.

2.3.11. Crosslinking of mitochondrial proteins

Crossliking during import of radiolabeled proteins into mitochondria was used in this study in order to check protein interactions. Import reactions were incubated with 200 µM DFDNB (1,5-Diuoro-2,4-dinitrobenzol), a membrane permeable lysine-specific crosslinker, for 20 min at 12°C or mock treated as control. Crosslinking was stopped by the addition of 100 mM glycine (pH 8.0) and incubation for 10 min on ice. After trypsin digestion of the non-imported preproteins, mitochondria were reisolated, washed, lysed and subjected to immunoprecipitation with Mia40-specific antibodies as described in 2.4.3. At the end, the samples were analysed by SDS-PAGE and autoradiography.

2.3.12. Pull-down assay

To demonstrate an interaction between Mia40 and Erv1 a pull-down assay was used. Mitochondria isolated from wild type or Mia40-His_6 cells (500 µg) were centrifuged (12000 x g, 5 min, 4°C) and the mitochondrial pellet was solubilized in 250 µl of lysis buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 2 mM PMSF) containing 1% (w/v) digitonin of 1 % Triton X-100 for 30 min at 4°C. To test whether the interaction is made via disulfide bonds, in one experiment 10 mM DTT was additionally present in the lysis buffer. Aggregates and insoluble membranes were removed by clarifying spin (20 min, 125 000 x g, 4°C) and the mitochondrial extract was added to 40 µl of preequilibrated NiNTA-agarose beads (Qiagen). After incubation for 60 min at 4°C, the beads were washed three times with lysis buffer (with 30 mM imidazole) and the bound proteins were eluted with sample buffer containing 300mM imidazole. The probes were resolved on SDS-PAGE gels under reducing or non-reducing conditions and analysed by immunodetection.

The same assay with small variations was applied to test the interaction between the different cysteine mutants of Mia40 and newly imported Tim13. The only changes were: (i) smaller amount of mitochondria used first for the import of radiolabeled Tim13 followed by the assay (200 µg) and (ii) lysis of mitochondria with 1 % SDS instead of digitonin or Triton X-100. At the end, the samples were analysed with SDS-PAGE and autoradiography.

2.4. Immunology methods

2.4.1. Purification of antibodies against Mia40

2.4.1.1. Coupling of peptides to the SulphoLink beads

The most often used antibodies in this study were the ones against the C-terminal peptide of yeast Mia40 (amino acids 292-403). The antigen (peptide CVKKEPLNEESKP) and the antibodies raised in rabbits were obtained from Pineda Antibody Service, Berlin. The additional cysteine inserted at the N-terminus of the peptide enabled its coupling to the SulfoLink gel (Pierce). One ml SulfoLink gel was packed into an appropriate column and the beads equilibrated with 6 ml 50 mM Tris-HCl, 5 mM EDTA, pH 8.5. After closing the column at the bottom, 1 mg of peptide dissolved in 1 ml of equilibration buffer was added. Column, closed at both ends, was gently rolled for 15 min at RT and then incubated for additional 30 min at RT without mixing. Upon buffer drainage, the beads were washed with 3 ml equilibration buffer and the excess of reactive groups was blocked with 1 ml 50 mM cysteine solution. Column was mixed with the cysteine solution for 15 min at RT and then incubated for another 30 min at RT without mixing. Finally, non-specifically bound peptide was removed by a washing step with 16 ml 1 M NaCl and the column was stored at 4°C in 10 mM Tris-HCl pH7.5 with 0.05% Na-azide.

2.4.1.2. Affinity purification of antibodies

To reduce the cross-reactivity of the antisera an affinity purification of the antibodies against C-terminal peptide of Mia40 was performed. The previously prepared column was equilibrated with 10 ml of 10 mM Tris-HCl pH 7.5 and then loaded with 6 ml of the antiserum diluted with 16 ml of 10 mM Tris-HCl pH 7.5 (supplemented with 130 µl 200 mM PMSF, 104 µl 1 mM EDTA, 104 µl 2 mM EGTA, 260 µl 0.5 M orthophenanthroline and 26 µl of 1 mg/ml leupeptin). The flow-through was reloaded twice. The column was washed with 20 ml of 10 mM Tris-HCl pH 7.5 followed by 20 ml of 10 mM Tris·HCl pH 7.5, 0.5 M NaCl. For the elution, column is subjected to different pH through application in given order of 10 ml of following buffers: 100 mM Na-citrate pH 4.0, 100 mM glycine-HCl pH 2.5 and 100 mM Na-phosphate pH 11.5. Fractions of 1 ml were collected and neutralized immediately with 200 µl 1 M Tris-HCl, pH 8.8 in the case of the first two buffers, and with 100 µl glycine pH 2.2 in the case of the phosphate buffer. The column was equilibrated to alkaline pH with 10 ml of 10 mM Tris-HCl pH 8.8 between the glycine and phosphate buffer elutions. At the end the column was washed with 10 mM Tris-HCl pH 7.5 and left in 10 mM Tris-HCl pH7.5 with 0.05% Na-azide at 4ºC. Several fractions (the loading solution, the flow-through, the wash and few elution fractions) were checked for specificity by immunodecoration on nitrocellulose membrane carrying yeast mitochondrial proteins. The majority of the specific antibodies were eluted with the glycine buffer in fractions 3-5. These fractions were aliquoted and stored at -20°C.

2.4.2. Immunodecoration (Immunoblotting)

Proteins immobilized on nitrocellulose membranes were visualized by immunodecoration with specific antibodies. The non-specific protein binding sites were blocked by membrane incubation with 5% (w/v) milk powder in TBS buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5) at RT for 30-60min. Then the membrane was incubated with the specific primary antibody (1:250 to 1:30000 dilutions in 5% milk in TBS) for 2 h at RT or overnight at 4ºC. The membrane was washed 3 times (each 5-10 min), once with TBS, then TBS with 0.05% (v/v) Triton X-100 and again with TBS, followed by an incubation for 50-90 min at RT with goat anti-rabbit antibodies coupled to horseradish peroxidase (diluted 1:10 000 in milk/TBS). After washing as described above, the membrane was treated with the chemiluminescent substrate of peroxidase (1:1 mix of ECL reagent 1 and 2) and the signals were detected on X-ray films (Fuji New RX). ECL reagent solutions were stable for 7-10 days when kept in light-protected bottles at 4°C.

For detection of His-tagged proteins, an anti-His antibody (Dianova, Hamburg) was used. Membranes were blocked in 3% (w/v) BSA in TBS buffer and anti-His antibody diluted 1:1000 in this blocking solution. Seco ndary antibodies (goat anti-mouse) were diluted 1:10000 also in 3% (w/v) in TBS buffer. Blots were developed as described above.

ECL reagent 1: 3 ml Tris·HCl, pH 8.5 (1M stock), 300 µl luminol (440 mg/10 ml DMSO), 133 µl p-coumaric acid (150 mg/10 ml DMSO), H_2O to 30ml

ECL reagent 2: 3 ml Tris·HCl, pH 8.5 (1M stock), 18 µl H_2O_2 (30%), H_2O to 30ml

2.4.3. Immunoprecipitation

Intermediates of Mia40 with the substrates were analysed by immunoprecipitations with purified antibodies against the C-terminal part of Mia40. Thirty µl of Protein A-Sepharose CL-4B (Amersham Biosciences) beads were washed 3 times with TBS, the

antibodies were added and incubated for 30-90min with overhead mixing. After washing off of the unbound antibodies, the beads were ready for incubation with proteins in the mitochondrial extract.

Following import of the radiolabeled preprotein, mitochondria were washed with 0.6 M sorbitol, 20 mM HEPES/KOH pH 7.3, 80 mM KCl and lysed in 1% SDS, 50 mM Na-phosphate pH 8.0, 300 mM NaCl, 1 mM PMSF for 10 min at RT. The extract was diluted 20-fold in TBS with 0.2 % Triton X-100 and non-solubilized material removed by an ultracentrifugation step (20 min, 45 000 rpm TLA-45, 4°C). The supernatant was added to the prepared Protein A-Sepharose beads and the mixture was incubated overhead for 1-2 h at 4°C. The beads were washed twice with 1 ml of TBS/0.2% Triton X-100 buffer and once with 1 ml of TBS before the bound proteins were eluted with either reducing or non-reducing Laemmli buffer (5 min at 95°C). Samples were then analyzed by SDS-PAGE and autoradiography.

3. RESULTS

3. 1. Identification and characterisation of Mia40

Despite the knowledge about import of precursor proteins into different mitochondrial compartments, little is known about the import into the intermembrane space (IMS), especially for proteins lacking presequences. A variety of IMS proteins are characterized by a small molecular size and a content of conserved cysteine residues forming specific motifs. The present study set out to identify components responsible for the import of these proteins into the IMS of mitochondria. Since some of the cysteine-rich proteins are essential for cell viability, the putative factors mediating their import should be also essential proteins. Furthermore, an import component is expected to possess conserved cysteine residues like the substrate proteins. From the set of essential and uncharacterized proteins with predicted mitochondrial location of yeast *Saccharomyces cerevisiae*, the protein Mia40 (mitochondrial intermembrane space import and assembly), encoded by the open reading frame *YKL195w*, was chosen as a potential candidate (Winzeler, Shoemaker *et al.* 1999; Sickmann, Reinders *et al.* 2003). Mia40 consists of 403 amino acid residues and contains at the N-terminus a typical mitochondrial targeting signal followed by a hydrophobic segment. Mia40 shows a high content of negatively charged residues, therefore the pI of the mature protein is 4.29. Mia40 possesses a C-terminal domain which is highly conserved in all homologues in the eukaryotic kingdom and harbours six conserved cysteine residues forming a CXC-CX_9C-CX_9C- motif (X represents non-cysteine amino acid residues) (Fig.5).

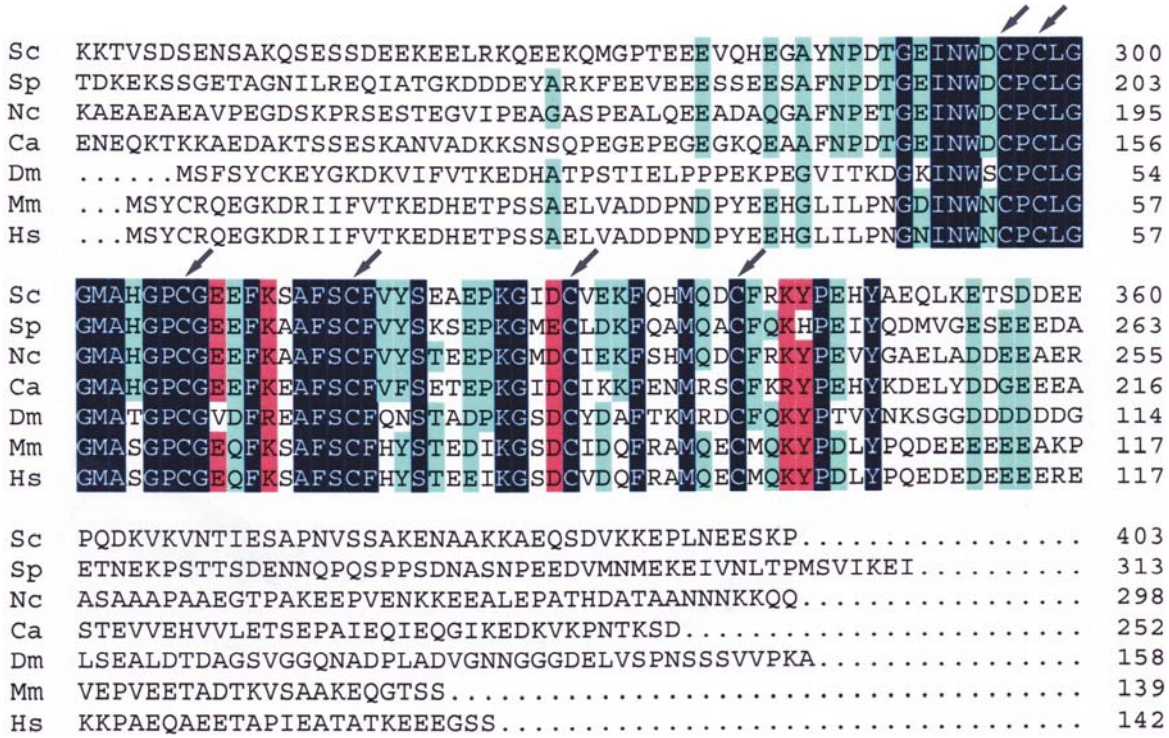

Figure 5. Mia40 C-terminal domain is conserved in all homologues from yeast to human. Sequence alignment of *Saccharomyces cerevisiae* (Sc) Mia40 (residues 241-403) with homologous proteins from *Schizosaccharomyces pombe* (Sp) (residues 144-313), *Neurospora crassa* (Nc) (residues 136-298), *Candida albicans* (Ca) (residues 97-252), *Drosophila melanogaster* (Dm), *Mus musculus* (Mm), *Homo sapiens* (Hs). The six highly conserved cysteines are indicated by arrows.

3.1.1. Mia40 exposes a large domain in the IMS of mitochondria

To confirm the mitochondrial location of Mia40, subcellular fractionation of yeast cells was performed. Mia40 was specifically detected in the mitochondrial fraction like the mitochondrial protein Tim50 (Fig.6A). Mia40 possesses a matrix targeting signal in front of a hydrophobic stretch, suggesting sorting of a large domain of the protein to the IMS by a stop-transfer mechanism. To verify whether Mia40 is in fact an IMS protein, mitochondria were treated with proteinase K, Triton X-100 and Na_2CO_3, followed by loading the samples on an SDS gel and immunobloting with Mia40-specific antibodies. Mia40 was protected against the activity of the protease in intact mitochondria, but was degraded by protease added to mitoplasts, whose outer membrane had been selectively ruptured (Fig.6B). Cytochrome b_2, a protein found in the mitochondrial IMS, showed the same behaviour. Upon alkaline treatment of mitochondria, a significant amount of Mia40 remained in the membrane pellet and Mia40 was only partially extracted from the membranes. The same fractionation pattern was reported before for other single-spanning membrane proteins such as Tim14 (Mokranjac *et al.*, 2003). These findings indicate that Mia40 is anchored by the N-terminal transmembrane region in the inner membrane and exposes a big C-terminal domain in the mitochondrial IMS (Fig.6C).

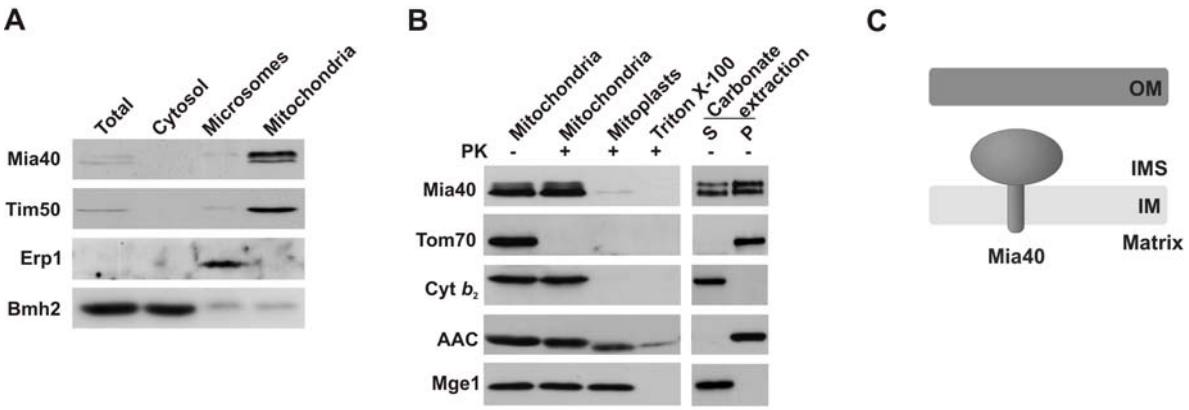

Figure 6. Mia40 is a mitochondrial protein in the intermembrane space. (A) Total yeast cell extract and subcellular fractions (each 50 µg protein) were analyzed by immunoblotting with antibodies against Mia40 and the marker proteins Tim50 (mitochondria), Erp1 (microsomes) and Bmh2 (cytosol). Interestingly, Mia40 showed a slower migration on SDS–PAGE than expected from the predicted molecular mass. This behaviour is characteristic for proteins showing a high content of acidic amino acid residues due to a reduced binding of SDS. In addition, Mia40 was detected as a double band under these conditions. **(B)** Mitochondria, mitoplasts and a mitochondrial Triton X-100 extract were incubated in the absence or presence of 50 µg/ml PK. Mitochondria were fractionated by carbonate extraction into soluble (S) and membrane pellet (P) fractions and analyzed as in (A). Tom70, outer membrane; cytochrome b_2 (Cyt b_2), IMS protein; ATP/ADP carrier (AAC), inner membrane; Mge1, matrix protein. **(C)** Model showing the topology of Mia40.

3.1.2. Mia40 binds copper and zinc ions

Many cysteine-rich proteins, e.g. small Tim proteins or Cox 17, bind cofactors, such as metal ions. To assess whether Mia40 has the potential to bind metal ions, the IMS domain of Mia40 (residues 68-403) was fused to the maltose-binding protein (MBP-Mia40ΔTM) and expressed in *E.coli*. After purification of the fusion protein an induction coupled plasma atomic emission spectroscopy (ICP-AES) was performed to determine the metal content. Significant amounts of zinc and copper ions were found in MBP-Mia40ΔTM (Fig.7A). Other metals, such as iron and nickel, were not detected. This suggests that Mia40 is able to specifically bind zinc and copper ions.

The presence of a metal cofactor in Mia40 was further supported by a strongly increased trypsin sensitivity of the endogenous protein when incubated with chelators (Fig.7B). In summary, these results suggest a stabilisation of the IMS domain of Mia40 by metal ions.

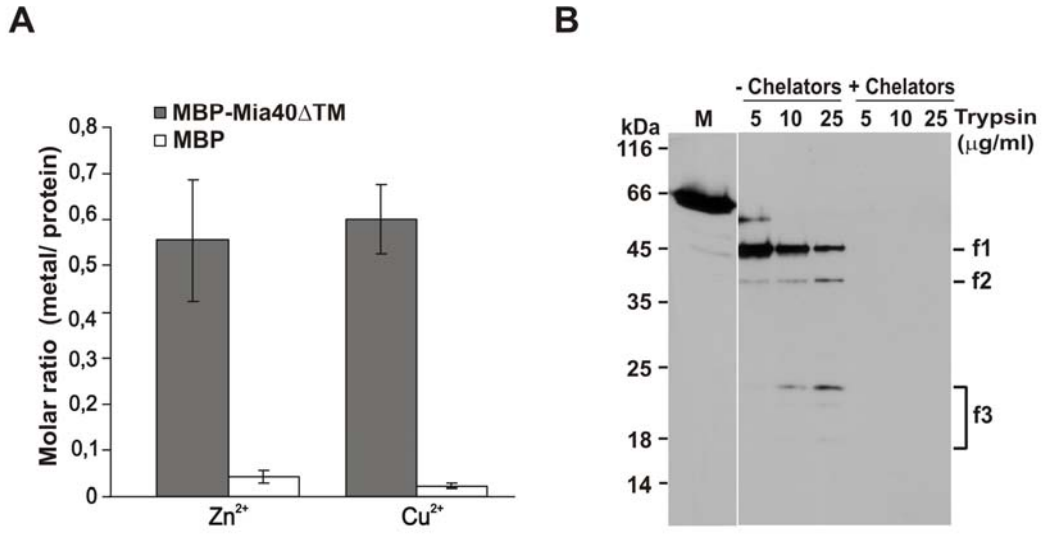

Figure 7. Mia40 binds zinc and copper ions. (A) A fusion protein of maltose-binding protein (MBP) and IMS domain of Mia40 (MBP-Mia40ΔTM) was purified. The zinc and copper ions content was determined by ICP-AES. The molar metal to protein ratio is shown. **(B)** Mia40 folding is stabilized by metal binding. Mitoplasts were incubated with the indicated amounts of trypsin in the absence or presence of chelators (10 mM EDTA, 2 mM bathophenanthroline-disulfonic acid). Protease-resistant fragments of Mia40 in the soluble fraction (f1 to f3) were identified by immunoblotting.

3.1.3. Mia40 is required for the import of small IMS proteins

Deletion of the *MIA40* gene is lethal for the yeast cells. Therefore a strain harbouring a glucose-repressible *GAL10* promoter in front of the *MIA40* gene on the chromosome, named GAL-MIA40, was constructed in order to study the function of Mia40. Depletion of Mia40 was achieved about 22 hours after the shift from galactose- to glucose-containing medium and the cell growth slowed down significantly (data not shown, Fig.8A). Mitochondrial extracts were prepared from wild type (WT) and Mia40-depleted cells (Mia40↓) and analyzed by immunoblotting for various mitochondrial proteins (Fig. 8B). The endogenous levels of the small IMS proteins containing cysteine motifs, like Tim13, Tim10, Cox17, were significantly reduced in mitochondria depleted of Mia40. Other IMS proteins, such as the ones harbouring heme as cofactor (cytochrome b_2, cytochrome *c* and cytochrome *c* heme lyase), were not affected. The levels of proteins found in the outer membrane (Tom40), the inner membrane (Tim23, AAC) and in the matrix space (Tim44, Mge1, Aco1) were also not altered. The steady-state level of the inner membrane protein Tim22 was slightly decreased, but this might be a secondary effect as the biogenesis of Tim22 depends on the presence of small Tim proteins in the IMS. These findings suggest that Mia40 is involved in the biogenesis or the maintenance of small IMS proteins containing cysteine motifs.

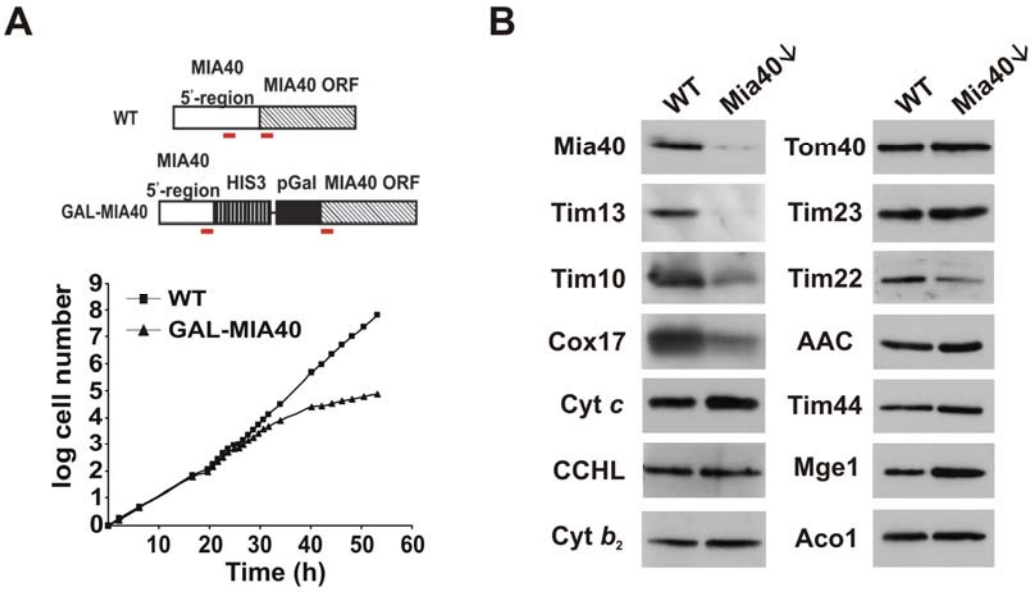

Figure 8. Mia40 is involved in the biogenesis of small IMS proteins. (A) Downregulation of Mia40 inhibits cell growth. Wild type (WT) or GAL-MIA40 cells were grown on galactose containing medium and then shifted to glucose containing medium. Cell number at time zero was set equal to 1. **(B)** Endogenous levels of the proteins indicated were analyzed by immunoblotting of mitochondria from wild type cells (WT) and cells depleted of Mia40 for 16 h (Mia40↓).

To assess the function of Mia40 in detail, the import of radiolabeled precursor proteins into isolated mitochondria was analyzed. Various precursors were synthesized in reticulocyte lysate ('radiochemical amounts') or purified from recombinant *E. coli* cells ('chemical amounts') and incubated with mitochondria isolated from wild type and Mia40-depleted cells (Mia40↓). Non-imported proteins were removed by proteinase K treatment. The import of Tim13, Tim10 and Cox17 was strongly impaired in Mia40-depleted mitochondria (Fig.9A). To check the specificity of the import defect observed in mitochondria depleted of Mia40, analysis of the import of proteins into the matrix (pSu9DHFR) and the outer membrane (porin) was performed. Additionally, the import of cytochrome *c* heme lyase (CCHL), another protein in the IMS, was tested. All three preproteins were efficiently imported in Mia40↓ mitochondria (Fig. 9B). Notably, when Mia40 was overexpressed (Mia40↑), the import rates of Tim13 were significantly increased suggesting that Mia40 is a rate-limiting factor for the import of Tim13 (Fig.9C). Control proteins (porin, pSu9DHFR) were imported into the Mia40↑ mitochondria as efficiently as in the wild type mitochondria. In conclusion, Mia40 is essential for the mitochondrial import of small IMS proteins that possess conserved cysteine motifs.

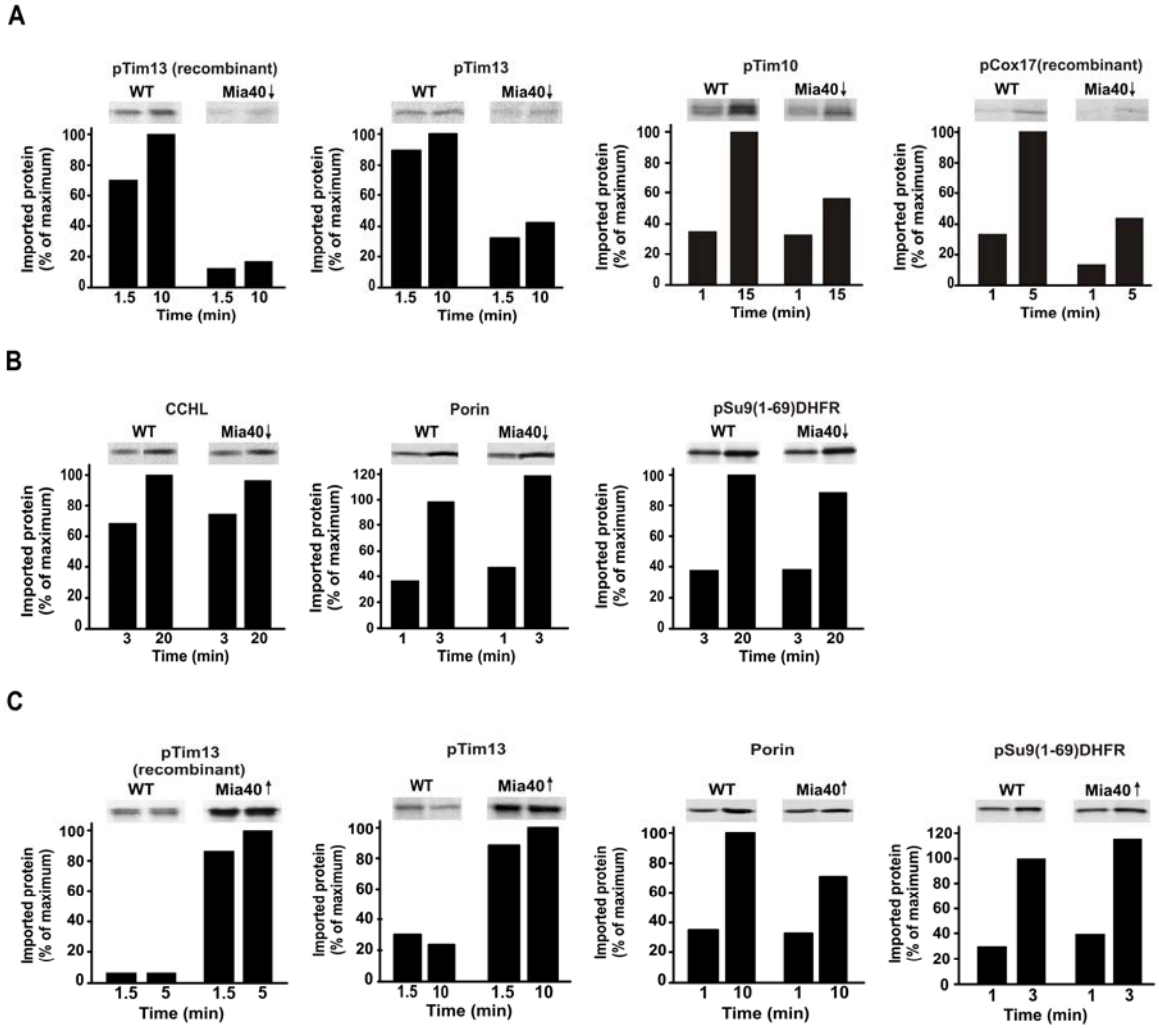

Figure 9. Mia40 is required for the import of small IMS proteins. (A, B) Chemical (recombinant) or radiochemical amounts of preproteins were imported into wild type and Mia40↓ mitochondria. Mitochondria were treated with proteinase K, reisolated and the amounts of imported proteins were analyzed by autoradiography and densitometric quantification. CCHL, cytochrome *c* heme lyase; pSu9(1-69)DHFR, presequence of subunit 9 of the F_1F_0-ATPase fused to dihydrofolate reductase. Import into wild type mitochondria at the longest time point was set as 100 %. **(C)** Precursor proteins were imported into wild type mitochondria and mitochondria with 10-fold increased Mia40 levels (Mia40↑) and analyzed as in (A, B).

3.1.4. Mia40 interacts with newly imported Tim13 via disulfide bridges

Does Mia40 directly act on the newly imported precursor? To test this, chemical crosslinking experiments were performed. Isolated mitochondria were incubated with the radiolabeled Tim13 in the presence or the absence of the crosslinking reagent 1,5-difluoro-2,4-dinitrobenzene (DFDNB) and then lysed under denaturing conditions. Mia40 was isolated by immunoprecipitation with specific antibodies. As shown in Fig.10, a crosslinked product of Tim13 of about 75 kDa was observed in the precipitation with Mia40-specific antibodies. It was absent in control precipitations with preimmune serum and when no crosslinker was added, therefore the product represents a specific interaction of Tim13 with Mia40. In addition, no crosslinking to Mia40 was detected with the $Tim13^{SSSS}$ mutant, which has the four conserved cysteine residues replaced by serine residues and does not stably accumulate in the IMS of mitochondria. Thus, Mia40 physically interacts with Tim13 during or shortly after its translocation into the IMS of mitochondria.

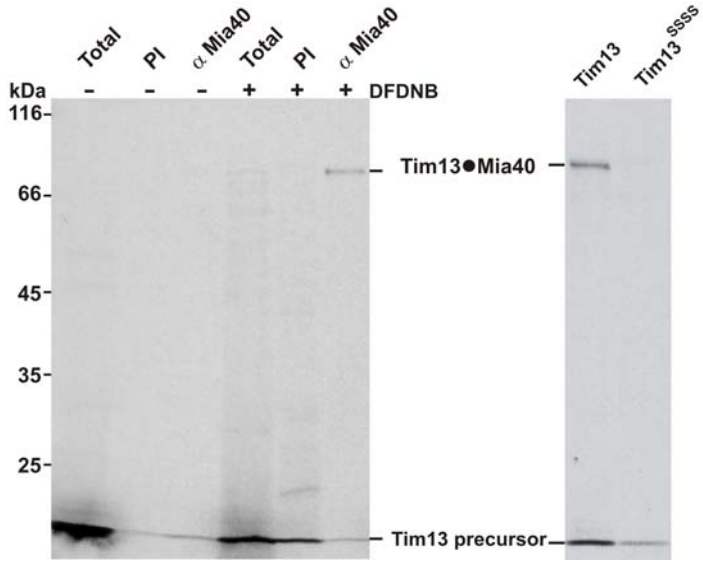

Figure 10. Mia40 interacts with newly imported Tim13. Radiolabeled precursor of Tim13 and of a cysteine-less mutant form of Tim13 ($Tim13^{SSSS}$) were imported into mitochondria for 20 min at 12 °C in the absence or presence of the chemical cross-linking reagent DFDNB (200 μM). After lysis the supernatants were subjected to immunoprecipitation with antibodies against Mia40 or preimmune serum. The proteins were finally separated on a reducing SDS gel, transfer onto nitrocellulose membrane and visualized by autoradiography. Total lane represents 5 % of the material used for the immunoprecipitation.

Interestingly, Tim13 co-precipitated with Mia40 under denaturing conditions in the absence of the crosslinker. This result suggests an interaction of Mia40 with Tim13 via disulfide bridges, which is dissociated by the reducing agent present in the SDS sample buffer. To confirm this, radiolabeled Tim13 was imported into isolated mitochondria in the absence of a crosslinking agent. After lysis of mitochondria, immunoprecipitation with Mia40-specific antibodies was performed and the proteins were resolved by SDS-PAGE under reducing and non-reducing conditions. The interaction of newly imported Tim13 with Mia40 was SDS-resistant and detected only under non-reducing conditions (Fig.11A). When β-mercaptoethanol was added to the sample loading buffer, the complex of Mia40 and Tim13 dissociated and Tim13 was resolved at its monomeric size (11kDa). A comparable interaction was observed between Mia40 and newly imported Cox17, another IMS protein with conserved cysteine motif and a substrate of Mia40 (Mesecke, Terziyska *et al.* 2005). In contrast, no interactions to newly imported cytochrome *c* heme lyase (CCHL) were detected (Fig.11B). Cytochrome *c* heme lyase contains four cysteine residues but it is not a substrate of the Mia40-dependent pathway. Therefore, the formation of the disulfide bonds between Mia40 and its substrate proteins appears to be a physiological reaction during the import process. In summary, Mia40 interacts covalently with newly imported substrate proteins, such as Tim13, via disulfide bridges.

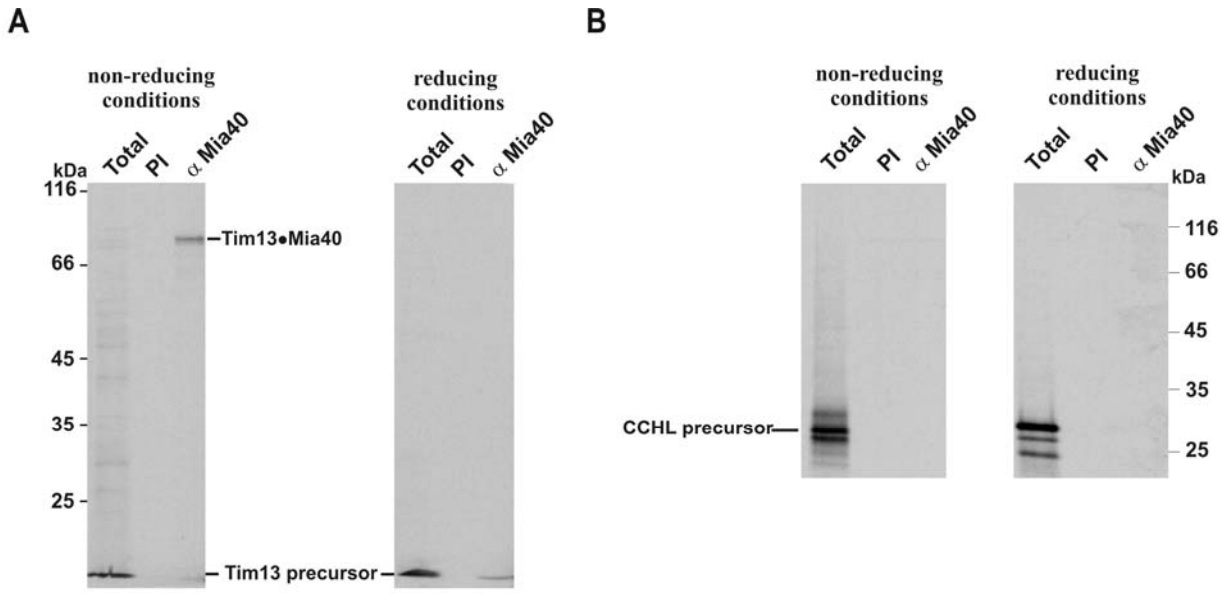

Figure 11. Mia40 forms disulfide bridges with newly imported Tim13. Mitochondria were incubated with radiolabeled Tim13 **(A)** or CCHL **(B)** protein for 20 min at 12°C. Non-imported material was removed by trypsin treatment, the mitochondria were washed and then lysed in SDS buffer. The resulting lysates were subjected to immunoprecipitation with antibodies against Mia40 (α-Mia40) or with preimmune (PI) serum. Proteins were resolved by SDS-PAGE under non-reducing or reducing conditions and visualized by autoradiography. Total lane represents 5 % of the material used for the immunoprecipitation.

3.2. Mia40 – a component of a disulfide relay system in the IMS of mitochondria

3.2.1. Import of Tim13 into isolated mitochondria is sensitive towards reducing agents

Mia40 is the first component specifically involved in the import of small cysteine-rich IMS proteins. Precursors of these proteins are translocated through the TOM channel in their reduced form and bind to Mia40 forming a mixed disulfide intermediates. If the formation of this mixed intermediate between Mia40 and its substrate is essential for the import of the small IMS proteins, conditions preventing any oxidation events should hinder the translocation of the precursors into mitochondria. To verify this, import of radiolabeled Tim13 into mitochondria was performed with addition of different amounts of dithiothreitol (DTT). The import of Tim13 was indeed inhibited by higher concentrations of DTT (Fig.12). Conversely, lower concentrations of DTT (below 5 mM) actually enhanced the import of Tim13, which is in line with a previous study (Lu, Allen *et al.* 2004) showing that oxidation of precursor proteins prior to import prevents translocation through the TOM channel. In conclusion, the sensitivity of the import reaction to reductants is consistent with the essential role of the mixed disulfide between Mia40 and Tim13 for the translocation of Tim13 into the IMS.

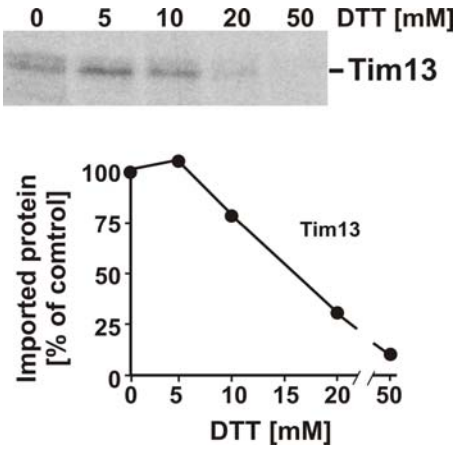

Figure 12. Import of Tim13 is inhibited by high concentrations of reducing agent. Radiolabeled recombinant Tim13 was imported into isolated mitochondria for 10 min in the presence of indicated concentrations of DTT at 25^0C. Non-imported material was removed by protease treatment. The imported Tim13 was visualized by autoradiography, quantified by densitometry and expressed in comparison to the protein imported without DTT.

3.2.2. Mia40 is present in two redox states: an oxidized and a reduced state

Since disulfide bonds are in general not formed *de novo* and oxidized proteins are often involved in these processes, it was addressed whether Mia40 can adopt an oxidized state. To analyze the redox state of Mia40, mitochondria were incubated in the absence or presence of DTT and then treated with N-ethylmaleimide (NEM) to prevent the reduced thiols from reoxidation during electrophoresis. Following separation on non-reducing SDS gel, two forms of Mia40 were detected by immunoblotting: a fast migrating oxidized form and a reduced form of lower mobility in the DTT-treated probe. Obviously, most of Mia40 is present in mitochondria in an oxidized form with at least one disulfide bond.

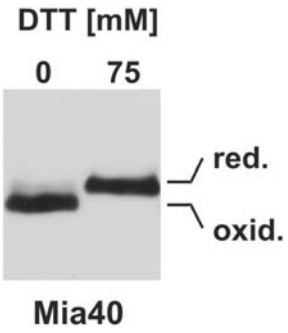

Figure 13. Mia40 can be detected in an oxidized and reduced form. Wild type mitochondria (50 µg) were incubated in the presence or absence of 75 mM DTT for 10 min at room temperature. Then, mitochondria were incubated with 50 mM NEM for 20 min to modify reduced thiol groups. Mitochondria were reisolated and lysed in sample buffer. The proteins were resolved on a non-reducing SDS gel and Mia40 was visualized by immunoblotting.

3.2.3. The presence of disulfide bonds in Mia40 is crucial for import of Tim13

To check whether a reduction of oxidized thiol groups in Mia40 might be the reason for the observed sensitivity of the import process towards reducing agents, mitochondria were preincubated with 10 mM DTT followed by mock or NEM treatment. After reisolation of the mitochondria and removal of DTT and NEM in a washing step, import of Tim13 into these mitochondria was performed. Interestingly, Mia40 was present in its oxidized state even when the mitochondria were preincubated with DTT and Tim13 was imported efficiently (Fig.14). The disulfide bond in Mia40 most probably was recovered before the Tim13 precursor was added to the reaction. In contrast, when the reduced thiol groups of Mia40 were modified by NEM, blocking any possible reoxidation, Mia40 was detected in its reduced form and the subsequent import of Tim13 was almost completely inhibited. The pretreatment of mitochondria only with NEM did not influence significantly the redox state and the function of Mia40. In summary, conditions that trap Mia40 in a reduced state block the import of Tim13 into the IMS of mitochondria.

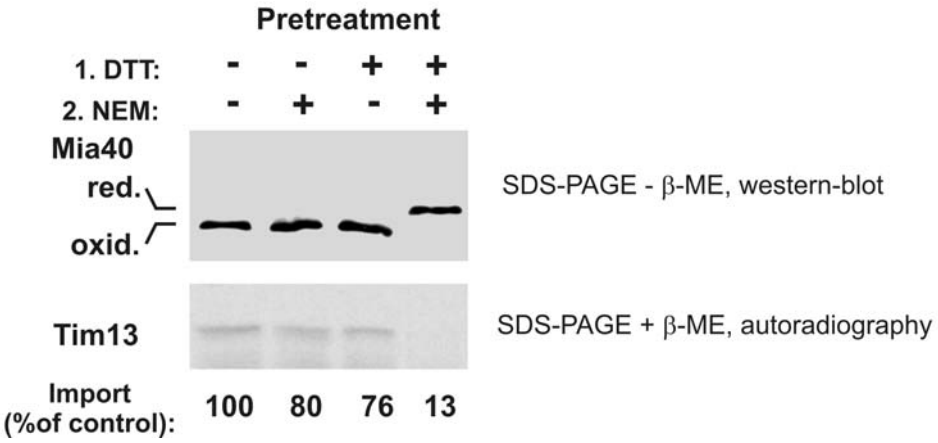

Figure 14. The presence of disulfide bonds in Mia40 is crucial for the import of Tim13. Two aliquots of mitochondrial suspension were incubated in the absence and two aliquots in the presence of 10 mM DTT for 10 min. To one of each set of aliquots 50 mM NEM was added in order to modify reduced thiol groups. After reisolation mitochondria were incubated in import buffer with radiolabeled Tim13 for 10 min. Non-imported material was removed by protease K treatment. At the end mitochondria were dissolved in sample buffer lacking β-mercaptoethanol. One half of the sample was directly loaded on a gel to determine redox state of Mia40 by immunoblotting. The other half was reduced by addition of β-mercaptoethanol and the amount of imported Tim13 was assessed by SDS-PAGE, autoradiography, and densitometry. The import efficiency without DTT and NEM was a control (100 %).

3.2.4. Mia40 becomes reduced after import of Tim13 into mitochondria

Mia40 interacts transiently via disulfide bridges with the newly imported precursor protein. Earlier studies of the import and assembly of Tim10 have already proposed an oxidative folding pathway in the IMS of mitochondria (Lu, Allen *et al.* 2004). Therefore, it is conceivable that upon release of the polypeptide Mia40 donates at least one disulfide bond to the precursor protein. The substrate would then become oxidized and Mia40 would be left reduced. To test this hypothesis, Mia40 redox state was monitored after the import of recombinant Tim13 precursor into wild type mitochondria. To trap thiol groups in their reduced conformation, mitochondria were incubated with NEM. Notably, a significant amount of Mia40 was found in a reduced state following import of chemical amounts of Tim13. In the mock treated mitochondria, in contrast, Mia40 remained in oxidized form. Hence, Mia40 becomes reduced after import of Tim13 into mitochondria.

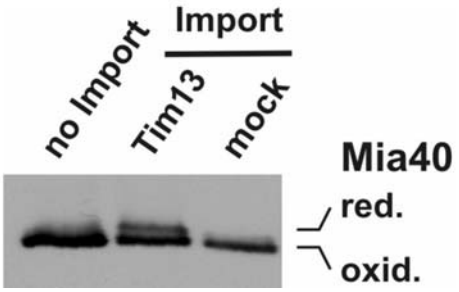

Figure 15. Mia40 becomes reduced after import of Tim13. Wild-type mitochondria (50 μg) were incubated with recombinant Tim13 or mock treated for 10 min. Import was stopped by diluting the reaction 10-fold in cold SH buffer. Then the mitochondria were incubated with 50 mM NEM for 20 min to trap the thiol groups in their reduced state. Finally the mitochondria were dissolved in sample buffer without β-mercaptoethanol and loaded on SDS gel together with non-treated mitochondria as a control. Reduced and oxidized species of Mia40 were detected by immunoblotting.

3.2.5. Depletion of Erv1 enhances the sensitivity of the Tim13 import to DTT and affects the formation of the mixed disulfide between Tim13 and Mia40

The presence of disulfide bridges in Mia40 is crucial for the import of small IMS proteins, enabling formation of the mixed disulfide between Mia40 and the precursor. After import Mia40 becomes reduced, therefore an oxidation reaction has to restore Mia40 in its active state and thereby ensure a next round of import. The IMS of mitochondria harbours a sulfhydryl FAD-linked oxidase, called Erv1, which is essential for the viability of yeast cells. The function of Erv1 had not been identified so far. A role of Erv1 in the oxidation of Mia40 and therefore in the biogenesis of the IMS proteins was assessed in collaboration with Nikola Mesecke and Dr. Johannes Herrmann (Mesecke, Terziyska *et al.* 2005). Depletion of Erv1 led to a reduction in the endogenous levels of small IMS proteins with cysteine motifs, such as Tim13 and Cox17, which are imported in a Mia40-dependent manner. Proteins from other mitochondrial subcompartments (Tom70, ATP/ADP carrier) and IMS proteins lacking cysteine motifs (CCHL, cytochrome b_2) were not affected in Erv1-depleted cells. When the import of the Tim13 was tested in the absence of DTT, no significant defect was observed in the Erv1-depleted mitochondria compared to the wild type. However, already very small concentrations of DTT present during the import reaction virtually blocked the Tim13 translocation in Erv1-depleted mitochondria (Fig.16). These results suggest that Erv1 counteracts the inhibitory effects of DTT on the Tim13 import.

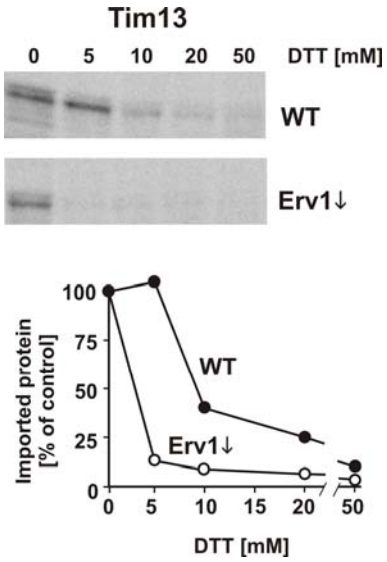

Figure 16. Sensitivity of Tim13 import to DTT is increased in Erv1-depleted mitochondria. Radiolabeled recombinant Tim13 was imported into wild type and Erv1-depleted mitochondria for 10 min in the presence of indicated concentrations of DTT at 25^{0}C. Non-imported material was removed by protease treatment. The imported Tim13 was visualized by autoradiography, quantified by densitometry and expressed in comparison to the protein imported without DTT (set as 100 %).

Next, the effect of Erv1 depletion on the functionality of Mia40 was analyzed, namely the generation of mixed disulfide with Tim13 precursor during import. The Mia40-Tim13 complex was efficiently formed in wild-type mitochondria, but strongly reduced in Erv1-depleted mitochondria (Fig.17). Thus, the function of Erv1 is required for the formation of the mixed disulfide of Mia40 with the newly imported Tim13 in the IMS of mitochondria. In summary, Erv1 is a second factor involved in the biogenesis of small cysteine-rich IMS proteins.

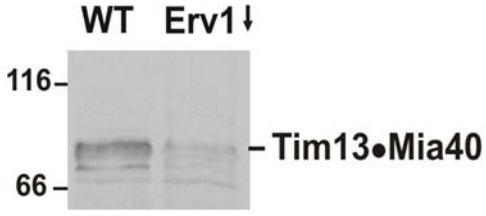

Figure 17. Depletion of Erv1 affects the formation of Mia40-Tim13 complex. Radiolabeled Tim13 was incubated with mitochondria from wild-type and Erv1-depleted cells for 10 min. Non-imported material was removed by treatment with proteinase K and the Tim13-Mia40 complex was detected by non-reducing SDS-PAGE, blotting and autoradiography.

3.2.6. Erv1 interacts with Mia40 maintaining it in its active state

Since both Mia40 and Erv1 are required for the import of the same group of IMS proteins and depletion of Erv1 affects the function of Mia40, it was addressed whether these two proteins interact with each other. Formation of transient mixed disulfides with the substrate protein has been shown for other sulfhydryl oxidases, like DsbB from the bacterial periplasm. To check if Erv1 can be found in a complex with Mia40 under non-reducing conditions, a strain was constructed, in which, in addition to the endogenous Mia40 protein, a C-terminally His-tagged Mia40 (Mia40-His_6) was expressed. Mitochondria were lysed with Triton X-100 and Mia40-His_6 protein was isolated by NiNTA affinity chromatography. A fraction of the endogenous Erv1 was found co-purified with Mia40-His_6 (Fig.18A, arrowheads). Under the experimental conditions about 5% of total Erv1 were detected in association with Mia40. The interaction of Erv1 with Mia40-His_6 was stable in the presence of SDS under non-reducing conditions (Fig.18B). This finding suggests a covalent interaction between the two proteins, probably via disulfide bonds. When DTT was added to the lysis buffer prior to NiNTA chromatography, Erv1 dissociated from Mia40-His_6 protein and was not detected anymore in the eluate (Fig.18C). Thus, Erv1 binds to Mia40 via disulfide bonds.

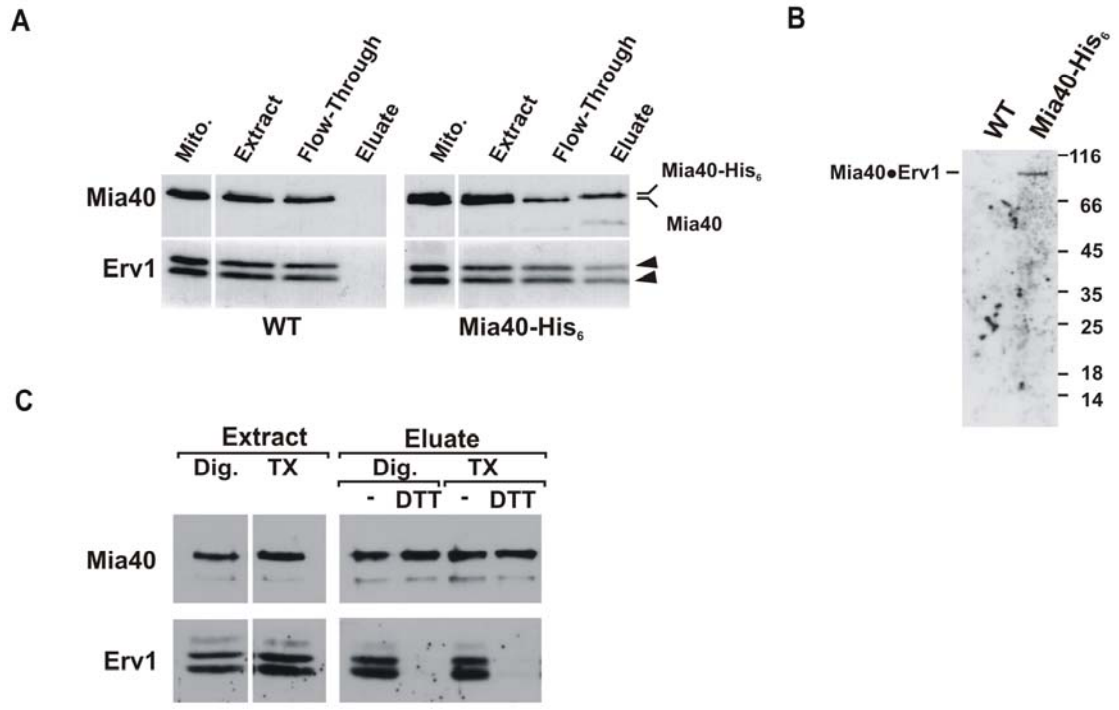

Figure 18. Erv1 interacts with Mia40. (A) Mitochondria (500 µg) isolated from wild type or Mia40-His_6 cells were lysed in 250 µl buffer consisting of 1 % TritonX-100, 300 mM NaCl, 20 mM imidazole, 2 mM PMSF, 50 mM sodium phosphate (pH 8.0). After centrifugation for 20 min at 125,000×g the extracts were incubated with NiNTA Agarose for 60 min at 4°C. Next the beads were washed three times with lysis buffer and the bound proteins were eluted with sample buffer containing 300 mM imidazole. Following SDS-PAGE under reducing conditions immunoblotting was performed with antibodies against Mia40 and Erv1. For control, 50 µg mitochondria were dissolved directly in sample buffer and loaded on an SDS gel (Mito.). Both the tagged and the untagged version of Mia40 found in the Mia40-His_6 strain are indicated. Arrowheads show the Erv1 protein copurified with Mia40-His_6. **(B)** Wild type and Mia40-His_6 mitochondrial extracts were obtained and incubated with NiNTA Agarose as described in (A). The bound proteins were resolved on an SDS gel under non-reducing conditions and transferred to nitrocellulose. At the end, immunodetection with Erv1-specific antibodies was performed. **(C)** Mitochondria from Mia40-His_6 cells were lysed with 1 % digitonin (Dig.) or 1 % Triton X-100 (TX) in the absence or presence of 10 mM DTT. To isolate Mia40-His_6 the extracts were incubated with NiNTA Agarose. Mia40 and Erv1 in the mitochondrial extract and the eluate were examined by immunoblotting.

Next, the effect of Erv1 on the redox state of Mia40 was analyzed. To this end, the redox state of Mia40 was assessed in the Erv1-depleted mitochondria. Notably, a significant amount of Mia40 was shifted to the reduced form in these mitochondria, in contrast to the wild type, where Mia40 was mainly present in the oxidized state (Fig.19). Hence, Erv1 affects the redox state of Mia40 and is required to keep Mia40 in its oxidized form.

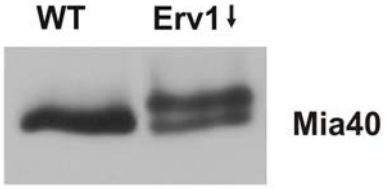

Figure 19. Erv1 maintains Mia40 in its oxidized state. Mitochondria were isolated from wild type and Erv1 depleted cells and treated with 50 mM NEM for 20 min at room temperature. The redox state of Mia40 was analyzed by non-reducing SDS-PAGE and immunoblotting.

In summary, Erv1 interacts in a reductant-sensitive manner with Mia40, maintaining it in an oxidized state and thereby enabling the import of small IMS proteins with cysteine motifs. The results indicate that both proteins form in the IMS a disulfide relay system which mediates oxidative folding in mitochondria.

3.2.7. The interaction of Mia40 with Tim13 and Erv1 is stable at physiological concentrations of glutathione

In the eukaryotic cell, the IMS is connected with the cytoplasm via pores formed mainly by porin. Since pores allow free diffusion of small molecules, such as glutathione, the redox conditions of the IMS are supposed to resemble the ones of the cytoplasm. In the yeast cytosol reduced glutathione was determined to be present at concentrations of about 13 mM (Ostergaard, Tachibana *et al.* 2004). This observation supports the notion that the IMS is rather a reducing environment. Thus, it is quite amazing to detect proteins containing disulfide bonds within the IMS of mitochondria. Therefore, Mia40 interactions with the substrate, Tim13, and the oxidase, Erv1, were tested under physiological concentrations of reduced glutathione. First, radiolabeled Tim13 was imported into mitochondria in the presence of 15 mM GSH, followed by immunoprecipitation with Mia40-specific antibodies. The Mia40-Tim13 complex was formed efficiently and detected on a non-reducing SDS gel (Fig.20A). Next, mitochondria from Mia40-His_6 cells were incubated with different GSH concentrations (0-20 mM), lysed and a NiNTA affinity chromatography was performed. The association of Erv1 with Mia40-His_6 protein was not inhibited by the presence of reduced glutathione, even at concentrations higher than the physiological concentration of GSH (Fig.20B). To conclude, the interactions of Mia40 with Tim13 and Erv1 are stable at physiological concentrations of reduced glutathione. This indicates that the disulfide relay system in the IMS of mitochondria can function efficiently in a reducing environment.

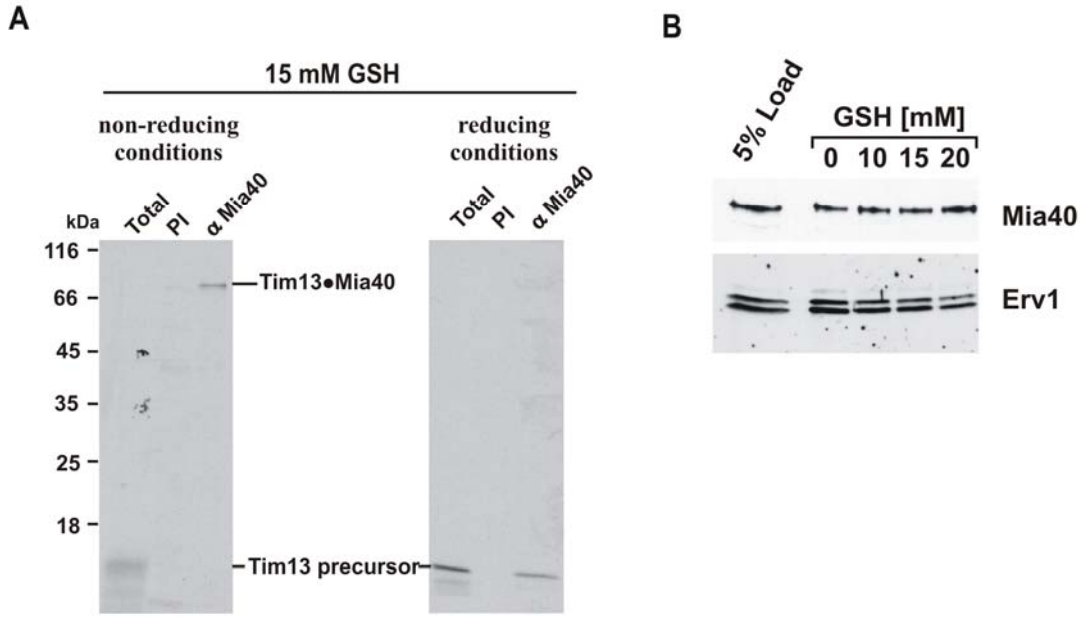

Figure 20. Mia40 interaction with Tim13 and Erv1 is stable at physiological glutathione concentrations. (A) Mitochondria were incubated with radiolabeled Tim13 protein for 20 min at 12°C in the presence of 15 mM reduced glutathione (GSH). To remove the non-imported material mitochondria were treated with trypsin and then lysed in SDS buffer. Next immunoprecipitation with preimmune serum (PI) and Mia40-specific antibodies was performed. Proteins were resolved on an SDS gel under non-reducing or reducing conditions and visualized by autoradiography. Total lane represents 5 % of the material used for the immunoprecipitation. **(B)** Mitochondria from Mia40-His_6 cells were preincubated for 5 min at 25°C with the indicated amounts of glutathione. After lysis the mitochondrial extracts were incubated with NiNTA Agarose beads for 1 hour at 4°C. Bound proteins were eluted with 300 mM imidazole and resolved by SDS-PAGE under reducing conditions. Mia40 and Erv1 were detected by immunoblotting.

3.2.8. Mia40-mediated import of small IMS proteins depends on the activity of the respiratory chain

Upon reoxidation of Mia40, the sulfhydryl oxidase Erv1 itself is reduced and needs to be reoxidized by a transfer of electrons to an electron acceptor. It was suggested that the molecular oxygen is the electron acceptor in the Erv1-mediated reaction, like in the case of many other sulfhydryl oxidases. However, recently a detailed study of the redox chemistry of the augmenter of liver regeneration (ALR), the human homologue of Erv1, showed that cytochrome *c* is a 100-fold better electron acceptor *in vitro* than oxygen (Farrell and Thorpe 2005; Bihlmaier, Mesecke *et al.* 2007). Thus, Erv1 may also be oxidized by cytochrome *c* in yeast. Based on the observation that yeast cells lacking cytochrome *c* ceased to grow under anaerobic conditions, Allen and colleagues suggested the reoxidation of Erv1 by cytochrome *c* in yeast (Allen, Balabanidou *et al.* 2005). The connection of the disulfide relay system via cytochrome *c* to the mitochondrial respiratory chain was studied in collaboration with Karl Bihlmaier and Dr. Johannes Herrmann (Bihlmaier, Mesecke *et al.* 2007). They observed that the redox state of Mia40 depends on the activity of the respiratory chain complexes. In the absence of oxidized cytochrome *c* or by inhibition of complex IV, Mia40 was shifted to its reduced form, whereas enhanced levels of oxidized cytochrome *c* or inhibition of complex III increased the oxidation of Mia40. Additionally, Erv1 could be efficiently oxidized *in vitro* by cytochrome *c*, thereby avoiding the oxidation of Erv1 by molecular oxygen and preventing the production of deleterious hydrogen peroxide. Thus, the disulfide relay system is directly linked to the respiratory chain by the communication between Erv1 and cytochrome *c*.

Results

Do cytochrome *c* and the activity of the respiratory chain affect not only the redox state of Mia40, but also Mia40-dependent protein import? To analyze this, import experiments into mitochondria isolated from wild type cells and from mutants of the respiratory chain complexes were performed under low-oxygen conditions in the presence of various concentrations of DTT. Already very low concentration of DTT blocked almost completely the import of Cox19 into mitochondria lacking cytochrome *c* oxidase (Δ*cox18*). On the contrary, the import of Cox19 into mitochondria from the cytochrome *c* reductase-deficient strain (Δ*rip1*) was even more efficient than the one into wild type mitochondria (Fig.21A). High sensitivity towards DTT was also observed for the import of Tim10 into Δ*cyc1*/Δ*cyc7* mitochondria, which are lacking both isoforms of cytochrome *c* (Fig. 21B). Hence, Mia40-dependent protein import in the absence of cytochrome *c* or cytochrome *c* oxidase is hypersensitive to DTT.

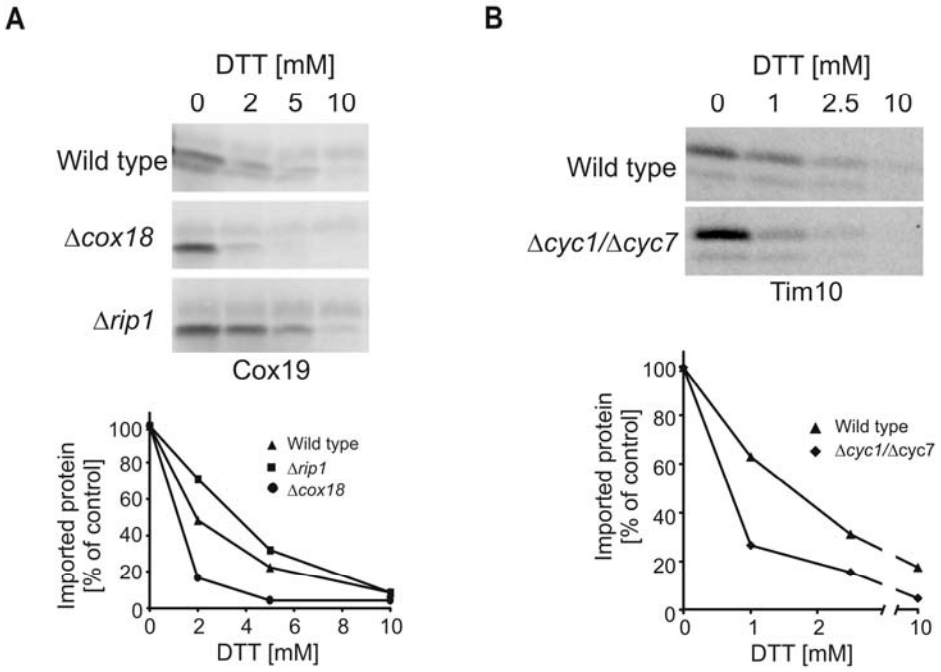

Figure 21. Mia40-mediated protein import depends on the activity of the respiratory chain complexes. (A) Mitochondria from wild type, Δ*rip1* and Δ*cox18* cells were incubated with radiolabeled Cox19 in the presence of the indicated concentrations of DTT for 10 min. Non-imported material was removed by protease treatment. Imported Cox19 was analyzed by SDS-PAGE and autoradiography, followed by quantification with densitometry. Import efficiencies without DTT were set as 100 %. **(B)** Radiolabeled Tim10 was imported into mitochondria of wild type and Δ*cyc1*/Δ*cyc7* mutant cells in presence of the different concentrations of DTT and examined as in (A).

Next, the import of Tim10 was tested in the presence of antimycin A or potassium cyanide to specifically block respiratory chain complexes in the wild type backgrund. Inhibition of the cytochrome *c* oxidase by potassium cyanide rendered the import of Tim10 more sensitive towards DTT, while the block of the cytochrome *c* reductase with antimycin A had an opposite effect (Fig.22). Furthermore, the import sensitivity to DTT correlated with the DTT sensitivity of the Mia40 interaction with the newly imported Tim10 (Mia40•Tim10).

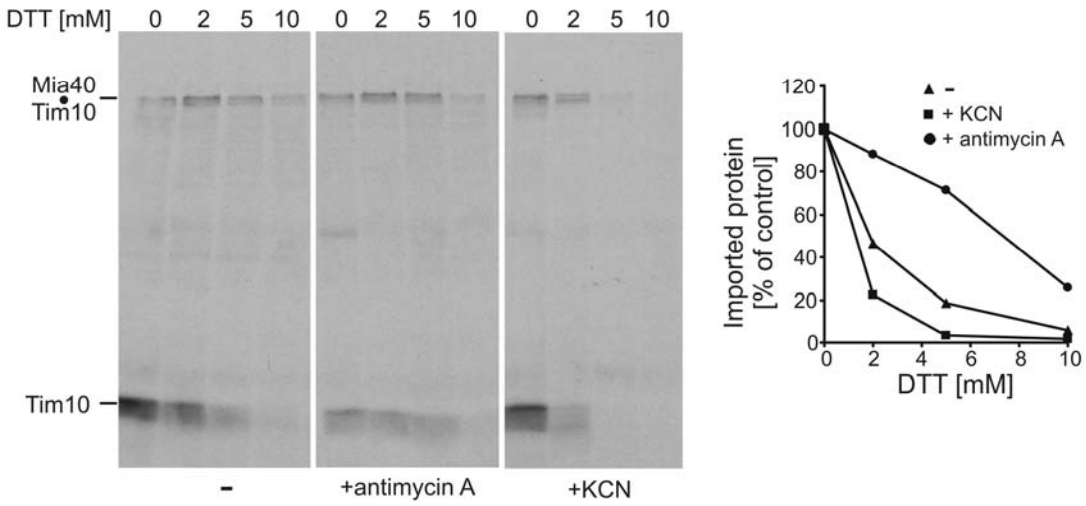

Figure 22. The interaction of Mia40 with Tim10 is influenced by the electron transport chain activity. Wild type mitochondria were preincubated for 3 min at 25°C in the absence or presence of antimycin A (100µg/ml) and potassium cyanide (10 mM). Then radiolabeled Tim10 was added and incubated with mitochondria for 10 min. Following treatment with 65 mM iodoacetamide, mitochondria were reisolated and proteins were loaded on a non-reducing SDS gel. Radiolabeled Tim10 associated with Mia40 (Mia40•Tim10) or Tim10 in its monomeric form were detected by autoradiography and quantified by densitometry.

In conclusion, the activity of the respiratory chain complexes influences the activity of the Mia40 and subsequently the efficiency of protein import into IMS of mitochondria. Increased levels of oxidized cytochrome *c* render the import more resistant to DTT, whereas a decreased oxidation of cytochrome *c* impairs the import process.

3.3. Erv1 – a novel substrate of Mia40-mediated pathway

The so far identified substrates of the Mia40-dependent import pathway, e.g. the small Tim proteins and the copper chaperone Cox17, have a low molecular mass in the range of 8–17 kDa. Their main feature is the presence of highly conserved cysteines which are organized in a twin CX_3C or a twin CX_9C motif. In yeast, Erv1 is a 22 kDa protein of the IMS and contains two CX_2C motifs: one in the flexible tail region and one in the highly conserved flavine adenine dinucleotide (FAD) binding domain. How Erv1 is imported into mitochondria had been not known untill recently and was a subject of the study done in cooperation with Barbara Grumbt (Terziyska, Grumbt *et al.* 2007).

3.3.1. Import of Erv1 into mitochondria depends on Mia40

To determine which import pathway the Erv1 follows, the Erv1 precursor was synthesized in reticulocyte lysate and imported into wild type mitochondria or mitochondria with altered levels of different import components. It was observed that the Erv1 precursor protein, in contrast to the matrix-targeted proteins, does not require ATP, $\Delta\psi$ or TIM23 complex for its import (Terziyska, Grumbt *et al.* 2007). However, the translocation of Erv1 has steps in common with the general import pathway of matrix-directed preproteins, most likely at the level of the TOM complex. To study if Mia40 plays a role in the import of Erv1,

the precursor of Erv1 was incubated with mitochondria from the GAL-MIA40 strain containing reduced or increased levels of Mia40 protein. Mitochondria depleted of Mia40 showed a significant defect in import of the radiolabeled Erv1 preprotein (Fig.23A). On the other hand, the import was strongly increased in mitochondria with enhanced levels of Mia40 (Fig.23B). These findings indicate that the import of the Erv1 precursor into IMS depends on the functional Mia40. Furthermore, this was supported by the results obtained *in vivo*. Similarly, the endogenous levels of Erv1 were strongly reduced in mitochondria from cells depleted of Mia40 as the ones of the other substrates of Mia40-dependent pathway, such as Tim13, Tim10 and Cox17, (Fig.23C). The amounts of proteins, not related to the Mia40 machinery, were not altered in those mitochondria.

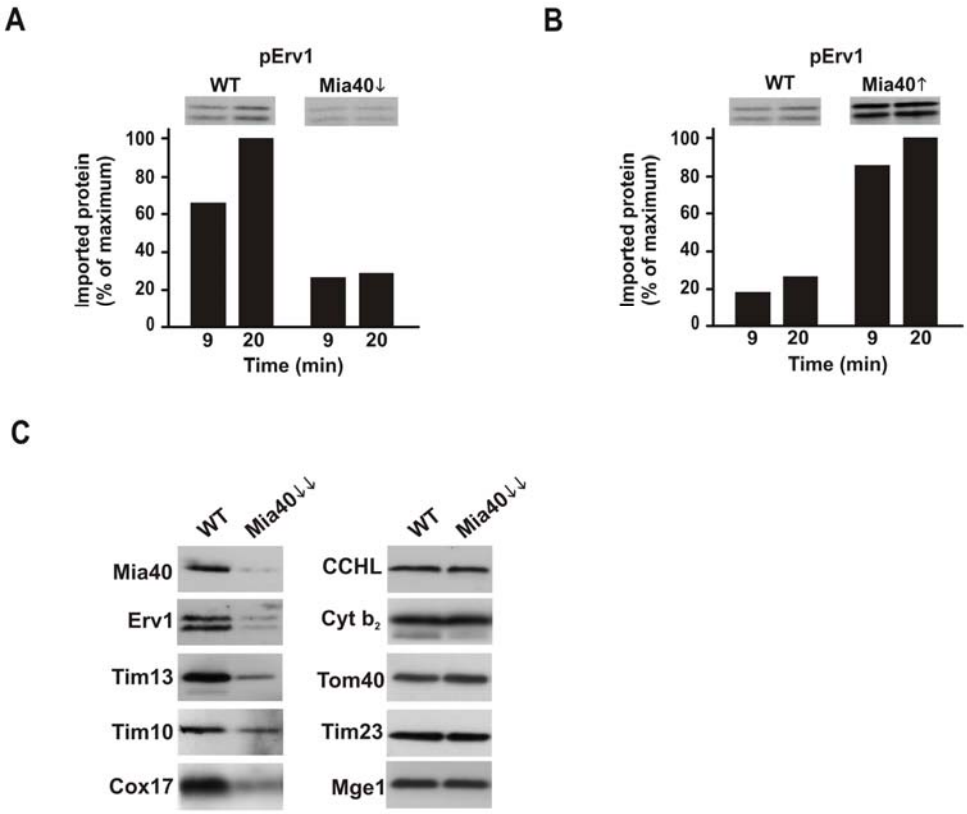

Figure 23. Mia40 is crucial for the import of Erv1. (A, B) The Erv1 precursor was imported for the indicated time into mitochondria isolated from cells with reduced amounts of Mia40 (Mia40↓), cells over-expressing Mia40 (Mia40↑) and from corresponding WT cells. Similar levels of endogenous Erv1 were found in all those cells. Imported Erv1 was analyzed by autoradiography and densitometry. The maximal value of the experiment was set to 100 %. **(C)** Endogenous levels of the proteins indicated were analyzed by SDS–PAGE and immunoblotting of mitochondria from wild type cells (WT) and cells depleted of Mia40 for 16 h (Mia40↓↓). Marker proteins for the matrix space (Mge1), the inner membrane (Tim23) and the outer membrane (Tom40) were tested as controls. Cytochrome *c* heme lyase (CCHL) and cytochrome b_2 (Cyt b_2) are IMS proteins that are not substrates of Mia40 pathway.

3.3.2. Imported Erv1 forms a mixed disulfide with Mia40

In addition, the import of Erv1 was sensitive towards reducing agents (Terziyska, Grumbt *et al.* 2007). Mildly reducing conditions stimulated the import efficiency of Erv1, while higher concentrations of DTT strongly inhibited it. This result suggests that a mixed disulfide of Erv1 with Mia40 is formed which is important for the efficient translocation of Erv1. To verify this, radiolabeled Erv1 was imported into wild type mitochondria, which were

subsequently lysed and subjected to immunoprecipitation with Mia40-specific antibodies. Following non-reducing SDS-PAGE and autoradiography, a complex of Mia40 with the imported Erv1 was detected (Fig.24). When β-mercaptoethanol was added to the sample loading buffer, monomeric Erv1 was released from the Mia40-Erv1 adduct (data not shown; Terziyska, Grumbt *et al.* 2007).

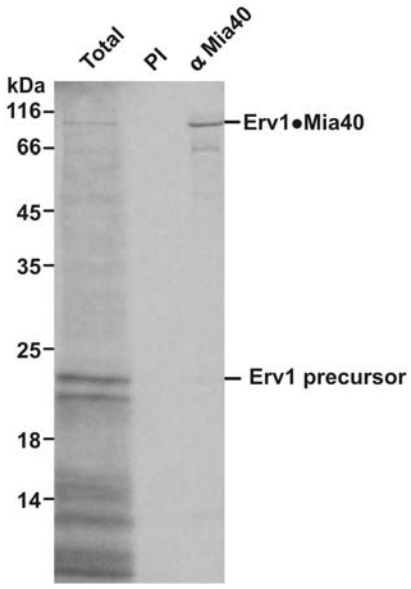

Figure 24. Mia40 interacts with the imported Erv1. Wild type mitochondria were incubated with Erv1 precursor for 10 min and then lysed with 1 % sodium dodecylsulfate. After addition of Triton X-100 containing buffer the probes were subjected to immunoprecipitation with Mia40-specific antibodies (αMia40) or with preimmune serum (PI). Finally the samples were analyzed by non-reducing SDS–PAGE and autoradiography. Total represents 5 % of the material used for the immunoprecipitations.

In summary, Erv1 is imported via the Mia40-dependent translocation pathway. Since Erv1 does not contain twin CX_3C or twin CX_9C motifs, it represents a novel type of substrate of the Mia40-mediated pathway. Moreover, Erv1 is the largest substrate of Mia40 identified so far.

3.4. Functional characterization of the conserved cysteine residues in Mia40

In yeast, the highly conserved C-terminal domain of Mia40 is sufficient for the function if it is targeted to mitochondria (data not shown). This domain of Mia40 contains six cysteine residues which form a $CXC\text{-}CX_9C\text{-}CX_9C$ motif and are found in all Mia40 homologues. It was shown before that pairs of conserved cysteine residues in Mia40 are crucial for its function but the exact role of single cysteine residues is not known (Naoe, Ohwa *et al.* 2004).

3.4.1. Analysis of the single cysteine mutants of Mia40

3.4.1.1. Single cysteine residues in Mia40 are essential for viability of yeast cells

To study the role of the individual cysteine residues in Mia40, single cysteine to serine (C→S) mutants of Mia40 were generated. First, their ability to functionally replace wild-type Mia40 *in vivo* was tested. As shown in Fig.25A the number in the name of the Mia40 mutants indicates the position of the particular cysteine residue in the conserved motif of Mia40. Plasmids carrying these mutant *MIA40* genes were introduced into a chromosomal *MIA40* disruption strain (Δ*mia40* strain) harbouring a URA plasmid with the wild type *MIA40* gene and tested for complementation by chasing out the URA plasmid. The empty vector and the plasmid containing the WT *MIA40* gene were used as controls. Replacement of the second

(C2S) and the third cysteine residues (C3S) in Mia40 with serine residues was lethal (Fig.25B). Exchange of the first cysteine residue (C1S) to a serine residue resulted in a severe growth defect compared to the wild type. Expression of the Mia40 variants containing serine replacements in the last three cysteine residues (C4S, C5S, C6S) complemented fully the deletion of the wild type *MIA40*. Since C2S and C3S mutants of Mia40 did not restore viability in the Δ*mia40* context, in order to examine biochemically all single cysteine mutants at the same time, the yeast GAL-MIA40 strain was used for further analysis. The growth phenotypes of the Mia40 variant strains after depletion of endogenous Mia40 were similar to the ones observed in the Δ*mia40* strains harbouring the Mia40 mutants (Fig.25C). The Mia40C6S variant showed a slightly slower growth compared to the wild-type in the drop dilution test, which allows a quantitative analysis of the growth rates. Taken together, the results of the growth analysis of the Mia40 single cysteine mutants suggest that the first three cysteine residues in Mia40 are important for its function in the cell.

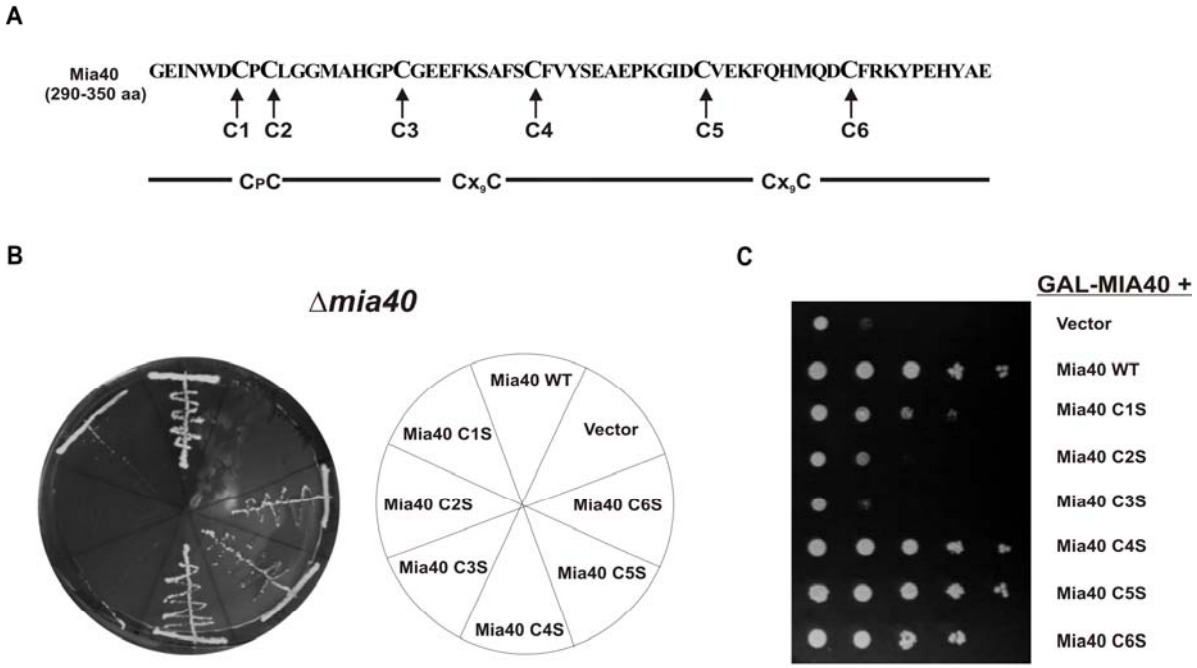

Figure 25. Analysis of the growth phenotypes of strains expressing the single cysteine mutants of Mia40. (A) Scheme presenting labeling of the single cysteine Mia40 mutants, where the number indicates the position of the cysteine in the conserved CXC-CX_9C-CX_9C motif of yeast Mia40 exchanged for serine. **(B)** Plasmids containing these mutants were introduced into Δ*mia40* strain harboring on URA plasmid wild-type *MIA40* gene and then tested for complementation by growth on 5-fluoroorotic acid containing medium. Empty vector and plasmid carrying WT *MIA40* gene were used as controls. **(C)** GAL-MIA40 strain carrying the *MIA40* gene under control of the *GAL10* promoter was transformed with the vectors containing the mutants of Mia40 described in (A) and then a drop dilution test was made for the resulting strains. To downregulate the endogenous levels of Mia40 protein, cells were grown on medium containing 2 % glucose for three days at 30°C.

3.4.1.2. Specific cysteine residues of Mia40 are crucial for the import of small proteins of the mitochondrial IMS

To examine the effect of every cysteine mutation on the function of Mia40 in detail, mitochondria were isolated from the GAL-MIA40 strains in which the different Mia40 single cysteine mutants with a C-terminal His_6-tag were expressed and endogenous Mia40 was strongly downregulated. As presented in Fig.26A, most of the Mia40 mutant proteins were expressed to similar extent, only the Mia40C1S and the Mia40C3S showed slightly reduced levels. The substrates of Mia40-dependent pathway, such as Tim13, Cox17, Erv1, were

significantly reduced in their endogenous levels in mitochondria of the Mia40C1S, the Mia40C2S and the Mia40C3S mutants and notably, also in the Mia40C6S mutant (Fig.26B). Mitochondria of the Mia40C4S and the Mia40C5S mutants contained expression levels of the Mia40 substrates similar to the ones in Mia40WT mitochondria. Proteins of the outer (Tom40) and inner membrane (Tim23, AAC) or the matrix (AcoI) were not affected in any of the Mia40 mutants indicating that the reduction in some mutants is a specific effect and not due to a general mitochondrial deficiency. Thus, the observed defects in the biogenesis of small IMS proteins in specific Mia40 mutants are consistent with their growth phenotypes.

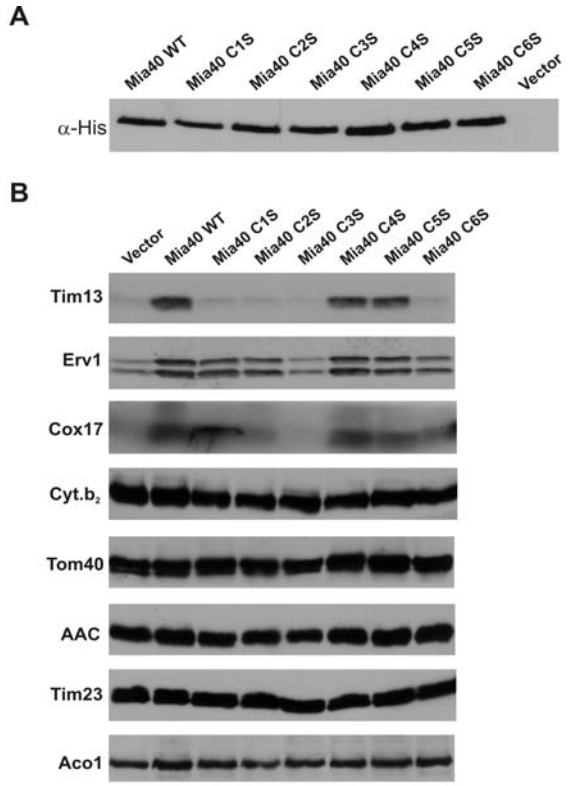

Figure 26. Endogenous levels of some mitochondrial proteins in mitochondria containing the single cysteine mutants of Mia40. **(A)** Mitochondria isolated from the GAL-MIA40 strain transformed with the empty plasmid or plasmids carrying single Cys to Ser mutations and depleted of endogenous Mia40 for 27 h were analyzed for the expression of the mutant Mia40 proteins by SDS-PAGE and immunoblotting with αHis antibodies. **(B)** Levels of the indicated proteins were analyzed by immunoblotting of mitochondria described in (A).

Next, the *in vitro* import of radiolabeled precursor proteins into mitochondria isolated from these Mia40 mutant strains was analysed. The import rates of Tim13 and Erv1 into mitochondria of the mutants, in which the first three cysteine residues or the sixth cysteine residue were replaced by serine residues, were greatly reduced (Fig. 27). Import of cytochrome c heme lyase (CCHL), an IMS protein that is not dependent on Mia40 pathway, was not impaired in any of the Mia40 mutants. Hence, the import results are consistent with the steady-state levels detected in the Mia40 cysteine mutants. In summary, the single cysteine residues, with the exception of the fourth and the fifth one, are crucial for Mia40 to function in the import of small proteins into the mitochondrial IMS.

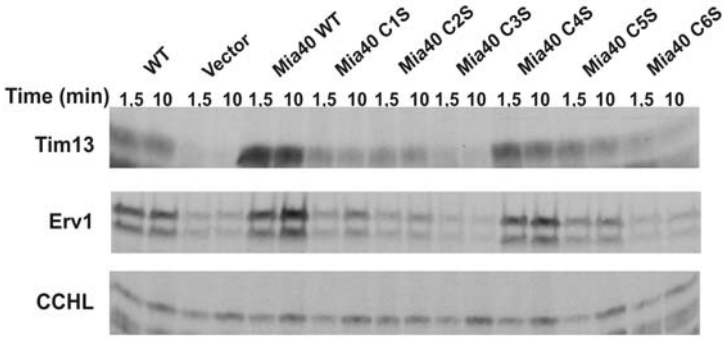

Figure 27. Import of precursor proteins into mitochondria harbouring the single cysteine mutants of Mia40. The indicated radiolabeled preproteins were imported into isolated mitochondria from the WT strain and into mitochondria as described in Fig.22. The samples were analyzed by SDS-PAGE and autoradiography.

3.4.1.3. Interaction with Tim13 is affected in specific single cysteine mutants of Mia40

Import of the precursor proteins into the IMS requires formation of the mixed disulfide with Mia40 protein. To determine if the import defects observed in the Mia40 mutants are due to the lack of the disulfide intermediates with Mia40, radiolabeled Tim13 was imported into mitochondria isolated from the Mia40 variant strains, then mitochondria were lysed and the His_6-tagged Mia40 mutant proteins were isolated via Ni-NTA agarose binding. Samples were analysed by non-reducing SDS-PAGE and autoradiography. The creation of the Tim13-Mia40 complex was not particularly affected in the Mia40C4S and the Mia40 C5S mutants compared to the wild type (Fig.28). Mitochondria of the Mia40C1S and the Mia40C2S mutants showed reduced formation of the Tim13-Mia40 intermediate, but the strongest reduction was found in the Mia40C6S mutant. Notably, the interaction of Tim13 with Mia40 could not be detected at all in the Mia40C3S mutant. In conclusion, the reduced accumulation of the newly imported substrates in the Mia40 mutant mitochondria correlates with the decreased formation of the mixed disulfide of the substrate protein with Mia40.

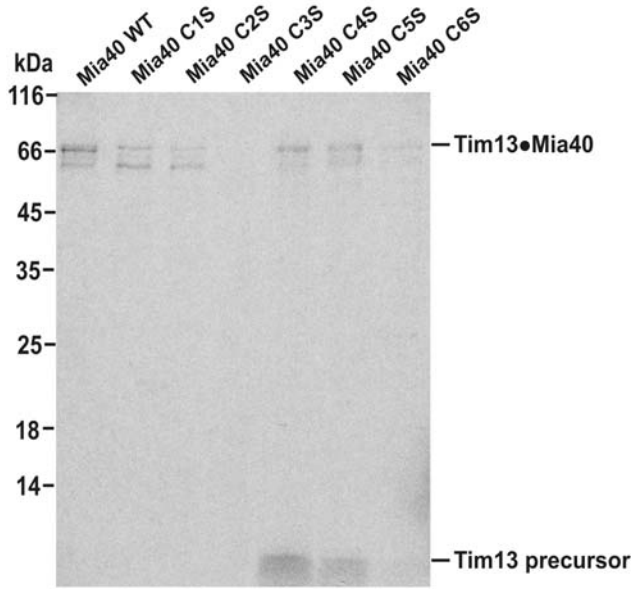

Figure 28. Analysis of the interaction of Mia40 with Tim13 in mitochondria containing the single cysteine mutants of Mia40. Mitochondria from cells expressing Mia40 WT and the single cysteine mutants with a C-terminal His_6 tag were incubated with radiolabeled precursor of Tim13 for 20 min at 12^0C and lysed afterwards with 1% sodium dodecylsulfate. To isolate the His_6-tagged proteins NiNTA affinity chromatography was performed and the eluates were analyzed by non-reducing SDS-PAGE and autoradiography.

3.4.1.4. Interaction with Erv1 is impaired in specific single cysteine mutants of Mia40

The oxidized cysteine residues in Mia40 are important for the interaction with the substrate and need to be reoxidized after the release of the substrate from Mia40. Erv1 maintains Mia40 in an import-competent oxidized state by introducing disulfide bonds in Mia40. The formation of Mia40-Erv1 complex was tested in the single cysteine mutants of Mia40. As shown in Fig.26, mitochondria isolated from cells expressing the Mia40WT and the different Mia40 variants contain diverse endogenous levels of Erv1. Therefore, different amounts of these mitochondria were used in the experiment to have comparable amounts of the total Erv1 protein. Mitochondria were first incubated for 20 min with iodoacetamide to accumulate the Mia40-Erv1 complex, then lysed and the mitochondrial extracts were loaded on a non-reducing SDS gel and finally analysed by immunoblotting with Erv1-specific antibodies. The formation of the Mia40-Erv1 complex was completely abolished in the Mia40C2S and the Mia40C3S mutants and strongly reduced in Mia40C6S mutant (Fig.29). Intriguingly, the Mia40C1S mutant did not show a difference compared to the Mia40WT,

despite its severe effects on the import of the substrate proteins. The Mia40C4S and the Mia40C5S mutants displayed no decrease in the creation of the Mia40-Erv1 complex. The results suggest that neither the cysteine residues four and the five in Mia40 nor the first cysteine residue are crucial for the interaction with Erv1.

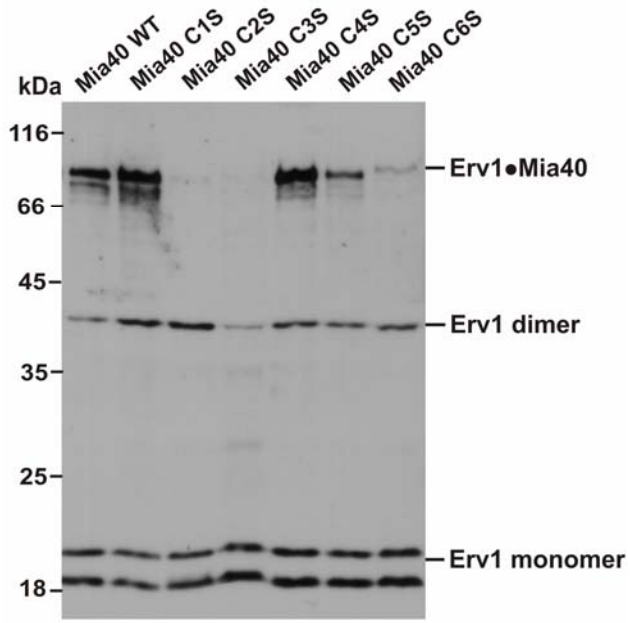

Figure 29. Analysis of the Mia40 interaction with Erv1 in mitochondria harbouring the single cysteine mutants of Mia40. Different amounts of mitochondria isolated from cells expressing Mia40WT and Mia40 mutant proteins were used (25 µg for Mia40WT, 50 µg for Mia40C1S, Mia40C2S, Mia40C4S and Mia40C5S, 100 µg for Mia40C6S and 250 µg for Mia40C3S). Mitochondria were incubated for 20 min at 25^0C with 80 mM iodoacetamide. After lysis of mitochondria, the proteins were resolved under non-reducing conditions by SDS-PAGE, transferred to nitrocellulose membrane and decorated with Erv1-specific antibodies.

3.4.2. Analysis of the double cysteine mutants of Mia40

The results obtained with the single cysteine variants of Mia40 suggest that the first, second, third and the sixth cysteine residues in Mia40 play important roles for the function of Mia40 in the disulfide-relay system in the IMS. To get more insight in their function, the results obtained with the single cysteine mutants of Mia40 were extended by studies using selected double cysteine to serine mutants of Mia40.

3.4.2.1. Double cysteine mutants of Mia40, apart from for the Mia40C4/5S, show defects in cell growth and the biogenesis of small IMS proteins

Firstly, the complementation of the *MIA40* deletion with the Mia40 double cysteine mutants, indicated in Fig.30, was tested. The Mia40C4/5S variant complemented fully the deletion of the wild type *MIA40*, which is in line with the results acquired from the Mia40C4S and the Mia40C5S mutants (Fig.30A). This indicates that the fourth and the fifth cysteine residues in Mia40 are not crucial for the Mia40 function and thus for the viability of yeast cells. In agreement with the results obtained for the Mia40C2S mutant, the Mia40C1/2S mutant failed to restore cell viability. The Mia40C5/6S mutant also did not complement the deletion of the wild type *MIA40*, pointing out that the sixth cysteine residue in Mia40 is essential when the fifth cysteine residue is absent. Surprisingly, the mutation of the third cysteine residue alone in Mia40 results in lethality, but an additional mutation of the sixth cysteine residue allows growth of yeast cells though with strongly reduced rates (Mia40C3/6S mutant). This finding reveals that the third cysteine residue is not absolutely essential for the function of Mia40. Similar effects on the cell growth were observed with the GAL-MIA40

strains expressing the double cysteine variants of Mia40 after depletion of endogenous Mia40 (Fig.30B). In summary, the results of the Mia40 double mutants confirm that the first three cysteine residues are important for the function of Mia40, though only the second cysteine residue appears to be strictly essential.

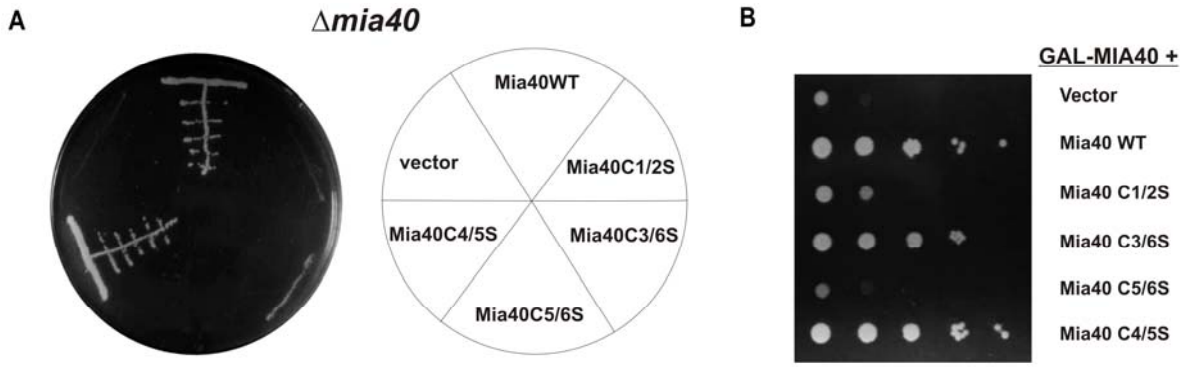

Figure 30. Analysis of the growth phenotypes of strains expressing the double cysteine mutants of Mia40. **(A)** Plasmids containing selected double cysteine mutants of Mia40 were introduced into Δ*mia40* strain harboring on an URA plasmid the wild-type *MIA40* gene and analyzed for complementation by growth on 5-fluoroorotic acid containing medium. Empty vector and plasmid carrying WT *MIA40* gene were used as controls. **(B)** The GAL-MIA40 strain carrying the *MIA40* gene under control of the *GAL10* promoter was transformed with plasmids encoding the double cysteine mutants of Mia40.The growth characteristics of the transformants were checked in a drop dilution test. The endogenous levels of Mia40 protein were downregulated by cell growth on medium containing 2 % glucose for three days at 30°C.

Next, to study the effects of the double cysteine mutations on the molecular function of Mia40, mitochondria were isolated from the GAL-MIA40 strains depleted of endogenous Mia40 and expressing the different double cysteine variants with a C-terminal His_6-tag. The expression levels of most of the Mia40 mutant proteins were comparable to the wild-type Mia40 levels, only the Mia40C5/6S mutant show slightly reduced levels, possibly due to higher protein instability (Fig.31A). Both the endogenous levels and the *in vitro* import rates of Tim13, the substrate of Mia40-dependent pathway, were considerably reduced in mitochondria of the Mia40C1/2S, the Mia40C3/6S and the Mia40C5/6S mutants (Fig.31B, C). In contrast, Mia40C4/5S variant showed no major effects in the biogenesis of the small cysteine-rich IMS proteins. The expression levels of the proteins of the outer membrane (Tom40), inner membrane (Tim23) or the matrix (AcoI) were not affected in any of the Mia40 double cysteine mutants. Similarly, the import of the IMS protein cytochrome *c* heme lyase (CCHL), which is imported independently of Mia40, was not impaired in these mutants. To summarize, the specific effects on the biogenesis of the small IMS proteins in the double cysteine mutants of Mia40 are in agreement with their growth phenotypes.

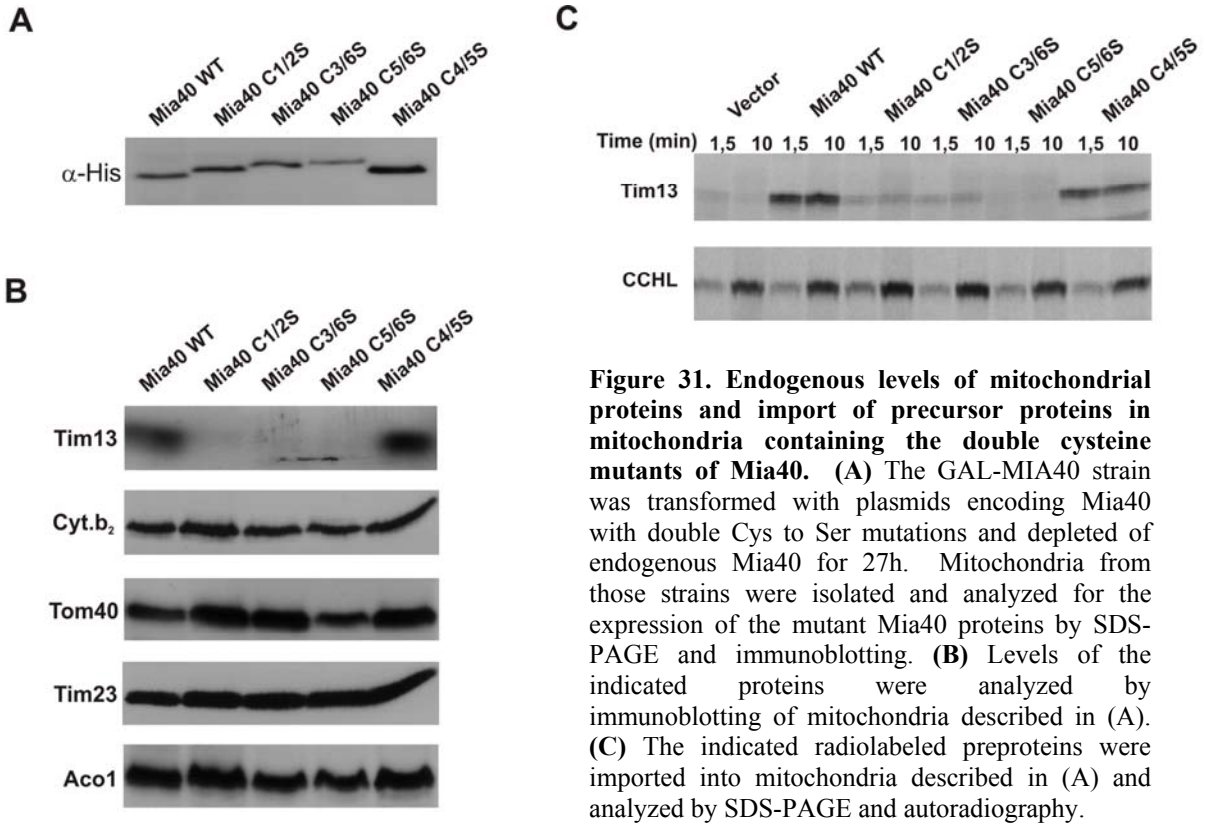

Figure 31. Endogenous levels of mitochondrial proteins and import of precursor proteins in mitochondria containing the double cysteine mutants of Mia40. **(A)** The GAL-MIA40 strain was transformed with plasmids encoding Mia40 with double Cys to Ser mutations and depleted of endogenous Mia40 for 27h. Mitochondria from those strains were isolated and analyzed for the expression of the mutant Mia40 proteins by SDS-PAGE and immunoblotting. **(B)** Levels of the indicated proteins were analyzed by immunoblotting of mitochondria described in (A). **(C)** The indicated radiolabeled preproteins were imported into mitochondria described in (A) and analyzed by SDS-PAGE and autoradiography.

3.4.2.2. Interactions of Mia40 with Tim13 and with Erv1 are affected in most of the double cysteine mutants of Mia40

Finally, the interactions of Mia40 double cysteine mutants with the substrate Tim13 and with Erv1 were analyzed. Consistent with the import results, the formation of the Tim13-Mia40 complex was not detected in the Mia40C1/2S and Mia40C5/6S variants (Fig.32A). In the Mia40C3/6S mutant this complex was formed, although in comparison to the wild type Mia40 a significant reduction was observed. The Mia40C4/5S variant showed no alteration in Tim13-Mia40 complex formation. The results obtained for the interaction of Mia40 with its oxidase Erv1 were comparable to the ones concerning the formation of the Tim13-Mia40 complex. The Erv1-Mia40 complex was absent in the Mia40C1/2S and the Mia40C5/6S mutants and present in the Mia40C4/5S variant (Fig.32B). In the Mia40C3/6S mutant the interaction with Erv1 could be detected but was strongly reduced. Taken together, the findings suggest that the observed defects in the cell growth and in the biogenesis of the small IMS proteins in the double cysteine mutants of Mia40 are due to the failure in the formation of the crucial interactions of Mia40 with its substrates and with Erv1.

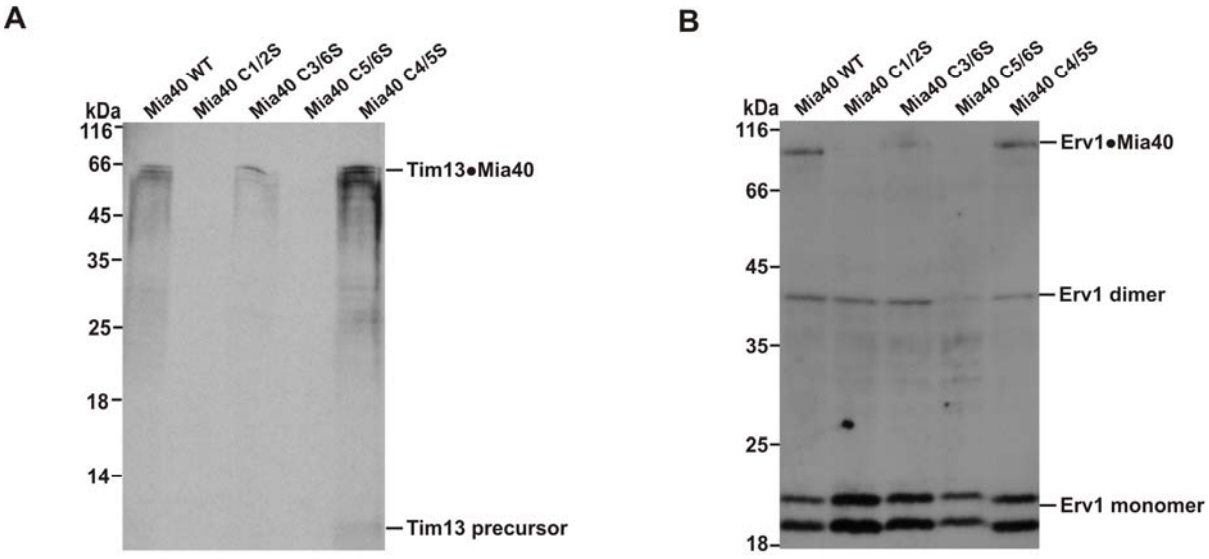

Figure 32. Analysis of the interaction of Mia40 with Tim13 and Erv1 in mitochondria harbouring the double cysteine mutants of Mia40. (A) Radiolabeled precursor of Tim13 was imported for 20 min at 12^0C into mitochondria isolated from cells expressing Mia40WT and the double cysteine mutants. After lysis with 1% sodium dodecylsulfate, the His_6-tagged proteins were isolated via NiNTA affinity chromatography and analyzed by non-reducing SDS-PAGE and autoradiography. **(B)** Different amounts of mitochondria (25 µg for Mia40WT and Mia40C4/5S, 100 µg for Mia40C1/2S, 200 µg for Mia40C3/6S and Mia40C5/6S) were incubated for 20 min at 25^0C with 80 mM iodoacetamide. Then mitochondria were solubilized and the probes were loaded on a non-reducing SDS-PAGE gel. After transfer to a nitrocellulose membrane, the Mia40-Erv1 complex was detected with Erv1-specific antibodies.

In conclusion, the double mutation of the fourth and the fifth cysteine residues in Mia40 led to no significant defects in the yeast cell viability and function, indicating that these residues do not crucially contribute to the Mia40 activity or stability. The replacement of the third and the sixth cysteine residues with serine residues at the same time resulted in strong impairment of the cell growth and the mitochondrial function. The simultaneous mutation of the fifth and the sixth cysteine residues in Mia40 showed even more severe defects. The results of these double cysteine mutants (Mia40C3/6S, Mia40C5/6S) as well as the findings of the Mia40C3S and the Mia40C6S mutants suggest that the third and the sixth cysteine residues play very important but not essential roles in the function or stability of Mia40 in the mitochondrial IMS. The failure to restore cell viability in the Δ*mia40* context and the biochemical data obtained from the Mia40C2S and the Mia40C1/2S mutants indicate that the second cysteine residue in the Mia40 motif is essential for the Mia40 function. The first cysteine residue also contributes greatly to the Mia40 activity, since the Mia40C1S mutant showed great defects in the cell growth and in the biogenesis of small IMS proteins. The observed viability of this Mia40 mutant strain might be explained by the remaining ability of the Mia40C1S mutant protein to interact with Erv1, in contrast to the Mia40C2S protein. In summary, the first three and the sixth cysteine residues in Mia40 motif play a significant role for the function of Mia40.

4.3. Characterization of the redox states of Mia40 and the Mia40 cysteine mutants

Mia40 can be separated in its oxidized and reduced form by different mobility on non-reducing SDS gels (see Fig.13). The observed oxidized form of Mia40 is important for its function in the transport of the cysteine-containing IMS proteins. Yet, the nature of the Mia40 oxidized state is unclear. To define how many disulfide bonds are present in Mia40 oxidized form, analysis of the wild type Mia40 and the Mia40 cysteine mutants was performed with the

use of reducing and thiol-modifying agents at different temperatures. When wild type mitochondria were incubated with DTT at room temperature and the free thiols were modified with iodoacetamide to prevent reoxidation, Mia40 migrated more slowly on the gel than the non-treated sample. In some cases, reducing agents under native conditions are not able to reduce proteins completely. Thus, incubation with DTT at 95^0C was carried out to denature the Mia40 protein, which resulted in even lower mobility of Mia40 on the gel (Fig.33). These findings suggest that Mia40 can be detected on non-reducing gels in three forms: (1) an oxidized form which migrates fast; (2) a partially reduced form that has a lower mobility and still contains disulfide bonds; (3) a fully reduced form that migrates the most slowly.

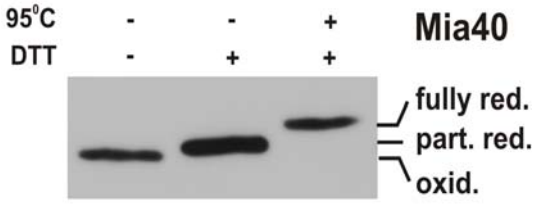

Figure 33. Three different redox states of wild type Mia40. Wild type mitochondria were treated with 10 mM DTT either at room temperature or at 95^0C for 20 min. Non-treated sample was used as control. Next, the free thiol groups were modified with 80 mM iodoacetamide, proteins were resolved on a non-reducing SDS gel and Mia40 was analyzed by immunoblotting.

To characterize which cysteine residues form the various disulfide bonds in the oxidized and the partially reduced forms of Mia40, analysis of the redox states of the different Mia40 cysteine mutants was performed (Fig.34A,B). First, mitochondria from cells expressing the Mia40 cysteine mutants were incubated in the absence or presence of 10 mM DTT for 10 min at room temperature followed by modification with iodoacetamide. The samples were loaded on a non-reducing gel and the proteins were detected with anti-His antibody. All Mia40 variants showed lower mobility compared to the Mia40WT, already without DTT treatment. Interestingly, all mutations, single or double, in the last four cysteine residues of Mia40 resulted in even more slow migration of the proteins and this additionally could be decelerated by DTT treatment. The Mia40C1S, the Mia40C2S and the Mia40C1/2S variants migrated at the size of the partially reduced form of wild type Mia40 and showed no sensitivity to the reducing agent. These results suggest that the first two cysteine residues in Mia40 are involved in a disulfide bond, which is opened by DTT to obtain the partially reduced form of Mia40. The other four cysteines in Mia40, present in the twin CX_9C motif, form disulfide bonds which are not easily accessible to the reducing agent.

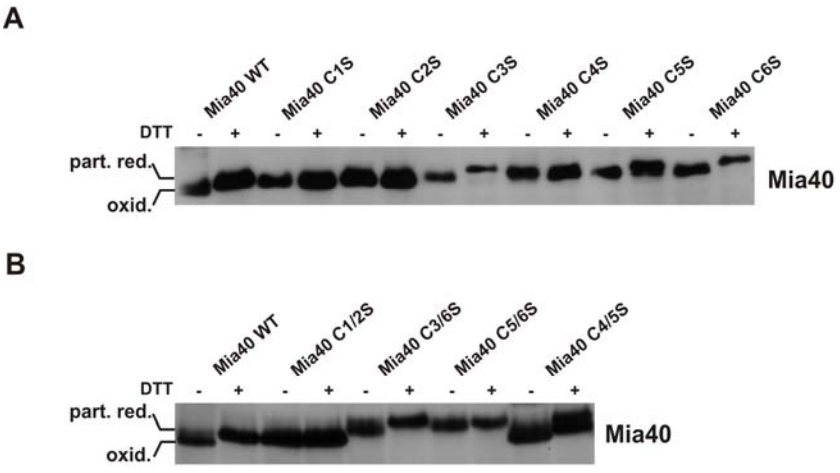

Figure 34. Analysis of the redox states of the Mia40 cysteine mutants. Mitochondria isolated from cells expressing the single **(A)** or the double **(B)** cysteine mutants of Mia40 were incubated in the absence or presence of 10 mM DTT at room temperature for 20 min. After modification with 80 mM iodoacetamide proteins were analyzed by non-reducing SDS-PAGE and immunoblotting with α-His antibody.

Results

Next, modification of the wild type Mia40 and the Mia40 single cysteine mutants with the $mPEG_{5000}$-maleimide (PEG-mal) was performed. PEG-mal is a thiol-specific alkylation reagent that attaches one PEG_{5000} moiety to each free cysteine residue of the target protein. Thereby, the modified protein migrates more slowly than the unmodified protein on SDS-PAGE. Mitoplasts generated from mitochondria of cells expressing the Mia40WT were incubated in the absence or presence of the reducing agent TCEP (tris carboxyethyl phosphine hydrochloride) on ice or at 95^0C, then modified with PEG-mal for 90 min and separated on a non-reducing gel. The modified Mia40 proteins were detected by antibodies against the hexahistidine tag. When Mia40WT was reduced and modified under native conditions, only two shifts in mobility were observed (Fig.35A). In contrast, upon denaturing Mia40WT protein could be modified by several PEG-mal molecules, which suggests accessibility to almost all cysteine residues. Similar experiment was carried out with the Mia40 single cysteine mutants. Under native conditions the Mia40C1S and the Mia40C2S mutants showed only one mobility shift. For the other four Mia40 variants modifications of three sulfhydryls were detected (Fig.35B). Thus, these findings confirm that the first and the second cysteine residues in Mia40 form an accessible disulfide bond, whereas the four remaining cysteine residues are involved in disulfide bonds that cannot be reduced upon native conditions.

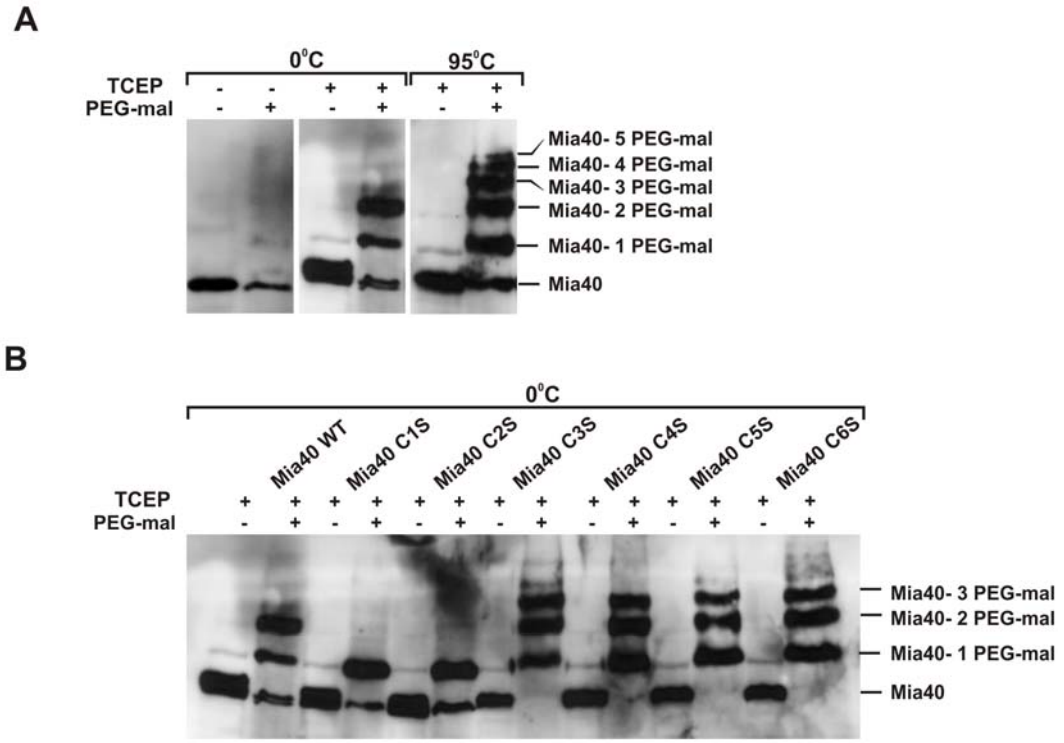

Figure 35. PEG-mal modification of wild type Mia40 and the Mia40 single cysteine mutants. Mitoplasts were generated from mitochondria isolated from cells expressing the Mia40WT (A) or the single cysteine mutants of Mia40 (B). Mitoplasts were then incubated in the absence or presence of 5 mM TCEP for 10 min on ice or at 95^0C. Next, PEG-mal at 4 mM final concentration was added and incubated with the samples for 90 min on ice. Finally, the probes were loaded on non-reducing SDS gel and immunodetection with α-His antibody was performed.

In summary, the first two cysteine residues in the Mia40 conserved motif form a disulfide bond that is accessible and sensitive to reducing agents. The disulfide bonds made by the other four cysteine residues in Mia40 are stable even under mild reducing conditions.

4. DISCUSSION

All intermembrane space (IMS) proteins are synthesized in the cytosol and need to be imported into mitochondria. The majority of IMS proteins lack N-terminal targeting signals. These proteins do not engage the inner-membrane TIM23 complex and depend neither on a membrane potential nor on ATP for their translocation into the IMS. In this study, the identification and characterization of novel machinery in the IMS of mitochondria that mediates import of small proteins with conserved cysteine motifs into the IMS has been presented.

4.1. Identification and characterization of Mia40

Mia40 is the first essential component of a specific translocation pathway of small IMS proteins. In this work, the mitochondrial location of Mia40 was confirmed and the submitochondrial location was assessed. Yeast Mia40 contains at the N terminus a classical mitochondrial targeting sequence followed by a hydrophobic segment, which suggests that the mature Mia40 is sorted to the inner membrane by the TIM23 translocase. Indeed, yeast Mia40 is imported into mitochondria via the TIM23 complex in a membrane potential-dependent manner (data not shown; (Chacinska, Pfannschmidt et al. 2004; Naoe, Ohwa et al. 2004). About the Mia40 topology, two different views have been reported. One proposed the anchoring of Mia40 to the inner membrane with the hydrophobic domain (Naoe, Ohwa *et al.* 2004). The second suggested that Mia40 is processed proteolytically twice and thereby released as a soluble form to the IMS (Chacinska, Pfannschmidt *et al.* 2004). As presented here, Mia40 fractionated mainly with the membrane pellet upon alkaline extraction of mitochondria. Additionally, determination of the N terminus of mature Mia40 purified from yeast mitochondria by Edman degradation showed a processing of the precursor N-terminally from the hydrophobic domain (Terziyska, Lutz et al. 2005). Thus, the topology of the mature Mia40 protein could be clarified. Mia40 from fungi is N-terminally anchored in the inner membrane and exposes a large C-terminal domain to the IMS. Notably, in higher eukaryotes Mia40 is significantly shorter and completely lacks the extended N-terminal segment of the fungal proteins. It was shown that human Mia40 protein is soluble within the mitochondrial IMS and that only the twin CX_9C motif is required for efficient import and accumulation of newly synthesized Mia40 in mitochondria (Hofmann, Rothbauer *et al.* 2005). Moreover, it was reported that the N-terminal anchor of the yeast Mia40 is not crucial for the function of the protein *in vivo*, when Mia40 is targeted differently to mitochondria (Naoe, Ohwa *et al.* 2004). Hence, during evolution the functional relevance of membrane anchoring of Mia40 has been apparently lost.

The C-terminal cysteine-rich domain is the highly conserved part of Mia40. There is 75% similarity between yeast and human homologues suggesting importance of this fragment for the protein function. Indeed, this domain is necessary and sufficient in yeast for the function of Mia40 as an import component in the IMS (data not shown). The C-terminal region of Mia40 harbours six highly conserved cysteine residues; Mia40 exists in different redox states, as shown here. On non-reducing SDS gels, three states of Mia40 can be detected: a fast migrating oxidized form, a partially reduced form migrating more slowly, and a fully reduced form of lowest mobility. In isolated yeast mitochondria, the majority of endogenous Mia40 was found in an oxidized state, containing intramolecular disulfide bonds. Similar results were obtained for the human Mia40 (Hofmann, Rothbauer *et al.* 2005). The mutational analysis of yeast Mia40 presented here and the mass spectrometric data for the recombinant

C-terminal domain of Mia40 indicate that all cysteine residues are present in intramolecular disulfide bonds in the oxidized form of Mia40 (Grumbt, Stroobant *et al.* 2007). The disulfide bond between the first two cysteine residues in Mia40 motif is redox-sensitive and becomes easily reduced, turning Mia40 from the oxidized to the partially reduced form. As described here, the reduced recombinant Mia40 can bind zinc and copper ions and it seems that the reduced state of Mia40 *in vivo* is stabilized by metal binding. All the redox states of Mia40, oxidized and partially or fully reduced, may represent physiological forms of Mia40 as reported for other proteins, such as metallothionein and the heat shock protein Hsp33 (Maret and Vallee 1998; Jakob, Eser *et al.* 2000).

In this study, Mia40 has been identified and characterized as the key player of a novel pathway for the import of small IMS proteins with conserved cysteine motifs. During the performance of this study, two other publications also identified Mia40 (named Tim40 in one of the reports) as a factor required for the biogenesis of small IMS proteins (Chacinska, Pfannschmidt *et al.* 2004; Naoe, Ohwa *et al.* 2004). Cells depleted of Mia40 showed strongly reduced endogenous levels of small IMS proteins with twin CX_3C or twin CX_9C motifs, e.g. Tim13 and Cox17. This was the result of impaired import of these proteins into mitochondria. Other proteins of the IMS, such as the cytochrome c heme lyase (CCHL) or cytochrome c, which coordinate heme as a cofactor, displayed no changes in their endogenous levels or import rates. The observed effect of Mia40 depletion was specific, as the mitochondrial import pathways that direct proteins to the matrix, inner membrane or outer membrane were not affected. Moreover, Mia40 could be detected bound to the newly imported Tim13 protein via disulfide bridges. The interaction between Mia40 and the substrate protein represents an import intermediate, since only the newly imported Tim13, but not the endogenous one, was co-immunoprecipitated with Mia40 (data not shown). Results obtained from the Blue native gels confirmed this (Chacinska, Pfannschmidt *et al.* 2004). The formation of the Mia40 intermediate with the precursor protein is crucial for the complete translocation into the mitochondrial IMS, as high concentrations of DTT inhibited the import of Tim13 or Cox17 almost completely (Mesecke, Terziyska *et al.* 2005). As observed here, Mia40 becomes partially reduced after import of chemical amounts of Tim13, indicating that the mixed disulfide bond is transferred to the substrate, which then undergo further folding and assembly. To recover its active state, Mia40 needs to become reoxidized in the IMS.

Taken together, Mia40 is the first protein of a novel translocation pathway mediating the mitochondrial IMS import of small cysteine-rich proteins. Mia40 interacts with the unfolded precursor proteins and promotes the completion of translocation into the IMS. The identification of Mia40 as an import factor for the small IMS proteins starts a new chapter in the studies of protein translocation into mitochondria.

2. Mia40 – a component of a disulfide relay system in the IMS of mitochondria

The two essential proteins, Mia40 and Erv1, cooperate in the mitochondrial IMS, promoting the import and assembly of small cysteine-rich proteins. In cells depleted of Erv1, the levels of mitochondrial IMS proteins, requiring Mia40 for import, were strongly reduced and Mia40 accumulated in a reduced conformation (Mesecke, Terziyska *et al.* 2005). In this work, a direct interaction between Erv1 and Mia40 was shown. The association of these proteins is sensitive to reducing agents, indicating that it occurs probably through disulfide bridges. So far, two functions of Erv1 have been reported: a role in the formation of cytosolic Fe/S proteins (Lange, Lisowsky *et al.* 2001) and a function as a sulfhydryl oxidase for generation of disulfide bonds by using oxygen as an electron acceptor (Lee, Hofhaus *et al.*

2000; Hofhaus, Lee *et al.* 2003). However, no physiological substrates or partner proteins of Erv1 has been known until now. Mia40 is the first physiological substrate of the sulfhydryl oxidase Erv1, which seems reasonable as Mia40 has to be reoxidized after donating a disulfide bond to its substrate.

Sulfhydryl oxidases, such as Erv1, were reported to catalyse formation of disulfide bonds using molecular oxygen and releasing hydrogen peroxide. Therefore, it was believed that molecular oxygen is the final acceptor of the electrons coming from the oxidized substrate protein during the Mia40-Erv1 action. However, a recent report on the redox chemistry of ALR, the human homologue of Erv1, showed that cytochrome *c* is a 100-fold better electron acceptor than oxygen (Farrell and Thorpe 2005). This result stimulated the idea that the yeast Erv1 may also be oxidized by cytochrome *c* and the first hint, pointing that this idea can be true, came from Tokatlidis and coworkers. They reported that yeast cells deleted in cytochrome *c* isoform Cyc1 cannot grow under anaerobic conditions (Allen, Balabanidou *et al.* 2005). As presented in this work, the import of small proteins into the IMS via the Mia40-mediated pathway depends on the energetic state of the respiratory chain. Protein import into mitochondria lacking cytochrome *c* or cytochrome *c* oxidase was hypersensitive to DTT, indicating that the reoxidation of Mia40 depends on the presence of oxidized cytochrome *c*. Furthermore, it was reported that Erv1 can efficiently reduce cytochrome *c in vitro* and that cytochrome *c* prevents the production of hydrogen peroxide by Erv1 (Bihlmaier, Mesecke et al. 2007; Dabir, Leverich et al. 2007). Therefore, the results suggest a direct interaction of Erv1 with cytochrome *c*, which links the disulfide relay system to the respiratory chain in the mitochondrial IMS. This matches the physiology of the eukaryotic cell. The oxidative equivalents required for disulphide bond formation are provided efficiently via cytochrome *c* from the respiration process. In higher eukaryotes, where low-oxygen conditions in tissues have been observed, it is likely that the link between the disulfide relay system and the respiratory chain is particularly important for the maintenance of Mia40 in the oxidized state in the highly reducing environment of the IMS. Moreover, the Erv1-cytochrome *c* connection prevents the production of the potentially damaging hydrogen peroxide in the mitochondrial IMS, which can be especially crucial for cells of higher eukaryotes. Notably, cytochrome *c* is imported via a Mia40-independent mechanism. The biogenesis of cytochrome *c* involves an unusual dependence on Tom proteins and the covalent attachment of heme by cytochrome *c* heme lyase (CCHL) in the IMS (Dumont, Cardillo *et al.* 1991; Diekert, Kroon *et al.* 2001). Import of cytochrome *c* independently from Mia40 is reasonable, since the presence of cytochrome *c* is required for the function of Mia40. Thus, Erv1 links functionally these two different import pathways in the mitochondrial IMS.

A model for a disulfide relay system mediating the import of small cysteine-rich proteins into the mitochondrial IMS can be proposed, based on the obtained data (Fig.36). Precursors of small IMS proteins in a reduced and unfolded conformation cross the outer membrane through the TOM complex (Lutz, Neupert *et al.* 2003; Lu, Allen *et al.* 2004). In the IMS, the preproteins interact via a disulfide bridge with oxidized Mia40. Isomerisation of this disulfide bond leads to a release of the oxidized polypeptide and reduction of Mia40. Erv1 reoxidizes Mia40 via thiol-disulfide exchange reaction, thereby supporting further rounds of import. Subsequently, Erv1 uses the oxidized cytochrome *c* (cyt *c*) as an electron acceptor to re-oxidise itself. Cytochrome *c* passes its electrons further to the cytochrome *c* oxidase (COX) complex. Thus, the mitochondrial IMS harbours a route for the transfer of electrons from the newly imported precursor to cytochrome *c* as an acceptor, linking the protein import process and the respiratory chain action. Under aerobic conditions the terminal acceptor is oxygen, while under anaerobic conditions the electrons flow to another unidentified so far acceptor.

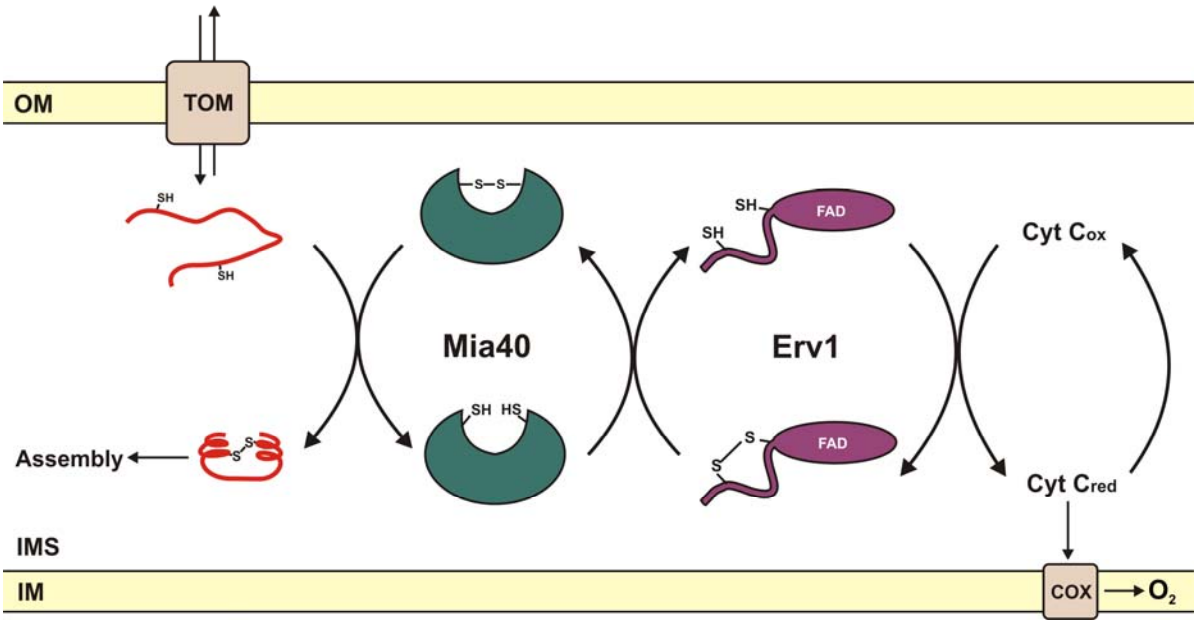

Figure 36. Model of the disulfide relay system mediating import of small cysteine-rich proteins into the IMS of mitochondria. For details see text.

It should be noted that the model described above remains still a quite speculative one and many important issues need to be elucidated. The experimental findings are in line with this model but alternative mechanisms of Mia40 and Erv1 function are possible and many details in the function of the relay system are open questions. Does Mia40 randomly bind to any free sulfhydryl groups on the imported precursor protein? Just very recently, it has been reported for the small Tim proteins Tim9 and Tim10 that only the most amino-terminal cysteine residue of the precursor is critical for the interaction with Mia40 *in organello* and *in vitro*, suggesting a site-specific recognition of Tim precursor proteins by Mia40 (Milenkovic *et al.*, 2007; Sideris and Tokatlidis, 2007). But if this is true also for the other type of Mia40 substrates, like Cox17 and Cox19, remains to be clarified. In case of Mia40 substrates with more than one disulfide bond in their native state, formation of all disulfide bridges in the correct arrangement is critical for the function. Thus, it will be important to verify how many disulfide bonds are introduced by Mia40 itself and if there are some other specific redox partners, required for the oxidative folding of the substrates with multiple disulfide bridges. Interestingly, Erv1 appears to play a role not only in the protein import but also in the assembly of small Tim proteins (Rissler *et al.*, 2005); however, virtually nothing is known about this second function of Erv1. In addition, the requirement of Erv1 in the maturation of cytosolic proteins containing Fe-S clusters (Lange *et al.*, 2001) may lead to the intriguing idea that the disulfide relay system may also be involved in the export of Fe-S cluster proteins from the mitochondrial matrix to the cell cytosol.

The model presented here is in line with the folding trap hypothesis, according to which the imported proteins are locked in the IMS as a result of their stable folding. To prevent the sliding of the polypeptide back into the cytosol, the newly imported proteins need to attain or preserve a folded conformation after their release from Mia40. In some cases, the disulfide bonds formed by the disulfide relay system stabilize folded conformations of imported IMS proteins, which is in agreement with the trap mechanism by oxidative folding. In other cases, binding of cofactors, e.g. metal ions, might be needed for the stabilization of the folded structures or their assembly into complexes. Certain proteins, depending on their redox potential, might dissociate from Mia40 directly in a reduced state. Thus, the redox state

and thereby the folding and the function of the imported IMS proteins might be regulated by the specific properties of each IMS protein.

Diverse substrates of the Mia40-Erv1 relay system have most likely different fates. In case of the small Tim proteins containing the twin CX_3C motif, it was until recently a subject of debate, if the conserved cysteine residues coordinate zinc ions or form disulfide bonds (Curran, Leuenberger *et al.* 2002; Allen, Lu *et al.* 2003; Lutz, Neupert *et al.* 2003; Lu, Allen *et al.* 2004) . Newly, it has been proposed that both mechanisms, zinc binding and disulfide bond formation, are involved in the biogenesis of small Tim proteins (Koehler 2004; Lu, Allen *et al.* 2004; Lu and Woodburn 2005). It seems that these proteins possess a more complex redox regulation than previously assumed. Notably, a component playing a role in the assembly and possibly recycling of the small Tim complexes, named "Helper Of Tim" (Hot13), was recently identified (Curran, Leuenberger *et al.* 2004). This non-essential IMS protein directly interacts with the imported small Tim proteins and facilitates their assembly into native complexes. Furthermore, Hot13 counteracts the negative effects of the oxidative agents on small Tim proteins' function, suggesting that Hot13 may have rather a reducing activity on small Tim proteins. In case of Cox17, a representative of the Mia40 substrates with the twin CX_9C motif, two redox-dependent isomeric forms have been observed: one conformer consisting of a single copper ion bound to a monomer stabilized by two disulfide bonds, and an other conformer forming an oligomeric complex and coordinating a polycopper-thiolate cluster with no disulfide bridges (Arnesano, Balatri *et al.* 2005). It is still not clear, however, which of the conformers is the physiological form of Cox17 in mitochondria. It has been proposed that the redox-controlled shifting from one isoform of Cox17 to the other determines the coordination and release of copper ions (Arnesano, Balatri *et al.* 2005). The central role of Mia40 in the import of the small cysteine-rich IMS proteins is in agreement with the possibility of different final redox states of the substrates, since both properties formation of disulfide bonds with the newly imported precursor and binding of zinc and copper ions were observed for Mia40. This way Mia40 shows some similarities to the copper chaperone of Sod1, CCS. This copper binding protein forms a transient disulfide bridge with the apoform of Sod1 and after the transfer of the copper ion to Sod1, the intermolecular disulfide bridge is converted into an intramolecular disulfide bond in Sod1. This results in the release of Sod1 in a copper-containing oxidized form (Lamb, Torres *et al.* 2001; Field, Furukawa *et al.* 2003).

The disulfide relay system presented in this study consists of a cascade of oxidoreductases, which shows functional similarity to the redox processes encountered in the endoplasmic reticulum (ER) of eukaryotic cells and the periplasm of bacteria (Kadokura, Katzen *et al.* 2003; Sevier and Kaiser 2006). Interestingly, some poxviruses contain also a protein relay system involving the Erv1-like sulfhydryl oxidase E10R that catalyzes the formation of disulfide bonds in capsid proteins in the cytosol of infected host cells (Senkevich, White *et al.* 2000). The mitochondrial Erv1 is homologous to the ER-resident protein Erv2, whose role is to oxidize the protein disulfide isomerase PDI, which oxidizes, in turn, secreted proteins. Likewise, in the periplasm of bacteria the membrane protein DsbB oxidizes the soluble DsbA, which then mediates the oxidative folding of substrates. Erv1 in mitochondria oxidizes Mia40, which further introduces disulfide bonds in the imported proteins. Thus, Erv1 exhibits a function corresponding to the one of Erv2 and DsbB. Mia40, instead, can be compared functionally to the DsbA or PDI, although it shares no obvious homology to these proteins and lacks the thioredoxin-like motif typical for many oxidoreductase systems. It may seem surprising that the IMS of mitochondria harbours a disulfide relay system, since the mitochondrial IMS is considered to be in equilibrium with the reducing cytosol. Interestingly, a number of proteins containing disulfide bridges can be found in the IMS, for example Rieske iron sulfur protein (Iwata, Saynovits *et al.* 1996), small Tim proteins (Curran,

Leuenberger *et al.* 2002; Lu, Golovanov *et al.* 2004), CCS , Sod1 (Lamb, Torres *et al.* 2001) and Sco1(Williams, Sue *et al.* 2005). Moreover, the connection of the Mia40-Erv1 relay system to the electron transport chain resembles the bacterial Dsb protein system (Bader, Muse *et al.* 1999). This similarity of the mitochondrial IMS to the bacterial periplasm can be explained by the origin of mitochondria from endosymbiotic prokaryotes. Apparently, during evolution the process of disulfide bonds formation was conserved from the bacterial periplasm to the IMS of current mitochondria. Considering some biochemical and physiological properties, still many parallels can be drawn between mitochondria and their bacterial ancestors. Nonetheless, some changes occurred in mitochondria in order to adapt to the specific requirements of an intracellular organelle. The principle of protein oxidation in the IMS has been obviously preserved from the bacterial periplasm and has been adapted to drive the vectoral translocation of proteins from the cytosol into the mitochondria. It also seems to act as a sorting mechanism to distinguish the IMS proteins from the protein pool streaming through the TOM complex. In the future, it will be interesting to understand how the systems found in different cell compartments developed during evolution and to gain a better knowledge of the mechanisms of the most recently discovered one, namely the disulfide relay system in the mitochondrial IMS.

4.3. Erv1 – a novel substrate of Mia40-mediated pathway

The precursor of Erv1 itself requires Mia40 for import into mitochondria as shown in this work. The newly imported Erv1 also interacts with Mia40 also via disulfide bonds. Interestingly, Erv1 does not need the two CX_2C motifs for its import into mitochondria (Terziyska, Grumbt *et al.* 2007). The fact that the biogenesis of Erv1 depends on Mia40 is quite surprising, since Erv1 protein has none of the characteristic cysteine signatures found in all so far known substrates of Mia40 pathway. The well studied group of Mia40 substrates are the small Tim proteins that contain the twin CX_3C motif (Paschen, Rothbauer *et al.* 2000; Curran, Leuenberger *et al.* 2002). Another group of Mia40 substrates is composed of proteins harbouring the twin CX_9C motifs. The best characterized representative is the copper chaperone Cox17 (Beers, Glerum *et al.* 1997). Twin CX_9C motifs are present in several additional IMS proteins, such as Cox19, Cox23, Mdm35, Mic14 (YDR031w), and Mic17 (YMR002w), which all depend on the Mia40-Erv1 relay system for their biogenesis (Chacinska, Pfannschmidt *et al.* 2004; Gabriel, Milenkovic *et al.* 2007). Erv1 does not contain a twin CX_3C or a twin CX_9C motif. Therefore it represents an unusual type of substrate of the Mia40-dependent import pathway. Moreover, with a molecular mass of 22 kDa Erv1 is the largest substrate of the Mia40-mediated route. The findings presented here suggest that Mia40-mediated machinery can import larger proteins than the substrates known so far and proteins with cysteines that are not organized in twin CX_3C or CX_9C motifs. Thus, the Mia40-mediated import pathway has a greater versatility considering the type and the size of the substrates than previously shown.

The typical substrates of Mia40 pathway consist of one domain with a simple fold which is stabilized by two interhelical disulfide bonds between the cysteine residues (Allen *et al.*, 2003; Webb *et al.*, 2006; Arnesano *et al.*, 2005). In contrast, Erv1 is characterised by a flexible tail segment and a catalytic core with a complex fold. The cysteine residues of the CX_2C motifs are involved in electron transfer and most likely not in the stabilization of the protein (Coppock and Thorpe 2006). It might be possible that the import of Erv1 by the Mia40-dependent pathway is different mechanistically from those of substrates with twin CX_3C and twin CX_9C motifs.

There are additional proteins in the IMS of mitochondria, which harbour disulfide bonds, but do not contain twin CX_3C and CX_9C signatures. Yet, in some of these proteins, similar cysteine patterns are formed. Cox12, for instance, is a subunit VIa of the cytochrome oxidase and contains a CX_9C-$CX_{10}C$ motif that can be functionally correlated with the twin CX_9C pattern. The copper chaperone CCS contains in its N-terminal domain a CX_2C signature, just like Erv1. On the other hand, in other proteins, such as the Rieske protein or copper/zinc superoxide dismutase (Sod1), only one pair of cysteine residues exists and these cysteine residues are not organized in a specific pattern. Whether the Mia40-dependent pathway plays a role in the oxidation of these proteins remains to be clarified.

To date, Erv1 is the only component of the Mia40-mediated pathway which uses Mia40 and pre-existing Erv1 for its import. Cytochrome *c*, which is the *in vivo* electron acceptor for Erv1, is imported in a Mia40-independent manner (Chacinska, Pfannschmidt *et al.* 2004). In fungi, Mia40 is synthesized with a presequence and is imported via the general import pathway (Chacinska, Pfannschmidt *et al.* 2004; Naoe, Ohwa *et al.* 2004). However, in higher eukaryotes, Mia40 homologues are much smaller in size and do not contain presequences. Since they hold the twin CX_9C motif, they are presumably itself substrates of the Mia40-dependent translocation pathway. This hypothesis can be supported by the observation that the expression of human Mia40 variants with mutations of the cysteine residues in the twin CX_9C motif in yeast mitochondria is largely impaired (Hofmann, Rothbauer *et al.* 2005). Thus, the Mia40-Erv1 machinery has the ability to import some of its components, certainly at least one of them: the Erv1 oxidase.

4.4. Functional characterization of the conserved cysteine residues in Mia40

It was reported before that the conserved cysteine residues in Mia40 are essential for its function but the precise role of the single cysteine residues has not been known (Naoe, Ohwa *et al.* 2004). Three different redox states of Mia40 can be observed: an oxidized, a partially reduced and a fully reduced form. In this study, the molecular basis of these redox states of Mia40 and the importance of each cysteine residue for the function of Mia40 in the disulfide relay system were analysed. The analysis of the redox state of the endogenous Mia40 and its cysteine mutants in yeast suggests that all cysteine residues are forming intramolecular disulfide bonds in the oxidized form of Mia40 (Fig.37). This is consistent with the mass spectroscopy results obtained from the recombinant C-terminal domain of Mia40 (Grumbt, Stroobant *et al.* 2007). The functional studies of the single cysteine mutants of Mia40 revealed that the first, the second, the third and the sixth cysteine residues in Mia40 play important roles for the function of Mia40.

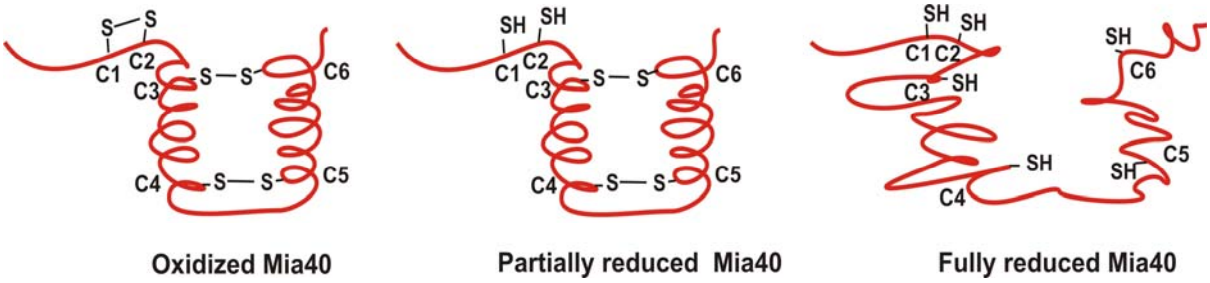

Figure 37. The redox states of Mia40. Schematic presentation of the three different redox forms of Mia40 with the pattern of disulfide bonds formed between specific cysteine residues. The labelling of the cysteine residues refers to their order in the Mia40 motif: the first cysteine residue as C1 (Cys296), the second C2 (Cys298), the third C3 (Cys307), the fourth C4 (Cys317), the fifth C5 Cys330) and the sixth C6 (Cys340).

Discussion

The first two cysteines in the CPC motif (C1 and C2) form a disulfide bridge in Mia40, which is easily accessible, redox-sensitive and necessary for the catalysis of redox reactions of Mia40. The findings shown here indicate that the partially reduced form of Mia40 is created by the opening of the disulfide bridge between these two cysteine residues. The close positioning of cysteine residues in a CXC motif might contribute to the lower stability of the disulfide bond linking these residues (Wilkinson and Gilbert 2004). In Mia40, the fairly unstable disulfide bond of the CPC fragment can be advantageous for the efficient transfer of disulfide bridges to substrate proteins. The mutational analysis of Mia40 indicates that the second cysteine residue in Mia40 is essential for the function and is presumably involved in the interaction with Erv1 and the substrate proteins.

The other two disulfide bridges in Mia40 connect the two CX_9C fragments present in a coiled-coil-helix-coiled-coil-helix (CHCH) domain, which is found in many other mitochondrial proteins, such as Cox17, Cox19, Mic14, Mic17 and Mdm35 (Beers, Glerum *et al.* 1997; Dimmer, Fritz *et al.* 2002; Nobrega, Bandeira *et al.* 2002; Gabriel, Milenkovic *et al.* 2006). These disulfide bonds of Mia40 are stable under mild reducing conditions. Similar observations were reported for Cox17 (Arnesano, Balatri *et al.* 2005). Thus stabilization by disulfide bonds might be characteristic for the proteins with CHCH domain harbouring a twin CX_9C motif (Milenkovic, Gabriel *et al.* 2007). The disulfide bond formed by C4 and C5 appears to be less important then the one between C3 and C6, since both the single and the double mutations of the fourth and the fifth cysteine residues showed no significant effects on the function and the stability of Mia40 and thereby on viability of the cells. The results obtained for the Mia40 C3S and the Mia40 C3/6S mutants appear not to be consistent. The single mutation of the third cysteine residue led to lethality of cells and no interactions with the substrates and with Erv1 were observed. On the other hand, the mutation of both the third and the sixth cysteine residues showed severe defects, but yeast cells were viable and the interactions of Mia40 were strongly impaired but still detectable. One explanation for the observed defects might be that disruptions of structural disulfide bonds in Mia40 affect the function of the protein. In case of the Mia40 C3S protein, the effect is more severe presumably due to the remaining unpaired cysteine residue that might generate non-native disulfide bonds. Another explanation for the observed discrepancy might be that the disulfide bond formed by the third and the sixth cysteine residues exhibits also a catalytic function in Mia40, though not a crucial one. In the absence of this disulfide bond it is possible that another disulfide bond can substitute its role but in the Mia40 C3S mutant protein this replacement might not be efficient enough. Taken together, the results demonstrate that the two disulfide bonds linking the two CX_9C fragments have a structural function for Mia40, though a catalytic role in the redox reactions of Mia40 cannot be completely excluded.

The mutational analysis of Mia40 enables a refinement of the model of the disulfide relay system in the IMS presented in Fig.36. First, the disulfide bond linking the first two cysteine residues in Mia40 is involved in the formation of the mixed disulfide intermediate with the substrate proteins. Following the transfer of the disulfide bond to the precursor protein, Mia40 is released in its partially reduced form, in which the first and the second cysteine residues are in their thiol state. Second, Erv1 binds to the partially reduced Mia40, forming a mixed disulfide via the second cysteine residue in Mia40. Mia40 transfers electrons to Erv1 and thereby recovers its catalytically active disulfide bond between C1 and C2 residues. Finally, the cysteine residues of the twin CX_9C motif form structural disulfide bonds which are required for the stability of Mia40; however a role of these cysteine residues in the catalytic reactions of Mia40 cannot be completely excluded. Future studies with purified components and the determination of the structure and the redox potentials of Mia40 and Erv1 will help to refine the model further and to provide more information about the reaction intermediates in the disulfide relay system.

5. SUMMARY

All intermembrane space (IMS) proteins are synthesized in the cytosol and have to be imported into mitochondria. Many proteins of the IMS lack typical N-terminal targeting signals and are characterized by a small molecular mass and highly conserved cysteine residues present in characteristic patterns. These proteins cross the outer membrane of mitochondria via the TOM complex and need their cysteine residues for the efficient retention in the IMS. The aim of this study was to analyse whether specific factors are required for the import of these proteins into the mitochondrial IMS. The candidate protein, later termed Mia40 (mitochondrial intermembrane space import and assembly), was structurally and functionally characterized.

The experiments presented here confirmed the mitochondrial location of Mia40 and determined its topology. Mia40 contains a classical N-terminal mitochondrial targeting signal followed by a hydrophobic segment. It is anchored in the inner membrane by a hydrophobic stretch and exposes a large C-terminal domain to the IMS. This domain harbours six highly conserved cysteine residues forming a CXC-CX_9C-CX_9C- motif (X represents non-cysteine amino acid residues). Since Mia40 is essential for viability of yeast, a strain harbouring the *MIA40* gene under control of the glucose-repressible *GAL10* promoter was used to study the function of Mia40 in mitochondria. Depletion of Mia40 resulted in strongly reduced levels of Tim13, Cox17 and of other IMS proteins with cysteine motifs, which was due to the impairment of their import into mitochondria. Mia40 is directly involved in the translocation of the small IMS proteins with conserved cysteine motifs: the newly imported IMS proteins form mixed disulfide intermediates with Mia40. In mitochondria, the majority of Mia40 is present in the oxidized state, thus allowing the formation of the mixed disulfide intermediates in an isomerization reaction. Subsequently, Mia40 transfers the disulfide bond from the mixed disulfide to the substrate proteins and thereby triggers the folding and the trapping of these proteins in the IMS. Mia40 is left in a partially reduced state and a reoxidation step is required for the next round of import. Erv1 is an essential FAD-containing sulfhydryl oxidase present in the IMS of fungi, plants and animals. The import of Tim13 was less efficient in mitochondria depleted of Erv1 and Mia40 interacted directly with Erv1 via disulfide bonds. In addition, the depletion of Erv1 affected the redox state of Mia40, which accumulated in a partially reduced state, suggesting that Erv1 is required for the recovery of the oxidized state of Mia40. Thus, Mia40 and Erv1 form a disulfide relay system mediating the import of small cysteine-rich proteins into the IMS. Erv1 passes its electrons further to cytochrome *c*, linking the import of small IMS proteins to the respiratory chain activity. Notably, Erv1 is not only a component but also a substrate of the disulfide relay system. It represents a novel type of substrate of the Mia40-mediated pathway. Thus, this pathway appears to be quite versatile and not limited to proteins with twin CX_3C or twin CX_9C motifs.

The conserved cysteine residues in Mia40 are crucial for its function. Using single and double cysteine mutants of Mia40, it was possible to assign specific roles to each cysteine residue. In the oxidized state of Mia40 all cysteine residues form intramolecular disulfide bonds. The first two cysteine residues in the CPC motif compose a redox-sensitive disulfide bridge and breaking of this disulfide leads to Mia40 in the partially reduced state. The disulfide bond formed by the first two cysteine residues in Mia40 seems to be involved in the interaction with Erv1 and the substrate proteins, suggesting that it is essential for the catalysis of redox reactions of Mia40. The two other disulfide bonds connect the two CX_9C fragments in Mia40 and most likely play a structural role.

Taken together, the essential protein Mia40 is the central component of a novel translocation pathway. Mia40 together with Erv1 forms a disulfide relay system required for the import of small cysteine-rich proteins into the IMS of mitochondria.

6. ABBREVIATIONS

AAC	ADP/ATP carrier
Aco1	aconitase 1
Amp	ampicillin
ALR	augmenter of liver regeneration
ATP	adenosine triphosphate
β-ME	β-mercaptoethanol
BSA	bovine serum albumin
ATPase	adenosine triphosphatase
C-	carboxy-
CBB	coomassie brilliant blue
CCHL	cytochrome *c* haem lyase
CCS	chaperone for superoxide dismutase
CHCH	coiled-coil-helix-coiled-coil helix
Cox	cytochrome *c* oxidase
DFDNB	1,5-difluoro-2,4-dinitrobenzene
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxid
dNTP	deoxyribonucleoside triphosphate
DNA	desoxyribonucleic acid
DTT	dithiotreitol
$\Delta\psi$	membrane potential
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetate
ER	endoplasmatic reticulum
Ero1	ER oxidoreductin
Erv1	essential for respiration and vegetative growth
FAD	flavin adenine dinucleotide
GSH	reduced glutathione
GST	glutathione S-transferase
HEPES	N-2 hydroxyl piperazine-N`-2-ethane sulphonic acid
Hsp	heat shock protein
IgG	immunoglobulin G
IM	inner membrane
IMS	intermembrane space
IPTG	isopropyl-β,D-thiogalactopyranoside
kDa	kilodalton
LB	Luria Bertani
m7G (5`)ppp(5`)G	7-methylguanosine triphospate
Mia40	mitochondrial intermembrane space import and assembly
MBP	maltose-binding protein
MPP	mitochondrial processing peptidase
MTS	matrix targeting signal

MW	molecular weight
N-	amino-
NADH	nicotine amide adenine dinucleotide
NADPH	nicotine amide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
OD x	optical density at x nm
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDI	Protein disulfide isomerase
PEG	polyethylene glycol
PEG-mal	$mPEG_{5000}$-maleimide
PI	preimmune serum
PK	Proteinase K
PMSF	phenylmethylsulfonyfluoride
pSu9(1-69) DHFR	presequence of subunit 9 of the F_1F_0-ATPase fused to DHFR
RNase	ribonuclease
RNasin	ribonuclease inhibitor
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
Sod1	Cu,Zn-superoxide dismutase
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TCEP	Tris (2-carboxyethyl) phopshine hydrochloride
TEMED	N,N,N‘,N‘-tetramethylene diamine
TIM	translocase of the inner mitochondrial membrane
TOB	translocase of outer membrane β-barrel proteins
TOM	translocase of the outer mitochondrial membrane
Tris	tris-(hydroxymethyl)-aminomethane
TX-100	Triton X-100
v/v	volume per volume
w/v	weight per weight
WT	wild type

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