The function of Mim1 in the biogenesis of the mitochondrial TOM complex

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Jelena Popov-Čeleketić
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1. Gutachter: Prof. Dr. Jürgen Soll

2. Gutachter: Prof. Dr. Ute Vothknecht

Sondergutachter: Prof. Dr. Dr. Walter Neupert

Мојој малој породици

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1. INTRODUCTION

1.1. Discovery, origin, structure and function of mitochondria

A typical cell of the human body has between hundred and several thousands mitochondria which are able to fuse and form a continuous network that permeates the entire cell. They were first described in 1857 by Swiss anatomist Rudolf Albrecht von Kölliker, and in 1890 Richard Altman proposed they were intracellular parasites. Eight years later German Carl Benda named them "mitochondria" (from the Greek mitos-thread and khondrion-granule), but it took almost another fifty years to isolate mitochondria from disrupted cells and show that they catalyze respiration. This work was done by Belgian biochemist Albert Claude who said that the mitochondria may be "considered as the real power plants of the cell" (Schatz, 2007). After Claude's remarkable discovery, the biochemistry of mitochondria became the focus of intense scientific investigation.

From a structural perspective, mitochondria and chloroplasts are unusual, compared to other membrane-bound organelles since they are bordered by two membranes. According to the endosymbiotic theory these organelles originated as separate prokaryotic organisms which were taken inside the eukaryotic cell as endosymbionts (Margulis, 1970). During the large time span that the mitochondria have co-existed with their hosts, genes and systems which were no longer necessary, were deleted, or transferred into the host genome instead. These transfers constitute an important way for the cell to regulate mitochondrial activity. Today, the vast majority of the mitochondrial proteins have to be imported into mitochondria since they are encoded by nuclear genes and synthesized in the cytosol (Lang et al., 1999). In the yeast *S. cerevisiae,* for example, out of 600-800 different

mitochondrial proteins only eight are encoded and synthesized in the mitochondria (Lithgow, 2000).

Each mitochondrion contains two membranes that define four distinct compartments: the outer membrane, the intermembrane space, the inner membrane, and the matrix. The outer mitochondrial membrane, which encloses the entire organelle, has a protein to phospholipid ratio similar to that of the eukaryotic plasma membrane (about 1:1 by weight). It contains highly abundant porins (also called voltage-dependent anion channels, VDACs) which form large channels (about 2-3 nm in diameter) that make outer mitochondrial membrane permeable to all molecules of 5000 Da or less. The outer membrane also contains the enzymes involved in metabolic activities and the protein complexes involved in translocation of newly synthesized proteins.

The inner membrane is highly folded into cristae carrying more then one fifth of the total mitochondrial protein. It is composed of approximately 20% lipids and 80% proteins, which makes the highest protein to lipid ratio in cellular membranes. Two topologically continuous inner membrane domains can be distinguished: the inner boundary membrane which together with the outer membrane forms the mitochondrial envelope, and cristae membranes, invaginations of the inner membrane that protrude into the matrix (Reichert and Neupert, 2002). Morphology of the cristae varies from tubular, lamellar to triangle-shaped depending on different mitochondrial activities. The inner mitochondrial membrane accommodates the MDa complexes of the electron transport chain, ATP synthase that control the basal rate of cellular metabolism, the protein import machinery, and the specific transport proteins that regulate the passage of metabolites into and out of the matrix.

Only around 5% of total mitochondrial proteins reside in the intermembrane space subcompartment. Those proteins are involved in the maintenance of mitochondrial morphology (like Mgm1p; (Herlan et al., 2003)), electron transport (cytochrome *c*; (Maneg et al., 2004)), apoptosis (Smac, AIF, cytochrome *c*; (Brdiczka et al., 2006)), copper transport (Cox17p; (Beers et al., 1997)) iron-sulfur cluster biogenesis (Erv1p, (Lange et al., 2001)), and protein translocation (small Tim proteins; (Neupert and Herrmann, 2007)).

Mitochondrial matrix is the site of many metabolic processes (oxidation of pyruvate and fatty acids and the citric acid cycle). The matrix contains a mixture of hundreds of enzymes, the mitochondrial ribosomes, tRNAs, and several copies of the mitochondrial DNA genome.

1.2. Protein translocation into mitochondria

Given that the vast majority of mitochondrial proteins is synthesized on free ribosomes in the cytosol, they have to be imported into the mitochondria (Fig. 1.1). Since most preproteins have N-terminal targeting sequences, mitochondrial protein import could start before the polypeptide chain is completed, but the definite proof for co-translational targeting mechanisms has been provided for only a few proteins, like fumarase (Neupert and Herrmann, 2007). However, there is some additional evidence supporting this theory. Cytosolic ribosomes were found to be associated with yeast mitochondria both *in vivo* and *in vitro* under certain conditions, and some biochemical data suggested a possibility of the co-translational insertion of nascent polypeptide chains into mitochondria (Fujiki and Verner, 1991; Fujiki and Verner, 1993; Verner, 1993). In fact, some recent studies propose that the co-translational process is involved in the mitochondrial import of at least some proteins (Karniely et al., 2006). A mechanism that directs mRNA molecules to the surface of the mitochondria followed by the protein translation on the ribosomes bound to the outer mitochondrial membrane was proposed as well (Corral-Debrinski et al., 2000). Still, the large body of evidence provides convincing support that the vast majority of precursor proteins can be imported post-translationally. Many mitochondrial precursor proteins synthesized in a cell-free system can be imported post-translationally into isolated mitochondria (Harmey et al., 1977; Neupert, 1997). Furthermore, mitochondrial precursor proteins that accumulate in the cytosol can be subsequently chased into mitochondria (Hallermayer et al., 1977; Reid and Schatz, 1982). The proteins that contain targeting sequence at their C-terminus have to be targeted into mitochondria after the synthesis of precursor protein was completed (Borgese et al., 2003; Fölsch et al., 1998; Suissa and Schatz, 1982)

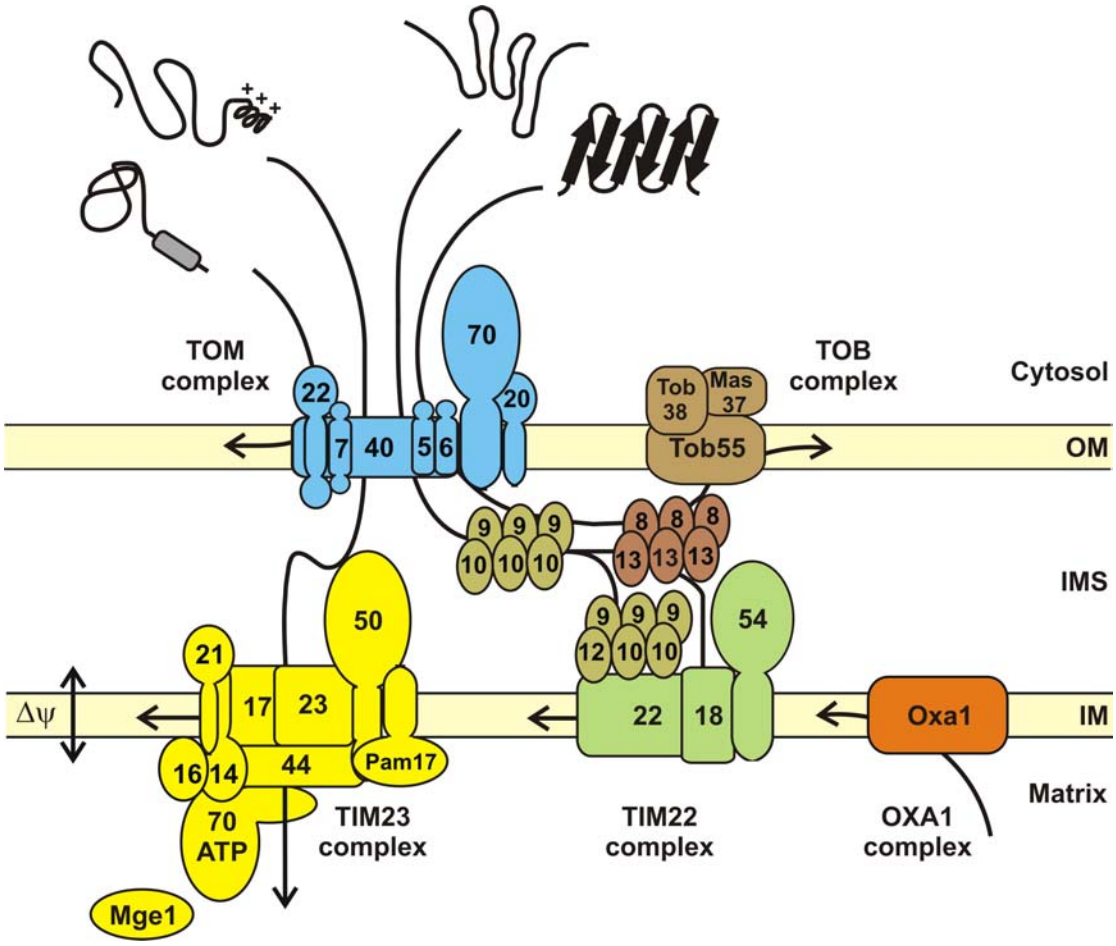


Figure 1.1. Protein translocation into mitochondria. Precursor proteins containing different targeting signals are imported into mitochondria and sorted into different mitochondrial compartments through the concerted action of protein translocases. OM - outer membrane, IMS - intermembrane space, IM - inner membrane, $\Delta\Psi$ - membrane potential across the inner membrane.

Precursor proteins are often present in the cytosol in the complexes with chaperones, which stabilize them as well as prevent their aggregation and degradation that precursor proteins are susceptible to since they are not in their final conformation. The precise signals that lead to binding and releasing processes between precursor proteins and cytosolic chaperones are still unclear. Those chaperones include members of the 70 kDa heat shock protein family (Hsp70), which maintain the newly synthesized preproteins in import-competent, unfolded or partially folded, state using an ATP-dependent mechanism (Mihara and Omura, 1996). Hsp70 is not specific only for binding to precursors targeted to mitochondria but also to

other organelles. It was shown that the chaperone Hsp90 in cooperation with Hsp70 mediate the targeting of a subset of mitochondrial preproteins in mammals (Young et al., 2003).

Cytosolic precursors of mitochondrial proteins contain the targeting and sorting sequences that determine the final destinations of the proteins within mitochondria. Matrix destined preproteins contain the N-terminal cleavable presequences also called matrix-targeting sequences (MTSs). The general properties of these presequences are conserved but there is no consensus in the primary structure even between closely related orthologs. Presequence usually consists of about 10 to 70 amino acid residues that have potential to form an amphipathic helix with one positively charged and one hydrophobic face (Roise, 1992; Roise and Schatz, 1988; Von Heijne, 1986; von Heijne et al., 1989). In most cases, the presequence is cleaved from precursors by the mitochondrial-processing peptidase (MPP) residing in the mitochondrial matrix as soon as the cleavage site reaches the matrix (Braun et al., 1992; Gakh et al., 2002).

Many mitochondrial precursors destined to all of the mitochondrial subcompartments contain the internal targeting sequences. Precursors for all proteins targeted to the outer membrane have internal signals. Those with single transmembrane domains (TMDs) contain mitochondrial targeting information in their hydrophobic anchors and the flanking positively charged residues (Rapaport, 2002), but internal targeting signals for β-barrel proteins remain unidentified up to date.

Some matrix destined proteins like rhodanese, 3-oxo-CoAthiolase, and chaperonin 10 (Hsp10) are synthesized with a non-cleavable N-terminal targeting signal, which has characteristics very similar to those of the cleavable signals (Hammen et al., 1996; Jarvis et al., 1995; Waltner and Weiner, 1995). Another matrix protein, DNA helicase Hmil, has a presequence-like targeting signal at its C-terminus suggesting that this precursor protein has to be imported in the reverse orientation (Lee et al., 1999).

Some intermembrane space (IMS) proteins have canonical targeting presequences, followed by a hydrophobic sorting sequence. Their import

depends on ATP and membrane potential across the inner membrane. These bipartite presequences are cleaved off at the outer surface of the inner membrane by the heterodimeric inner membrane peptidase (Imp1-Imp2) and the mature proteins are released into the IMS (Glick et al., 1992).

The inner membrane proteins Tim23, Tim17, Tim22 and members of the carrier family contain several internal targeting and sorting signals.

1.3. The inner membrane translocases

1.3.1. The TIM23 translocase

The TIM23 complex is the major translocase in the inner mitochondrial membrane. It is involved in the import of all precursors of matrix proteins, most of the proteins destined to the inner membrane, and many proteins of the IMS. The translocation by the TIM23 complex requires both membrane potential across the inner membrane and energy obtained from ATP hydrolysis. The complex is composed of two cooperating subcompartments: the membrane sector (protein conducting channel) and the import motor.

The membrane sector is composed of three essential subunits Tim50, Tim23, and Tim17, and two non-essential ones, Tim21 and Pam17, which have regulatory functions. Tim23 and Tim17 form the 90 kDa core of the TIM23 translocase. These two proteins have phylogenetically related transmembrane domains with four predicted transmembrane segments, which, though being homologs, cannot substitute for each other (Emtage and Jensen, 1993; Kübrich et al., 1994; Maarse et al., 1994). Tim23 additionally exposes a hydrophilic amino terminal domain to the IMS. This region consisting of 100 amino acid residues can be divided into two parts. The N-terminal part was found to span the outer membrane and might have a role in the positioning of the TIM23 translocase in proximity to the TOM complex, thereby increasing the efficiency of protein import (Donzeau et al., 2000). The second part of the N-terminal domain (residues 50-100) contains an essential coiled-coil domain specific for dimerization of Tim23 and substrate binding in the IMS (Bauer et al., 1996; Geissler et al., 2002;

Yamamoto et al., 2002). Tim17 has very short N-terminal domain exposed to the IMS. Even though it is only 11 to 14 residues long, it contains two conserved negative charges crucial for protein import. The function of Tim17 is not clear yet, but it was suggested that it plays a role in gating of the TIM23 pore (Meier et al., 2005).

Tim50 is a receptor of the TIM23 translocase, anchored by its N-terminus into the inner mitochondrial membrane exposing a large domain to the IMS (Geissler et al., 2002, Yamamoto et al., 2002). It interacts with presequence-containing proteins when they reach the *trans* site of the TOM complex and directs them to the TIM23 translocase (Mokranjac et al., 2003a; Geissler et al., 2002; Yamamoto et al., 2002).

Tim21 and Pam17, recently discovered components of the TIM23 complex, seem to be involved in the regulation of the translocase during protein import. It was observed that Tim21 interacts with IMS domain of Tom22 suggesting that it might play a role in interaction between the TOM complex and the TIM23 complex (Chacinska et al., 2005; Mokranjac et al., 2005).

The membrane sector of the TIM23 complex translocates the presequence to the matrix side of the inner membrane in a process, which is dependent on membrane potential. Then, the import motor takes over and mediates further translocation steps of preproteins. This part of the import pathway requires ATP. The components of the import motor are Tim44, Tim14 (Pam17), Tim16 (Pam16), mitochondrial heat shock protein mtHsp70, and the co-chaperone Mge1. Tim44 is a hydrophilic matrix protein, which in fungi is fully attached to the inner membrane. It contains one hydrophobic pocket believed to be a membrane binding site (Josyula et al., 2006). Tim44 functions as a docking site for other import motor components and binds the incoming preproteins before it passes them to mtHsp70 in the ATP bound state. MtHsp70 has two domains – an N-terminal ATPase domain and a C-terminal peptide binding domain. When ATP is bound, the substrate binding pocket is opened and Hsp70 is ready to grasp the arriving polypeptide, while when ADP is bound, the pocket is closed and mtHsp70 loses affinity for Tim44. It seems that upon ATP hydrolysis Hsp70 is released from Tim44 (Mokranjac et al., 2003b; Schneider et al., 1996; Liu et al., 2003). The

exchange of ATP and ADP requires the action of the nucleotide exchange protein Mge1.

Binding of incoming preproteins to Hsp70 is regulated by two import motor subunits with DnaJ-like structures, Tim14 (Pam18) and Tim16 (Pam16). Two of them are believed to form a complex (Mokranjac et al., 2006). Whereas Tim14 stimulates hydrolysis of ATP in the mtHsp70, Tim16 does not influence ATPase activity *in vitro*. Tim16 is not a functional DnaJ protein because it does not contain HPD motif important for interaction with Hsp70. Recently published crystal structure of Tim14-Tim16 complex suggested that Tim16 bound to Tim14´s HPD motif and therefore functioned as a negative regulator of Tim14 function by physically blocking the contact site of Tim14 and Hsp70.

To summarize, precursor proteins after passing through the TOM complex are directed to the TIM23 translocase by binding to IMS domains of Tim50 and Tim23. When MTS is translocated across the import channel of the TIM23 translocase, Tim44 binds it and passes it to mtHsp70 in ATP bound state. Tim14 stimulates ATP hydrolysis which leads to the tight binding of Hsp70 to the preprotein and to dissociation of Hsp70 from Tim44. From this moment on, preprotein can only slip into the matrix because backsliding is prevented by bound Hsp70.

1.3.2. The TIM22 translocase

The TIM22 complex is involved in the insertion pathway of multiple membrane-spanning domain proteins like Tim23, Tim17, Tim22, and the metabolite carrier proteins family. This 300 kDa complex is composed of three membrane proteins, Tim22, Tim 54, and Tim18; and three associated small Tim proteins, Tim9, Tim10, and Tim12. While the exact functions of Tim54 and Tim18 are not known, Tim22 is essential, and is the pore forming subunit of the complex. It can support import of carrier proteins, although at reduced levels, even in the absence of the two other membrane components of the translocase (Kovermann et al., 2002). The TIM22 translocase inserts the proteins into the lipid bilayer of the inner membrane in a membrane potential-dependent manner (Kerscher et al., 1997; Kerscher

et al., 2000; Sirrenberg et al., 1996). Small Tim proteins bind to the precursor proteins when they reach IMS after passing through the TOM complex. They function in a chaperone-like manner preventing aggregation of the imported precursors and are required for further translocation from the outer membrane to the TIM22 complex. The essential 70 kDa Tim9-Tim10 complex is required for the transport of carrier proteins and specifically binds to their hydrophobic loops. Non-essential Tim8-Tim13 complex of the same size was found to specifically interact with precursors that contain hydrophilic extensions like Tim23 when it binds to the N-terminal part of Tim23 whereas Tim9-10 complex interacts with the hydrophobic loop of the membrane embedded region (Bauer et al., 2000; Neupert and Herrmann, 2007). It is believed that Tim9-10 complex can functionally replace the nonessential Tim8-13 complex to some extent.

1.3.3. The Oxa1 translocase

The OXA1 translocase of the inner mitochondrial membrane facilitates the insertion of both mitochondrial and nuclear-encoded proteins from the matrix into the inner membrane. This process is called mitochondrial protein export. Eight mitochondrial proteins in yeast, seven of which are highly hydrophobic, are encoded by mitochondrial genome. These are cytochrome b, Cox1, Cox2, Cox3, Atp6, Atp8, and Atp9.

Oxa1 is an evolutionarily conserved protein and its homologues are found throughout prokaryotes and eukaryotes (Kuhn et al., 2003). It spans the inner membrane five times, exposing a long α-helical C-terminal domain to the matrix. This domain forms α-helical coiled-coil domain that binds mitochondrial ribosomes (Szyrach et al., 2003). In addition, Oxa1 was reported to interact with newly synthesized mitochondrial proteins (Hell et al., 2001). Taken together, these data indicate that OXA1 translocase can insert proteins into the inner membrane in a co-translational manner.

Several proteins, including Oxa1 itself, that are synthesized in the cytosol and imported into the matrix via the TIM23 translocase, have to be inserted into the inner membrane using the export machinery (Hell et al., 2001). This

pathway resembles insertion reactions of polytopic membrane proteins of bacterial origin and is called the conservative sorting pathway (Stuart, 2002).

Mba1 is an additional component of the mitochondrial export machinery and it is also found to bind mitochondrial ribosomes (Ott et al., 2006). It shares substrate specificity with Oxa1 but it either cooperates with or functions independently of Oxa1 (Preuss et al., 2001).

1.4. The outer membrane translocases

1.4.1. The TOM complex

The translocase of the outer membrane (TOM complex) mediates the import of almost all nuclear encoded mitochondrial proteins. The composition of the TOM complex was studied in detail in the fungi, *N. crassa* and *S. cerevisiae*. The structure and function of the TOM complex in other organisms, like plants or animals, is very comparable to that in fungi. The translocase is a multi-subunit complex of 600 kDa composed of seven subunits: Tom70, Tom40, Tom22, Tom20, Tom7, Tom6, and Tom5 (Neupert, 1997; Pfanner and Geissler, 2001) which are grouped according to their function into receptor and pore components (Fig. 1.2). The TOM complex has several binding sites for precursor proteins. Cytosolic domains of Tom20, Tom70, and Tom22 represent *cis*-binding site while IMS domains of Tom22, Tom40, and Tom7 seam to contribute in binding of precursor proteins in IMS and they are referred to as *trans*-binding site (Bolliger et al., 1995; Esaki et al., 2004; Mayer et al., 1995b).

The two major receptors are Tom20 and Tom70. Both are anchored to the outer membrane with their N-terminal transmembrane segments exposing hydrophilic C-terminal domains to the cytosol. These two receptors show different substrate specificities but partially overlap in function, therefore they can partially substitute for each other (Lithgow and Schatz, 1995). Single deletion of either receptor can be tolerated, but double deletion is lethal (Ramage et al., 1993).

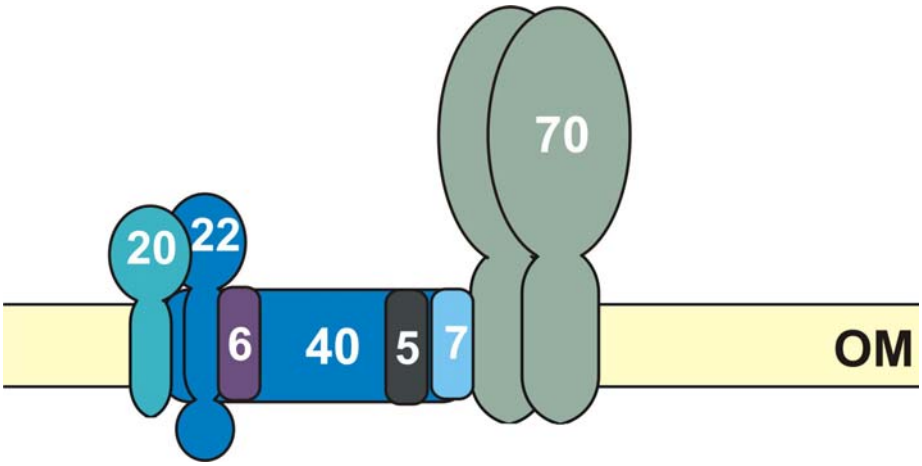


Figure 1.2. The TOM complex. The TOM complex in the outer mitochondrial membrane (OM) is composed of the TOM core complex (containing the pore forming protein Tom40 and proteins Tom22, Tom5, Tom6 and Tom7) with associated receptors Tom20 and Tom70.

Tom20 is the main receptor for preproteins containing N-terminal presequence. It was demonstrated by NMR analyses that the cytosolic part of Tom20 contains a binding groove for the hydrophobic face of the MTS (Abe et al., 2000). It was also reported that Tom20 can interact with some proteins that lacked a mitochondrial presequence, like the outer membrane proteins porin (Schleiff et al., 1999), Tom40 (Rapaport and Neupert, 1999), and the intermembrane space protein cytochrome *c* heme lyase (Diekert et al., 2001). Tom20 cooperates with Tom22, another receptor of the TOM complex, in binding and unfolding of a precursor protein. Different surfaces of the presequence are recognized by different receptors of the TOM complex. The hydrophobic side is recognized by Tom20, and the positively charged side by Tom22 (Brix et al., 1999). Tom22 spans the outer membrane once, exposing its negatively charged N-terminal domain to the cytosol and a smaller C-terminal part to the IMS. It also connects Tom20 to the central import pore and it is critical for general integrity of the TOM complex (Mayer et al., 1995a; van Wilpe et al., 1999).

Tom70 is a dimeric receptor for hydrophobic preproteins with internal targeting information, especially the carrier protein family (Brix et al., 1999; Schlossmann et al., 1994). Its cytosolic domain contains 11 tetratricopeptide repeat motifs (TPR) (Wu and Sha, 2006) that might have a role in protein–protein interaction (Haucke et al., 1996). The TPR motifs interact not only

with precursor proteins but also with cytosolic chaperones, like Hsp70 and, in animals, Hsp90 (Young et al., 2003).

The TOM core complex, also called general import pore (GIP), is composed of the central, pore-forming component, Tom40, three small associated subunits Tom5, Tom6, and Tom7, and the receptor protein Tom22. Its size, as estimated by size-exclusion chromatography, is approximately 400 kDa. Tom40 is the only component of the TOM complex essential for yeast viability. It is a membrane embedded protein composed of series of antiparallel β-strands forming a β-barrel. Purified Tom40 is able to form ion channels in artificial membranes (Ahting et al., 2001). However, it is still not clear whether the pore of the TOM complex is formed by one or more Tom40 molecules. Single particle imaging of negatively-stained isolated TOM holo complex showed particles with two or three pores like structures while TOM core complex contains two pores.

Small Tom proteins are all tail-anchored, composed of 50 to 70 amino acid residues. They have one α-helical TM domain with very few residues exposed to the IMS. Deletion of either of small Tom proteins shows only minor effects but deletion of all three proteins is lethal in yeast (Dekker et al., 1998; Dietmeier et al., 1997; Sherman et al., 2005). Their individual functions remained unclear up to date but they appear to be involved in stabilization of the TOM complex.

For the import of the β-barrel outer membrane proteins, the TOM complex cooperates with the other outer membrane protein translocation machinery, the TOB complex (for topogenesis of mitochondrial outer membrane beta-barrel proteins, also known as the SAM complex (sorting and assembly machinery) (Paschen et al., 2003; Wiedemann et al., 2003).

1.4.2. The TOB complex

The precursors of β-barrel proteins use the TOM complex in the first step of their import pathway and also require the TOB complex in order to get inserted into the outer mitochondrial membrane.

This 250 kDa complex is composed of one membrane embedded component Tob55 and two hydrophilic proteins, Tob38 and Mas37, which are peripherally associated with the outer membrane. The main component of the TOB complex is Tob55, also called Sam50, which together with Tob38 forms functional TOB core complex. Both proteins are essential for cell viability in yeast and *N. crassa*. Tob55 is composed of two domains: a hydrophilic N-terminal part facing the IMS and a membrane embedded C-terminal domain that forms β-barrel structure with 14-16 transmembrane β-sheets.

The N-terminal part forms characteristic structure called the POTRA domain (polypeptide-transport-associated domain) which is supposed to have a chaperone-like function (Gentle et al., 2005; Sanchez-Pulido et al., 2003). Therefore, this domain was proposed to present the interaction site for β-barrel precursors with the TOB complex after they were imported via the TOM complex into IMS (Habib et al., 2007; Pfanner et al., 2004). Both domains are conserved among Tob55 bacterial (Omp85) and eukaryotic homologous. In yeast, depletion of both Tob55 and Tob38 leads to impaired insertion and assembly of newly imported β-barrel proteins (Kozjak et al., 2003; Paschen et al., 2003; Waizenegger et al., 2004). The exact functions of three components of the TOB complex are still not clear but according to high conservation of Tob55 one could assume that this protein plays the most important role in the β-barrel assembly pathway. It might be that the two other proteins, Tob38 and Mas37, have somewhat of an accessory function.

1.4.3. Biogenesis of the TOM complex

The biogenesis of membrane proteins and especially of multisubunit complexes that reside in membranes is a fascinating process. Several studies addressed the biogenesis of the TOM complex (Fig.1.3). The interesting point is that the TOM complex is also involved in its own biogenesis.

Components of the TOM complex, like other outer membrane proteins, do not contain cleavable presequences. Their targeting information is contained

in the protein sequence itself. Each of the components has to be recognized by preexisting TOM complex in the outer mitochondrial membrane. Tom22, Tom6, and Tom40 (Dembowski et al., 2001; Keil et al., 1993) need to be recognized by receptor proteins while newly synthesized Tom70 and Tom20 interact directly with the translocation pore (Schlossmann and Neupert, 1995; Schneider et al., 1991; Waizenegger et al., 2003).

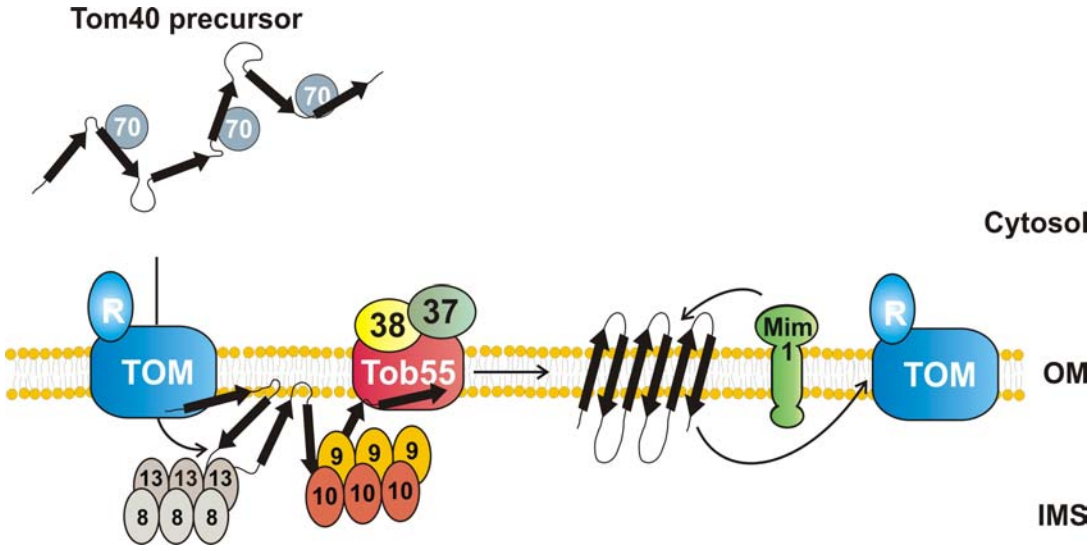


Figure 1.3. Biogenesis of the TOM complex. Precursor of Tom40 protein is imported into mitochondria by the TOM complex and then conveyed to the TOB complex with the assistance of the small Tim proteins in the intermembrane space (IMS). The TOB complex inserts Tom40 in the outer membrane (OM) where other Tom proteins sequentially associate with Tom40, in a process requiring Mim1 protein, leading to the fully assembled TOM complex.

The assembly pathway of Tom40 is studied in some detail using different experimental approaches (Fig.1.3). On its way to the outer membrane it is recognized by Tom20 and possibly also by the Tom70 receptor (Rapaport and Neupert, 1999). After the initial recognition step, Tom40 precursor passes the outer membrane via the TOM complex and then associates with the TOB complex to form an intermediate of approximately 250 kDa referred to as the assembly intermediate I. After the TOB-mediated insertion into the outer membrane, Tom40 forms 100 kDa intermediate complex called assembly intermediate II. This intermediate is probably composed of Tom40 dimer and one Tom5 subunit. The final step in the biogenesis process is the

formation of the 400 kDa, mature TOM core complex by the sequential addition of Tom6, Tom7, and Tom22 to the 100 kDa complex.

Recently, a novel 14 kDa outer membrane protein was identified and named Mim1 (for mitochondrial import) (Mnaimneh et al., 2004). This protein, also known as Tom13, was characterised as an additional assembly factor of the TOM complex (Ishikawa et al., 2004; Waizenegger et al., 2005). Deletion of Mim1 resulted in accumulation of non-imported mitochondrial precursor proteins (Mnaimneh et al., 2004), but also in changes in mitochondrial morphology (Altmann and Westermann, 2005). These effects are believed to be secondary to the main function of Mim1 in the biogenesis of the TOM complex. Mim1 has one putative transmembrane segment (TMS), highly conserved among fungi. Its N-terminal domain faces the cytosol and its C-terminal domain is exposed to the IMS. Depletion of Mim1 abrogates assembly of the TOM complex and results in accumulation of Tom40, the major constituent of the TOM complex, as a low molecular mass species. Mim1 is not a component of the TOM complex or of the TOB complex (Ishikawa et al., 2004; Waizenegger et al., 2005, Meisinger et al., 2007); but rather is a subunit of another, yet unidentified, complex in the outer mitochondrial membrane.

1.5. Aim of the present study

Recently discovered protein named Mim1 has been characterized as an assembly factor of the TOM complex. Specifically, it was found to play a role in the assembly of Tom40 into the TOM complex in the step after the Tom40 precursor interacted with the TOB complex.

The aim of this study was to investigate the structural and functional characteristics of Mim1. Several questions were addressed:

i) What are the domains that are crucial for the function of Mim1?

ii) What are the interaction partners of Mim1?

Iii) How is Mim1 inserted by itself into the outer membrane?

iv) How does Mim1 promote the assembly of the TOM complex?

2. MATERIAL AND METHODS

2.1. Molecular biology methods

2.1.1. Standard polymerase chain reaction (PCR)

DNA sequences were amplified by polymerase chain reaction (PCR), using thermostable DNA polymerase as described previously (Sambrook et al., 1989). *Taq* (isolated from *Thermus aquaticus*), and *Pfu* (isolated from *Pyrococcus furiosus* polymerases were used. *Taq* DNA polymerase has no proofreading ability, and therefore *Pfu* DNA polymerase was added when the PCR product needed to be used for subsequent cloning.

PCR mix contained (total 100 µl): 1-2 U DNA polymerase (*Taq*-polymerase and/or *Pfu*-polymerase), 10 µl PCR-buffer (1% Triton X-100, 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.8), 2 µl dNTPs (10 mM stock), 50 pM primers and 200 ng plasmid DNA template or 1 µg genomic DNA template.

The following program was used:

<table>
<tr><td>1) 94°C, 5 min</td><td colspan="2">Nuclease inactivation and complete DNA denaturation</td></tr>
<tr><td rowspan="4">2) 30-35 cycles</td><td colspan="2">DNA amplification:</td></tr>
<tr><td>94°C, 1 min</td><td>DNA denaturation</td></tr>
<tr><td>45-65°C, 1 min</td><td>Annealing of primers</td></tr>
<tr><td>72°C, 1-6 min</td><td>DNA synthesis*</td></tr>
<tr><td>3) 72°C, 5-20 min</td><td colspan="2">Completion of the last reaction</td></tr>
</table>

The duration of this step is determined by the length of the DNA fragment to be amplified and DNA polymerase used (*Taq* polymerase 1 min/1kb; *Pfu* polymerase 2.5 min/1kb).

The amplified DNA fragments were analyzed by agarose gel electrophoresis.

2.1.2. Site directed mutagenesis

For inserting point mutations in DNA sequence QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used.

PCR conditions:

Sample reaction:

5 µl of 10x reaction buffer

5-50 ng of dsDNA template

125 ng primer #1

125 ng primer #2

1 µl of dNTP mix

ddH_2O to a final volume of 50 µl

1 µl of PfuTurbo DNA polymerase (2.5 U/ µl)

The following program was used:

Cycles	Temperature	Time
1	95°C	30 sec
12-18	95°C	30 sec
	55°C	1 min
	68°C	1 min/ kb of plasmid length

Upon the termination of PCR, 1 µl of the DpnI restriction enzyme was added to the reaction and incubated at 37°C for 1 h to digest the parental

supercoiled dsDNA. Then, MH1 *E. coli* cells were transformed with 1 µl of the DpnI treated DNA.

2.1.3. Analytical and preparative gel electrophoresis

DNA fragments were separated by electrophoresis in a horizontal agarose gel (0.8-2%) according to their molecular mass. Samples were mixed with loading buffer (6% (v/v) glycerol, 0.05% bromphenolblue, 0.05% xylencyanol) and electrophoresis was performed in TAE-buffer (4.84 g/l Tris-Base, 1.14 ml/l acetic acid, 1 mM EDTA, pH 8.0). The agarose solution contained 0.5 µg/ml ethidium bromide to allow visualization of DNA in gel under UV light. The agarose was stored at 65°C until use. The 1 kb and the 100 bp DNA markers were used (New England Biolabs, Beverly, USA). DNA fragments to be further processed were excised from the gel with a sterile scalpel under UV light and the DNA extracted from the gel using the "Gel extraction kit" (Qiagen). Extracted DNA was routinely stored at –20ºC.

2.1.4. DNA and RNA concentration measurement

For DNA concentration measurements the absorption of DNA solutions was measured at 260 nm. One optical unit (OD) corresponds to a concentration of 50 µg/ml of double stranded DNA, 33 µg/ml single stranded DNA, 40 µg/ml RNA or 20 µg/ml oligonucleotides.

2.1.5. Enzymatic manipulation of DNA: restriction and ligation reactions

Digestion of DNA with restriction endonucleases

For analytical and preparative purposes PCR product and plasmid DNA were digested with specific restriction endonucleases (up to 5 U of enzyme for 1 µg DNA). The buffer, temperature (usually 37ºC) and incubation time (1-3 h) for every reaction were chosen according to the manufacturer's recommendations. The digested fragments were analyzed by agarose gel electrophoresis or directly isolated using anion-exchange chromatography

(Qiagen). For preparative purposes, digested DNA fragments were extracted from gels using “Gel extraction kit” (Qiagen).

When plasmid DNA was cut with a single restriction enzyme, it was treated with shrimp alkaline phosphatase (SAP) (Roche). This enzyme removes 5’-phosphate groups on linearized plasmid DNAs thereby preventing recircularization of the vector.

Ligation

Linearized DNA vector (50-200 ng) and a 5 fold molar excess of DNA fragment to be inserted, were incubated in a 10 µl reaction mixture containing 1 µl of 10x ligation buffer (10 mM $MgCl_2$, 5% (w/v) PEG-8000, 1 mM DTT, 1 mM ATP, 50 mM Tris-HCl, pH 7.6), and 0.5 µl (1 U) T4 DNA ligase (Gibco-BRL). Reactions were incubated at 14ºC overnight or at RT for 5 h and 1 µl of the reaction mixture was used for transformation of *E. coli* cells.

2.1.6. Preparation of *E. coli* competent cells

A single colony of *E. coli* strain (MH1 or XL-1 Blue) was inoculated in 50 ml of LB-medium containing Ampicillin (LBamp) and grown overnight at 37ºC under moderate shaking conditions. The following day, 1 l of liquid LBamp medium was inoculated with the overnight culture. The bacterial cells were grown further until they reached OD_{600} ~ 0.5. Then, they were incubated on ice for 30 min, harvested by centrifugation (4,400 x g, 5 min, 4ºC) and washed sequentially with 500 ml, 250 ml, and 50 ml of 10% (v/v) glycerol. The competent cells were finally resuspended in 500 µl 10% (v/v) glycerol, aliquoted and stored at –80ºC.

2.1.7. Transformation of *E. coli*

Ligation reaction mixture (1 µl) was added on ice to 50 µl of *E. coli* competent cells. The mixture was transferred to an ice-cold cuvette and the cuvette was introduced into the electroporation apparatus (Gene Pulser, BioRad). The instrument was set at 2.5 kV, 400 Ω, and 25 µF. The obtained time constant was 7-8 ms. After a short application of a high electric voltage

to the cells, the suspension was diluted with 1 ml LB-medium, and incubated for 45 min at 37°C under moderate shaking to allow cell recovery. The transformed cells were harvested by centrifugation (10,000 x g, 15 sec, RT) and plated on LB-amp medium. The plates were incubated overnight at 37°C.

2.1.8. Small and large scale isolation of plasmid DNA from E. coli

Small scale preparation of plasmid DNA was performed through alkaline lysis according to a published procedure (Birnboim and Doly, 1979). LB-amp medium (2.5 ml) was inoculated with a single bacterial colony picked out from a Petri dish, and incubated overnight at 37ºC, while shaking (140 rpm). The next day bacteria were harvested by centrifugation (8,000 x g, 30 sec, RT) and the pellet was resuspended in 300 µl of buffer E1 (10 mM EDTA, 50 mM Tris-HCl, pH 8.0) containing 100 mg/ml RNase. Cells were lysed by adding of 300 µl of buffer E2 (0.2 M NaOH, 1% SDS). Samples were mixed by inverting the tubes 5 times and incubated 5 min at RT. Neutralization step was performed by adding 300 µl of buffer E3 (3.1 M K-acetate, pH 5.5) and mixing the samples immediately afterwards. In the next step samples were centrifuged (10,000 x g, 10 min, 2ºC), the DNA-containing supernatant was transferred to new tubes and the DNA was precipitated by the addition of 600 µl of 96% isopropanol. Samples were then centrifuged again (10,000 x g, 40 min, 2ºC) and the resulting pellets were washed with 85% cold ethanol, dried at RT, resuspended in 30 µl water and stored at –20ºC.

For large scale preparation of plasmid DNA a “PureYield” Plasmid Midiprep System (Promega) was used. LB-medium (50 ml) supplemented with ampicillin was inoculated with bacteria carrying the plasmid of interest and incubated overnight at 37ºC while shaking at 140 rpm. The bacteria were harvested the next day by centrifugation (10000 x g, 10 min, RT) and resuspended in 6 ml of Cell Resuspension Solution. Cells lysis was performed by adding 6 ml of Cell Lysis Solution. Tubes were inverted 5 times and left for 3 min at RT. After neutralization with 10 ml of Neutralization Solution, samples were mixed by inverting the tubes 5 times and incubated for 3 min at RT to ensure thorough clearing. Samples were

centrifuged (10000 x g, 10 min, 4ºC), and then the supernatants were applied onto a clarifying column standing on top of an anion-exchange column placed onto a vacuum manifold. When the entire volume of the sample passed under vacuum through column stack, the clarifying column was removed. The anion-exchange column was washed with 5 ml of Endotoxin Removal Wash and then with 20 ml of the Column Wash Solution. The column was left to dry for 30 sec under vacuum. Plasmid DNA was eluted from the column with 500 µl of sterile deionized water (ddH_20). DNA isolated this way was stored at –20ºC.

2.1.9. Overview of used plasmids

Plasmid	**Reference**
pMal cRI	New England BioLabs
MBP-Mim1	(Waizenegger et al., 2005)
pGEM4	Promega
pGEM4-Su9(1-69)-DHFR	(Pfanner et al., 1987)
pGEM4-Mim1	This thesis
pGEM4-*N.c.*Tom70(1-38)39Met-Tom20	(Ahting et al., 2005)
pRS426	(Mumberg et al., 1995)
pRS426-Mim1*S.c.*	This thesis
pRS426-Mim1*N.c*.	This thesis
pRS426-Mim1*S.p.*	This thesis
pRS426-Mim1(ΔC)	This thesis
pRS426-Mim1(ΔN)	This thesis
pRS426-Mim1(TM)	This thesis

pRS426-Mim1-G61L	This thesis
pRS426-Mim1-LII	This thesis
pRS426-Mim1-7His	(Waizenegger et al., 2005)
pRS315	(Sikorski and Hieter, 1989)
pRS315- Mim1*S.c.*	This thesis
pRS315- Mim1*N.c.*	This thesis
pRS315- Mim1*S.p.*	This thesis
pRS315- Mim1(ΔC)	This thesis
pRS315- Mim1(ΔN)	This thesis
pRS315- Mim1(TM)	This thesis

2.1.10. Cloning strategies:

pGEM4-Mim1

The DNA sequence encoding full length Mim1 was amplified by PCR using yeast genomic DNA as a template. The following primers were used:

Primer name	Included cutting site	Primer sequence
YOL-N-termBamHI	BamHI	5´-AAGGATCCATGACAGAG GTTGTGGGATTCTGG-3´
YOL-C-termHindIII	HindIII	5´-AAAAGCTTTTAAAGGAAC TTGGACGCAACCCT-3´

pRS426-Mim1*S.c.*:

The DNA sequence encoding full length Mim1 was amplified by PCR using the pGEM4-Mim1 as a template. Primers used were Mim1NtermEcoRI and YOL-C-termHindIII.

Primer name	Included cutting site	Primer sequence
Mim1NtermEcoRI	EcoRI	5´-AAGAATTCATGACAGAG GTTGTGGGATTCTGG-3´

pRS426-Mim1(ΔC):

The DNA sequence encoding Mim1 lacking the last 114 bp was amplified by PCR using Mim1NtermEcoRI and Mim-wo-IMS-rev. pGEM4-Mim1 was used as a template for PCR reaction.

Primer name	Included cutting site	Primer sequence
Mim1-wo-IMS-rev	HindIII	5´-AAAAAGCTTTTAGTTAAAC CAATTGAATCTCCAGCAG-3´

pRS426-Mim1(ΔN):

The DNA sequence encoding Mim1 lacking first 102 bp was amplified by PCR using EcoRIdeltaN Mim1 and YOL-C-termHindIII primers. pGEM4-Mim1 was used as a template for PCR reaction.

Primer name	Included cutting site	Primer sequence
EcoRIdeltaN Mim1	EcoRI	5´-AAAGAATTCATGCTTG TGCAGAGCCTTGTATCC-3´

pRS426-Mim1(TM):

The DNA sequence encoding Mim1 lacking first 102 and last 114 bp was amplified by PCR. Primers EcoRIdeltaN Mim1 and Mim-wo-IMS-rev were used. pGEM4-Mim1 was used as a template for PCR reaction.

pRS426-Mim1*N.c.*:

Mim1 open reading frame from *N. crassa* was amplified by PCR from *N. crassa* cDNA library using following primers.

Primer name	Included cutting site	Primer sequence
EcoRIMim1n.c. Forv.	EcoRI	5´-AAGAATTCATGTCCGC TGAGGAGATATCGAAC-3´
HindIIIMim1n.c.Rev.	HindIII	5´-AAAAAGCTTTCACTCCA AGCTGGTCAGCTC-3´

pRS426-Mim1-G61L:

pRS426-Mim1-G61L was generated using QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. The plasmid pGEM4-Mim1 containing Mim1 wt gene was used as a template for the PCR reaction. Glycine in the position 61 was exchanged for leucine. The following primers were used:

Primer name	Primer sequence
61Leu mim1f	5´-CAACGGCATGATGCTCTTGTTCGGCGAGC TATTTGC-3´
61Leu mim1r	5´-CAAATAGCTCGCCGAACAAGAGCATCATGC CGTTG-3´

pRS426-Mim1-LII:

This construct was generated using QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Two

amino acids were changed, glycine in the position 63 into isoleucine and alanin in the position 67 into isoleucine. The plasmid pGEM4-Mim1 containing Mim1-G61L gene was used as a template for the PCR reaction to generate Mim1 construct carrying point mutations in positions 61 and 63. This construct was then used as a template for the PCR to generate Mim1-LII. The following primers were used:

Primer name	Primer sequence
63 F	5´-GGCATGATGCTCCTCTTCATCGAGCTATTTGCTC ACGAGC-3´
63 R	5´-GCTCGTGAGCAAATAGCTCGATGAAGAGGAGCA TCATGCC-3´
63.67 F	5´-CTCTTCATCGAGCTATTTATTCACGAGCTCTGCTG GAGATTC-3´
63,67 R	5´-GAATCTCCAGCAGAGCTCGTGAATAAATAGCTCG ATGAAGAG-3´

pRS315- Mim1*S.c.*:

This construct was generated by subsequent cloning of promoter (300 bp, cutting sites XbaI and BamHI), Mim1 (pGEM4-Mim1 was digested with EcoRI and HindIII and Mim1 fragment was subcloned into the pRS315 vector containing promoter encoding sequence) and 3´ untranslated region (UTR, 300 bp downstream of the Mim1 gene). UTR sequence was amplified using following primers:

Primer name	Included cutting site	Primer sequence
Mim1-3´UTR-Forv.	HindIII	5´-AAAAAGCTTACGATCACCA AAGCATTAGCAAC-3´
Mim1-3´UTR-Rev.	HindIII	5´-AAAAAGCTTGGATCCGCTT GACGTCTTGTCACACATG-3´

pRS315- Mim1*N.c.*

Mim1 open reading frame from *N. crassa* was amplified by PCR from *N. crassa.* cDNA library using BamHIMim1n.c.Forv. and HindIIIMim1n.c.Rev. primers. The construct was generated with the 3´and 5´UTR of *S.c.* MIM1 as described for pRS315- Mim1*S.c*.

Primer name	Included cutting site	Primer sequence
BamHIMim1n.cForv.	BamHI	5´-AAAGGATCCATGTCCGC TGAGGAGATATCGAAC-3´

pRS315- Mim1*S.p.*

Mim1 open reading frame from *S. pombe* was amplified by PCR from *S. pombe* cDNA library. The construct was generated with the 3´and 5´UTR of *S.c.* MIM1 as described for pRS315- Mim1*S.c*. Following primers were used:

Primer name	Included cutting site	Primer sequence
Mim1pombe-fw	BamHI	5´-AAAGGATCCATGGAGAA AAATACAGTTACAGTTC-3´
Mim1pombe reverse	HindIII	5´-AAAAAGCTTTTAGGCTTG AATATACTGATGTC-3´

pRS315-Mim1(ΔC):

The DNA sequence encoding Mim1 lacking last 114 bp was amplified by PCR using YOL-N-termBamHI and Mim1-wo-IMS-rev primers and pGEM4-Mim1 as a template for PCR reaction. The construct was generated with the 3´and 5´UTR of S.c. MIM1 as described for pRS315- Mim1*S.c*.

pRS315- Mim1(ΔN):

The DNA sequence encoding Mim1 lacking first 102 bp was amplified by PCR using BamHIdN-Mim1 and YOL-C-termHindIII primers and pGEM4-

Mim1 as a template for PCR reaction. The construct was generated with the 3´and 5´UTR of S.c. MIM1 as described for pRS315- Mim1*S.c*.

Primer name	Included cutting site	Primer sequence
BamHIdN-Mim1	Bam HI	5´-AAAGGATCCATGCTTG TGCAGAGCCTTGTATCC

pRS315- Mim1(TM):

The DNA sequence encoding Mim1 lacking first 102 and last 114 bp was amplified by PCR using pGEM4-Mim1 as a template. YOL-N-termBamHI and Mim-wo-IMS-rev primers were used. The construct was generated with the 3´and 5´UTR of S.c. MIM1 as described for pRS315- Mim1*S.c*.

2.1.11. Used yeast strains:

Strain	**Genotype**	**Reference**
YPH499	*MATa ade2-101 his3-* Δ200 *leu2-Δ1 trp1-* Δ*63 ura3-52 lys2-801*	(Sikorski and Hieter, 1989)
GAL-Mim1	YPH499, *mim1::HIS3-pGAL-MIM1*	(Waizenegger et al., 2005)
Mim1-3HA	YPH499, *mim1::MIM1-3HA-HIS3*	(Waizenegger et al., 2005)
Δmim1	YPH499, *mim1::HIS3*	(Waizenegger PhD thesis)

2.2. Cell biology methods

2.2.1. *E. Coli* – media and growth

Media for *E. coli*

LB-medium: 0.5% (w/v) yeast extract, 1% (w/v) bacto-tryptone, 1% (w/v) NaCl.

LB-Amp medium: LB-medium supplemented with 100 µg/ml of ampicillin.

Described media were used for preparing the liquid cultures. For the preparation of LB or LB-Amp plates, 2% (w/v) bacto-agar was added to the liquid media solutions. Bacto-agar, glucose and liquid media were autoclaved separately (120ºC, 20 min) and subsequently mixed. The ampicillin was added after media cooled down to 50ºC.

Cultivation of E. coli

LB-Amp liquid medium (50ml) was inoculated with the single colony from the plate and incubated overnight at 37°C while shaking at 140 rpm. If necessary, cells were grown for longer time (24h) at lower temperatures (30 or 24°C).

2.2.2. Preparation of yeast DNA

The isolation of yeast DNA was performed as described previously by Rose et al*.,* 1990. *S. cerevisiae* was inoculated in 10ml YPD medium and incubated over night at 30ºC while shaking (140rpm). Cells were harvested by centrifugation, washed with 25 ml of sterile water and resuspended in 200 µl of breaking buffer (2% Triton-X100, 1% SDS, 100 mM NaCl, 1mM EDTA, 10 mM Tris-HCl, pH 8.0). In the next step, 200µl phenol/chloroform/isoamyl alcohol (25:24:1) mix and 0.3 g glass beads were added, and the samples vortexed for 2 min. The probes were then centrifuged (36,670 x g, 5 min, RT) and the aqueous phase of the supernatant was transferred to new tubes. DNA was precipitated by adding 2.5 vol. of cold 100% ethanol. Samples were than incubated for 10 min at –

20°C, centrifuged (36,670 x g, 10 min, 2°C), and washed with 70% ethanol. Pellets were dried at RT, resuspended in 30 µl ddH_2O and stored at –20°C.

2.2.3. Cultivation of *S. cerevisiae* strains

Media for *S. cerevisiae*

YP-medium: 10 g yeast extract, 20 g bacto-pepton, H_2O to 930 ml, pH 5.5. After autoclaving YP medium was usually supplemented with 2% glucose (YPD), 2% galactose (YPGal) or 3% glycerol (YPG).

Lactate medium: 3 g yeast extract, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2$ x 2 H_2O, 0.5 g NaCl, 1.1 g $MgSO_4$ x 6 H_2O, 0.3 ml 1% $FeCl_3$, 22 ml 90% lactic acid, H_2O to 1 l, pH 5.5 (adjusted with 10 M KOH). The medium was usually supplemented with 0.1% glucose or 0.1% galactose.

S-medium: 1.7 g yeast nitrogen base, 5 g ammonium sulfate, 1.5 g “Dropout mix” powder” (mix containing equal weight of all amino acids; for selecting one auxotrophic marker, the corresponding amino acid was left out), H_2O to 900 ml. After autoclaving 66.6 ml 30% galactose (SGal) or 50 ml 40% glucose (SD) or 100 ml 30% glycerol (SG) was added. The total volume was complemented to 1000 ml.

To prepare plates with solid media, 2% (w/v) agar was added before autoclaving. 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; with the exception of tryptophan (10 mg/ml) which was filter sterilized. The amino acids were added to the mixture before pouring the plates.

S. cerevisiae growth

S. cerevisiae growth was performed as described in Sambrook et al*.,* 1989, in YPD, YPGal or lactate medium supplemented with 0,1% glucose. The cells were grown on SD medium when a selection on the auxotrophic marker was necessary. The cells were incubated at 30ºC, under shaking conditions (140 rpm). For isolation of mitochondria, cells were propagated for 3 days while the OD_{600} never exceeded 1. For depletion of Mim1, yeast strain harboring the corresponding gene under *GAL* promoter was grown for

2 days on lactate media supplemented with 0.5% galactose. Cells were then collected, washed with sterile water and resuspended in lactate medium supplemented with 0.1% glucose. The cells were then grown in the latter medium for 15 h till Mim1 was hardly detectable.

2.2.4. Transformation of *S .cerevisiae* (lithium acetate method)

The corresponding yeast strain was grown overnight in YPD-medium and diluted in the morning to 50 ml medium with an OD_{600} of 0.2. Cells were grown further, till they reached an OD_{600} of 0.5. The yeast culture was then transferred to a sterile centrifuge tube and cells were harvested by centrifugation (1,000 x g, 3 min, RT). Pellet was washed with 25 ml of sterile water and then the cells were recollected by centrifugation, resuspended in 400 µl of 100 mM lithium acetate solution and transferred to an Eppendorf tube. For each transformation 50 µl of the cell suspension was centrifuged (7,500 x g, 5 min, RT) and the supernatant removed. The following mixture was added to the cells in this order: 240 µl PEG 3350 (50% v/v), 36 µl 1 M lithium acetate, 5 µl single stranded salmon sperm DNA (10 mg/ml; previously incubated for 5 min at 95ºC), 70 µl H_2O containing 0.1-10 µg of DNA to be transformed. The mixture was vortexed for 1 min and incubated for 20-30 min at 30ºC and then 20-25 min at 42ºC with moderate shaking. The cells were harvested by centrifugation (7,000 x g, 15 sec, RT), washed with sterile water, resuspended in 100 µl of sterile water and spread on plates with the appropriate selective media. The plates were incubated for 3-5 days at 30ºC to recover transformants.

2.2.5. Large scale isolation of yeast mitochondria

Isolation of mitochondria from *S. cerevisiae* was performed following a previously described method (Daum et al., 1982). Yeast cells were grown to OD_{600} of 0.8-1.2, collected by centrifugation (4,400 x g, 5 min, RT) and washed with water. Pellets were then resuspended in a buffer containing 10 mM dithiotreitol (DTT), 100 mM Tris, pH unadjusted, to a final concentration of 0.5 g/ml. Cell suspension was incubated for 15 min at 30°C with moderate shaking, followed by a repeated centrifugation step and resuspended in 100

ml of 1.2 M sorbitol. To digest the cell wall and to obtain spheroplasts, cells were collected by another centrifugation step 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. The cell suspension was shaken at 140 rpm for 30-60 min at 30ºC. Efficiency of spheroplasts generation was checked after 30 min by diluting 25 µl of suspension in either 1 ml water or 1 ml 1.2 M sorbitol. Formation of spheroplasts was stopped if the OD_{578} of the water suspension was 10-20% of the sorbitol one. All subsequent steps were performed at 4ºC.

The spheroplasts were isolated by centrifugation (3,000 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 (homogenizer) on ice. The cell remnants and unopened cells were sedimented by centrifugation performed twice (2,000 x g, 5 min). The supernatant was centrifuged (17,400 x g, 12 min, 4ºC) to pellet down mitochondria. Sedimented mitochondria were resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS·KOH, pH 7.4). After two centrifugation steps at 2,000 x g for 5 min, mitochondria were separated from the supernatant by centrifugation at 17,400 x g for 12 min. Final mitochondrial pellet was resuspended in 0.5-1 ml SEM buffer. Protein concentration was determined by Bradford assay. Mitochondria were usually diluted to 10 mg/ml, aliquoted (300 µg per aliquot), frozen in liquid nitrogen and stored at -80°C till use.

2.2.6. Isolation of crude yeast mitochondria (“fast mito prep”)

The yeast strains were inoculated in 50 ml YPD or selective medium and incubated overnight at 30°C while shaking at 140 rpm. The cells corresponding to 10-20 OD units were harvested by centrifugation (3,000 x g, 5 min, RT), washed with water and resuspended in 400 µl SEM buffer containing 1 mM PMSF. Upon addition of 0.3 g glass beads (diameter 0.3 mm) the samples were vortexed four times for 30 sec each, with 30 sec break intervals on ice. After centrifugation (1,000 x g, 3 min, 4ºC), the supernatants were transferred to a new tube and the protein concentration

was determined. Mitochondria were sedimented by centrifugation (17,400 x g, 10 min, 4ºC) and cytosolic proteins from the supernatants (50 µl) were precipitated using trichloroacetic acid. Crude mitochondrial pellets were resuspended in 30 µl 2 x sample (Laemmli) buffer, shaken for 5 min at 95°C, and analyzed by SDS-PAGE and immunodecoration.

2.2.7. Dilution assay

Dilutions assay was performed to determine the growth characteristics of yeast strains. Cells were grown to exponential phase in synthetic medium lacking either leucine (SD-Leu) or uracil (SD-Ura) and diluted in sterile water to an OD_{600} of 0.5. Cells were then diluted in water in 10-fold increments, and 3 µl of each dilution was spotted onto the indicated solid media. Plates were incubated at 30°C and 37°C for 2-5 days.

2.2.8. Immunofluorescence microscopy

WT and *Δmim1* cells were grown to exponential phase in liquid YPD medium at 30°C. Formaldehyde was added to 10 ml of culture to final concentration of 3.7% and then the mixture was incubated at 30°C while shaking. After 1 h the cells were spin down and resuspended in spheroplasting solution (1.2 M sorbitol, 0.1 M K-phosphate buffer pH 7.4, 0.5 mM $MgCl_2$, 2 µl mercaptoethanol/ml, 100 µg previously freeze dried and aliquoted zymolyase 100T/ml). After 15-60 min (until spheroplasting was sufficient) cells were centrifuged at 3,000 rpm and pellets were gently washed with spheroplasting premix (1.2 M sorbitol, 0.1 M K-phosphate buffer pH 7.4, 0.5 mM $MgCl_2$), resuspended in 300 µl of the same solution and frozen at -80°C.

A slide containing 15 wells was coated with 0.02% polylysine (400K, Sigma) by adding 5 µl of solution to each well and then washing off with distilled water. After wells were dried, drops of desired cell solutions were added and after 5 min they were aspirated and wells were washed with BSA-PBS-NaAzid solution (1% BSA, 0.04 M K_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl, 0.015 M NaN_3). In the next step the primary antibodies were added (diluted

1: 500 in BSA-PBS-NaAzid solution) and incubated for 2 h in a moist and dark place at RT. After the primary antibodies were washed off (3x with BSA-PBS-NaAzid-Triton-X100 solution) the secondary antibodies (fluorescein- isothiocyanate conjugates, Sigma) in a dilution 1:1:50 were added and incubated for 1 h at moist and dark place. After washing step (as described for primary antibodies), DAPI (10 µg/ml in PBS) was shortly added to the wells (DAPI enables visualization of DNA) and then washed off. Slide was mounted with 80% glycerol and coverslip sealed with nail polish.

The samples were analyzed by Olympus Bx-60 microscope with camera Hamamatsus Photonics.

2.3. Biochemical methods

2.3.1. Pull-down experiments

For pull-down assays, isolated mitochondria were centrifuged (36,600 x g, 10 min, 4°C) and the mitochondrial pellet was solubilized in lysis buffer (20 mM Tris-HCl, 20 mM KCl, 1 mM PMSF, 10 mM imidazole, 1% digitonin, pH 8). After a clarifying spin (20 min, 125,000 x g, 4°C), the supernatants were incubated with Ni-NTA beads. The beads (30 µl) were previously washed with 3 x 1 ml TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.5) and in the final washing step with 400 µl solubilization buffer containing 0.05% instead of 1% digitonin. Mitochondrial extract was incubated with the beads for 1 h at 4°C. Then the beads were washed three times with 400 µl solubilization buffer containing 0.05% digitonin. Bound proteins were eluted with sample buffer containing 300 mM imidazole. Samples were incubated at 95°C for 5 min, and then analyzed by SDS-PAGE and immunodecoration.

2.3.2. Chemical crosslinking experiments

For chemical crosslinking experiments, mitochondria were resuspended in import buffer (250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS-KOH, 2 mM NADH, 2 mM ATP, pH 7.2.) with addition of 2 mM NADH, 1 mM

ATP, 10 mM creatine phosphate and 100 µg/ml creatine kinase and then incubated with the chemical crosslinkers disuccinimidyl glutarate (DSG), disuccinimidyl suberate (DSS), or 1,5-Difluoro-2,4-dinitrobenzene (DFDNB) on ice. The crosslinking reagents were added from 100-fold stock solution in DMSO. After 30 min of incubation glycine (0.1 M, pH 8.8) was added to quench excess of crosslinker and mitochondria were reisolated and analysed by SDS-PAGE and immunodecoration.

2.3.3. *In vitro* synthesis of radioactive labeled proteins

For *in vitro* synthesis of ^{35}S labeled proteins, the constructs cloned into pGEM4 (Promega) plasmids first had to be transcribed into mRNA using SP6-RNA-polymerase (Melton et al., 1984; Sambrook et al*.,* 1989). Transcription mixture (100 µl) contained: 20 µl 5 x transcription buffer (200 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM spermidine, pH 7.5), 10 µl 0.1 M DTT, 4 µl RNasin (40 U/µl), 20 µl 2.5 mM rNTP, 5.2 µl 2.5 mM m7G(5')ppp(5')G, 3 µl of SP6-Polymerase (25 U/ml) and 10-20 µg DNA. The mixture was incubated at 37°C for 1 h. The RNA was precipitated by adding 10 µl of 10 M LiCl and 300 µl of absolute ethanol, centrifuged, and subsequently washed with 70% ethanol. RNA pellets were dried at room temperature and then were resuspended in sterile water supplemented with 1 µl RNasin (40 U/µl), aliquoted and kept at – 80°C till use.

For *in vitro* protein translation rabbit reticulocyte lysate was used. The mix containing 25 µl RNA, 3.5 µl amino acid mix (without methionine), 7 µl 15 mM Mg-acetate, 12 µl ^{35}S (10 mCi/ml) and 100 µl rabbit reticulocyte lysate (Promega) was incubated at 30ºC for 1 h. At the end of the translation reaction 5 mM of cold methionine and 250 mM sucrose were added. The probe was then centrifuged (90,700 x g, 45 min, 2ºC) to pellet down ribosomes, and 30 µl aliquots of the supernatant were frozen at –80ºC.

2.3.4. Import of radiolabeled preproteins into mitochondria

Mitochondria were resuspended at 0.5 mg/ml in F5 import buffer containing 0.03-3% (w/v) fatty acid-free BSA, 250 mM sucrose, 80 mM KCl, 5 mM

$MgCl_2$, 10 mM MOPS-KOH, 2 mM NADH, 2 mM ATP, pH 7.2. Upon addition of lysate (1-3% (v/v)) import reactions were incubated for various time periods at different temperatures (15°C-25°C). Import was stopped by adding ice cold SEM buffer (1:10 dilution) with or without proteinase K (100-400 µg/ml). Protease treatment was stopped after 15 min of incubation on ice by addition of 2 mM PMSF. After centrifugation step (36,600 x g, 12 min, 4°C), mitochondrial pellets were resuspended in 30 µl 2 x sample buffer, shaken for 5 min at 95°C and then analyzed by SDS-PAGE and autoradiography.

2.3.5 Purification of recombinant proteins expressed in *E. coli*

Purification of recombinant maltose binding protein (MBP, MW = 42 kDa) fused to Mim1 (MBP-Mim1) from *E. coli* was performed as described before (Guan et al., 1987). The MH1 *E. coli* colony containing the MBP-Mim1 fusion protein cloned into pMalcRI vector was inoculated in up to 50 ml of liquid LB medium supplemented with ampicillin and incubated overnight at 37°C with moderate shaking. The next morning, 5 ml of the overnight culture was diluted into 500 ml of the same medium. The culture was further shaken until it reached an OD_{600} of 0.5. At this stage, 1 ml of the culture was taken for analysis of uninduced cells. The cells were pelleted (10,000 x g, 15 sec, RT) and resuspended in 100 µl of sample buffer to a concentration of 1 OD unit/ml. The rest of the cells were induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Bacteria were grown further for 2-3 hours, OD_{600} was measured again and 1 ml was taken for analysis of the induced cells. Further treatment was as described above.

The rest of the bacterial cells were harvested by centrifugation (3,000 x g, 10 min, 4ºC), washed with H_2O, and resuspended in 15 ml of column buffer (200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 20 mM HEPES-NaOH, pH 7.4). To degrade the cell walls lysozyme was added to the bacterial suspension to a final concentration of 1 mg/ml and then the mixture was incubated at 0ºC for 30 min, while rolling. The obtained spheroplasts were sonicated on ice, 10 times for 12 sec, with 48

sec breaks in between, utilizing Branson sonicator 450 (settings: *timer*: hold; *output control*: 4; *duty cycle*: 80 %).

A column was packed with 5-10 ml of amylose resin (New England Biolabs) depending on the expression levels of the protein, washed with several column volumes (CV) of water, and then with 7 CV of column buffer. The sonicated suspension was centrifuged (39,000 x g, 25 min, 4ºC) and the supernatant was applied onto the equilibrated amylose column with a flow rate of 1 ml/min. Flow-through was collected, column washed with 10 CV of column buffer and the bound proteins eluted with 2 CV of elution buffer (10 mM maltose in column buffer). Fractions of 1 ml were collected and protein concentration was determined in all the fractions before freezing at –80ºC.

2.3.6. Determination of protein concentration.

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

2.3.7. Protein precipitation with trichloroacetic acid (TCA)

Proteins from aqueous solutions were precipitated by adding 72% TCA to a final concentration of 12% (w/v). The samples were incubated for 20-30 min on ice or at -20ºC, and then centrifuged (36,700 x g, 20 min, 2ºC). The precipitated proteins were washed with cold acetone (–20ºC), and re-centrifuged (36,700 x g, 10 min, 2ºC). Protein pellet was dried for 5-10 min at RT and dissolved in 2 x sample buffer.

2.3.8. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated under denaturing conditions according to their molecular weights via one-dimensional vertical slab SDS-Polyacrylamide gel

electrophoresis (SDS-PAGE) (Laemmli, 1970). The concentrations of acrylamide and bis-acrylamide in the separating gel were chosen according to the molecular sizes of proteins to be separated. The amount of the loaded protein was between 10 and 100 µg per lane. The samples were resuspended in 20-30 µl 1 x sample buffer and incubated at 95ºC for 5 min before loading.

The electrophoresis was performed at 30-35 mA for 90-120 min for the gels of dimensions of approximately 14 cm x 9 cm x 0.1 cm. Protein molecular mass markers of 116, 66, 45, 35, 25, 18, and 14 kDa (Peqlab) were usually used.

Buffers for SDS-PAGE:

Bottom gel: 2% (w/v) agar in running buffer

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.

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

1 x sample (Laemmli) buffer: 60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromphenol-blue.

2.3.9. Blue-Native gel electrophoresis (BNGE)

Blue-Native gel electrophoresis (BNGE) was used for separation of proteins under native (non-denaturing) conditions (Schägger et al., 1994). For this purpose, 50-100 µg of pelleted mitochondria were resuspended in 30 µl of buffer N (20 mM Tris-HCl, 80 mM KCl, pH 8.0) containing 1-1.5% digitonin and 1 mM PMSF. The solubilization was performed at 4ºC for 15 min. After a clarifying spin (36,670 x g, 10 min, 4°C), 5 µl of sample buffer (5% (w/v) Coomassie-Brilliant-blue G-250, 100 mM bis-Tris, 500 mM 6-amino-n-capronic acid, pH 7.0) were added to the soluble fraction and loaded onto a 6-13% gel. The electrophoresis was performed at 4ºC. For the first step of

electrophoresis, cathode buffer containing Coomassie-Brilliant-blue and anode buffer were used. The voltage was set to 100 V and after 1 h shifted to 500 V. When the blue front had migrated to about half of the separation distance, the cathode buffer was replaced by a cathode buffer without Coomassie-Brilliant-blue. Marker proteins like apoferritin (440 kDa), alcohol dehydrogenase (ADH) (monomer: 50 kDa, dimer: 100 kDa, trimer: 150 kDa), and bovine serum albumin (BSA) (monomer: 66 kDa, dimer: 136 kDa) were used.

Buffers for BN-PAGE:

Bottom gel: 20% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 0.5 M 6-amino-n-capronic acid, 50 mM bis-Tris-HCl, pH 7.0, 0.05% (w/v) APS, 0.025% (v/v) TEMED.

Stacking gel: 3.8% (w/v) acrylamide, 0.12% (w/v) bis-acrylamide, 0.5 M 6-amino-n-capronic acid, 50 mM bis-Tris-HCl, pH 7.0, 0.08% (w/v) APS, 0.08% (v/v) TEMED.

Running gel: 0.15-0.3% (w/v) bis-acrylamide, 5% (v/v) glycerol, 0.5 M 6-amino-n-capronic acid, 50 mM bis-Tris-HCl, pH 7.0, 0.04% (w/v) APS, 0.04% (v/v) TEMED.

Cathode buffer: 15 mM bis-Tris, pH 7.0, 50 mM Tricine, with or without 0.02% Coomassie-Brilliant-blue G250

Anode buffer: 50 mM bis-Tris, pH 7.0

2.3.10. Transfer of proteins onto nitrocellulose or PVDF membrane (Western-blot)

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes and the ones separated by BNGE were transferred onto PVDF membranes. Modified semi-dry method was used (Kyhse-Andersen, 1984). The membrane, six sheets of Whatman filter paper (3mm) and the gel were incubated in transfer buffer (20 mM Tris, 150 mM glycine, 20% (v/v) methanol, 0.08% (w/v) SDS) for few minutes prior to the transfer procedure. The membrane was placed onto three sheets of Whatman filter paper lying on the graphite anode electrode. The gel was placed on the membrane and

then covered with another three filter papers and the cathode electrode. The electro transfer was performed at 250 mA for 1 h. After protein transfer the nitrocellulose membranes were stained with Ponceau S solution (0.2% (w/v) Ponceau S in 3% (w/v) TCA) to verify transfer efficiency and visualize the marker proteins' bands. The membranes were then immunodecorated, or the radioactive material visualized by autoradiography.

PVDF membrane had to be activated before blotting by short incubation (couple of min) in methanol. It was then shortly washed in water, and 5 min (or longer) in transfer buffer. Prior to blotting, separating part of the blue native gel was equilibrated by shaking for 2-5 minutes in transfer buffer. Electro transfer onto PVDF membranes was performed at 220 mA for 1 h at 4ºC. Before immunodecoration, the PVDF membrane were destained with methanol and then washed in TBS buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5).

2.3.11. Autoradiography and quantification

Radiolabeled proteins were detected by autoradiography. Dry nitrocellulose membranes were exposed to X-ray films (Kodak Bio Max MM) for different time periods depending on signal intensities (few hours to few weeks). The films were developed in a developing machine (Gevamatic 60, AGFAGevaert), then scanned and the intensity of bands of interest were quantified by densitometry using Image Master 1D Elite software (Amersham).

2.4. Immunological methods

2.4.1. Immunodecoration

Proteins immobilized on nitrocellulose or PVDF membranes were visualized by immunodecoration with specific antibodies. Subsequently to blotting, membranes were first incubated for 45 min in 5% (w/v) milk powder in TBS to block all non-specific binding sites. The immunodecoration was done for 1 h at RT or over night at 4°C, with specific primary antibody (1:200 to

1:10.000 dilutions in 5% milk in TBS). The membrane was then washed with TBS (5 min), TBS/0.05% (w/v) Triton X-100 (10 min) and again with TBS (5 min), and subsequently incubated with goat anti-rabbit antibodies coupled to horseradish peroxidase (diluted 1:10.000 in 5% milk in TBS) for 1 h at room temperature. The membrane was again washed (as above) and treated with the chemiluminescent substrate of peroxidase: luminol (2.5 mM 3-aminophtalhydrazide and 0.4 mM p-coumaric acid in 0.1 M Tris-HCl, pH 8.5), mixed with equal volume of H_2O_2 (0.018% (v/v) in 0.1 M Tris-HCl, pH 8.5). The luminescence reactions were detected with Roentgen films (FujiNewRX).

For detection of HA-tagged Mim1 and His-tagged Mim1 commercially available anti-HA antibody or anti-His antibody (Santa Cruz, Roche) were used. Blots were blocked in the supplied blocking solution and secondary antibody (goat anti-mouse) was diluted 1:5000 in the blocking reagent in TBS. Further treatment was performed as described above.

2.4.2. Co-immunoprecipitation

The desired amount of Protein A Sepharose CL-4B (PAS) (Amersham Biosciences) beads slurry was washed with TBS (3 x 5 min x 1 ml) and then the appropriate amounts of purified antibodies or antisera were added (enough antibodies to immunodeplete the corresponding antigen from the extract) and incubated for 1.5 h at 4ºC, while rotating the cups overhead. Beads were then washed from the unbound antibodies (3 x 5 min x 1 ml TBS). Isolated mitochondria were centrifuged (17,400 x g, 10 min, 4°C) and the mitochondrial pellet was resuspended at concentration of 2 mg/ml in solubilization buffer (20 mM Tris, 80 mM NaCl, pH 8.0 containing 10% (v/v) glycerol, 1% (w/v) digitonin and 1 mM PMSF) for 30 min at 4°C. After a clarifying spin (90,700 x g, 20 min, 2ºC), mitochondrial extract was added to antibodies prebound to PAS and the mixture was incubated overhead for 1.5-2 h at 4°C. Beads were washed twice with 20 mM Tris, 80 mM NaCl, pH 8.0 containing 10% (v/v) glycerol, 0.05% (w/v) digitonin and 1 mM PMSF. Bound proteins were eluted with sample buffer without β-mercaptoethanol

and incubated for 5 min at 37°C. Samples were analyzed by SDS-PAGE and immunodecoration.

2.4.3. Affinity purification of antibodies against Mim1

Affinity purification of Mim1 antibodies was performed in order to reduce the background of the antisera. The affinity column was made by coupling the purified MBP-Mim1 protein which served as an antigen to a CNBr-activated Sepharose 4B (Amersham). Prior to coupling the MBP-elution buffer was exchanged with a bicarbonate one on a PD-10 column in order to remove all substances which contain amino groups from the solution. The PD-10 column was equilibrated with 30 ml 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 and 2.5 ml of solution containing 5 mg protein was loaded on the column by the gravity flow. First 2.5 ml of the eluate was discarded and the protein was collected from the column in the following 3.5 ml. CNBr-Sepharose was prepared by placing 0.3 g of the beads in 5 ml 1 mM HCl, pH 2.8. The suspension was left 1h to swell and then was mixed again and poured onto a sintered glass filter connected to a vacuum-pump and then was washed with 100 ml of 1 mM HCl. The matrix was transferred into a disposable 10 ml plastic column (Biorad) and, while taking care not to get dry, drained from HCl. The column outlet was then closed with the supplied cap. MBP-fusion protein in carbonate buffer was added, the column closed with parafilm and incubated for 1 h at RT, while rolling. It was then positioned into a vertical position; buffer was allowed to pass through and than analyzed for protein content with the Bradford method to check the efficiency of coupling. Remaining active groups were blocked by loading 6 ml of 0.1 M ethanolamine, pH 8.0. When 4 ml of ethanolamine buffer has passed through, the outlet was closed and the column left for 2 h at RT. Subsequently, ethanolamine was allowed to pass through and all nonspecifically bound proteins were removed by 3 washing cycles of alternating pH. Each cycle consisted of 6 ml 0.1 M Na-acetate, 0.5 M NaCl, pH 4.5 followed by 6 ml 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0. Finally, the column was washed with 10 ml of 10 mM Tris-HCl, pH 7.5, and it was ready for affinity purification of antibodies. If the antibodies were not purified the

same day, 3 ml of 0.05% NaN_3 solution was added to the column before it was stored at 4°C.

The next day, the column was left at RT for 1 h and then equilibrated with 10 ml of 10 mM Tris-HCl, pH 7.5. Antiserum (18 ml) were diluted up to 90 ml with 10 mM Tris-HCl, pH 7.5 and loaded on the corresponding affinity column under gravity flow. The column was washed with 10 ml 10 mM Tris-HCl, pH 7.5 followed by 10 ml 10 mM Tris-HCl, 0.5 M NaCl, and pH 7.5. For the elution, column was subjected to alternating pH through application of the following buffers (10 ml of each) in the given order: 10 mM Na-citrate, pH 4.0, 100 mM glycine-HCl, pH 2.5 and 100 mM Na_2HPO_4, 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 one. 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. It was then washed with 10 mM Tris-HCl, pH 7.5, and left in 0.05% NaN3 at 4ºC. The collected fractions were checked for specificity by immunodecoration on nitrocellulose membrane carrying yeast mitochondrial proteins. The specific Mim1 antibodies were eluted with the glycine buffer in fractions 2-4. These fractions were aliquoted and stored at -20°C.

3. RESULTS

Mim1 is an outer membrane protein which was recently discovered in a high-throughput screen (Mnaimneh et al., 2004). Depletion or deletion of this protein results in a growth phenotype, accumulation of non-imported mitochondrial precursor proteins and in changes in mitochondrial morphology (Altmann and Westermann, 2005). Mim1 is an additional factor for the assembly of the TOM complex. In the absence of Mim1, the TOM complex is not fully assembled and Tom40 accumulates as a low molecular mass species. Mim1 is not a component of any known complex in the outer membrane (the TOM or the TOB complex), rather it appears to be a subunit of another, yet unidentified, complex (Ishikawa et al., 2004; Waizenegger et al., 2005). The aim of this work was to obtain more insight into the function of Mim1, its oligomeric state, and the contribution of its domains to the overall function.

3.1. Antibodies against Mim1

To study the function of Mim1, it was necessary to raise antibodies against this protein. The DNA sequence encoding Mim1 was cloned into pMAL vector. Recombinant MBP-Mim1 protein was expressed in MH1 *E. coli* cells and then purified by using an amylose column. Rabbits were injected with recombinant MBP-Mim1 fusion protein and serum was obtained. Antibodies were affinity-purified according to an established protocol (see 2.2.). The best signal was obtained with antibodies which were purified and eluted with glycine buffer at pH=2.2. Two bands were detected with these purified antibodies. The first at an approximate molecular mass of 15 kDa belongs to Mim1 (predicted mass of Mim1 is 12.8 kDa), whereas the second one which migrated at approximately 7 kDa is a degradation product of Mim1 (Fig. 3.1).

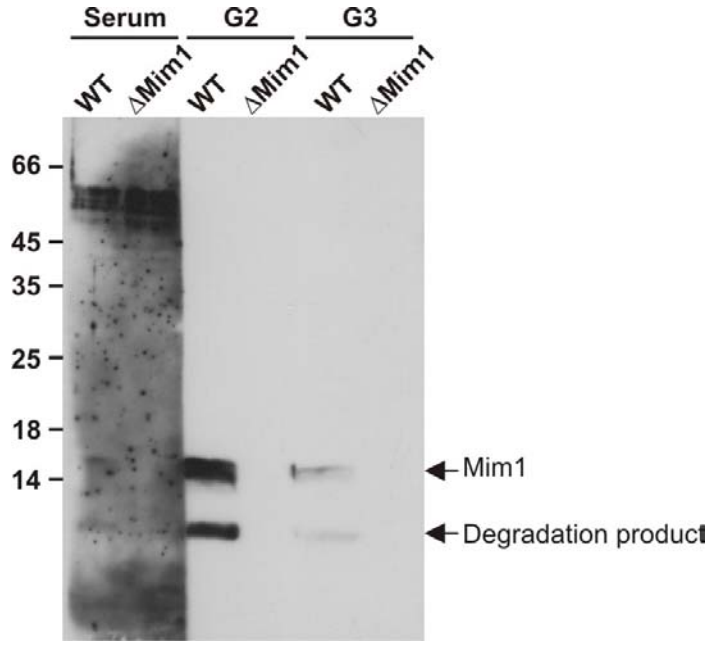


Figure 3.1. Purification of antibodies against Mim1. WT and ΔMim1 mitochondria were loaded on the SDS gel to test different fractions of antibody purification. G2 and G3 – fractions eluted with glycine buffer pH 2.2.

3.2. The transmembrane domain of Mim1 is highly conserved among fungi

Sequence homology analysis has shown that the transmembrane domain (TM) of Mim1 is highly conserved among fungi, whereas the N- and the C-terminal domains are variable (Fig. 3.2).

```
S.c.   ............MTEVVGFWESVSDDESEDKDCMEVQNTVSADESPLVQ
N.c.   MSAEEISNPLAESGVTISSDSEQYSAPESASPQSPSSS..SPAVVLYQPP
S.p.   ..............................MEKN..TVTVPKTLFS

S.c.   SLVSFVGSCSINLLLPFLNGMMLGFGELFAHELCWRFNWFNHRNKGYKVY
N.c.   TVWSLFRSAVINLFLPFVNGMMLGFGELFAHEAARRLGWSNT.....KVF
S.p.   QVIHIFKYAAINLGLPFLNGVMLGFGEIFAHAFIHSLGWAPG.....HTR

S.c.   PESR....KIAALKEISSPGTRGRVASKFL.......
N.c.   PVSRRDARPIGPGVEVVERPRRRVDLDDHLDELTSLE
S.p.   IYSIQRHQYIQA.........................
```

Figure 3.2. Mim1 is highly conserved among fungi. Amino acid sequences of *S. cerevisiae (S.c.), N. crassa (N.c.)* and *S. pombe* (*S.p.*) are presented. Identical residues are in bold letters. The putative transmembrane segment is boxed.

The conservation of the transmembrane (TM) domain suggested the possibility that it is important for the function of Mim1. One of the tasks of this study was to investigate whether the TM segment is the functional domain of Mim1.

3.2.1. Mim1 from *N. crassa* and *S. pombe* can complement the deletion of Mim1 in *S. cerevisiae*.

To analyze whether the conservation levels of different domains of Mim1 reflect their importance for the function of the protein, complementation assays were performed. It was tested whether Mim1 variants from *S. pombe* and *N. crassa* can rescue the deletion phenotype in S. cerevisiae. The cDNAs encoding either *N. crassa* Mim1 or *S. pombe* Mim1 were introduced into yeast expression vectors and these plasmids were transformed in the S. cerevisiae strain lacking Mim1. As a control, a plasmid encoding S. cerevisiae Mim1 was also transformed into ∆mim1 cells. The expression of these proteins was under the control of either the endogenous *S. cerevisiae MIM1* promoter (in pRS315 vector) or the strong *TPI* promoter (in pRS426 vector). The capacity of these proteins to complement the deletion phenotype of Mim1 was analysed by drop dilution assay. The deletion strain, ∆mim1, carrying an empty plasmid served as a negative growth control.

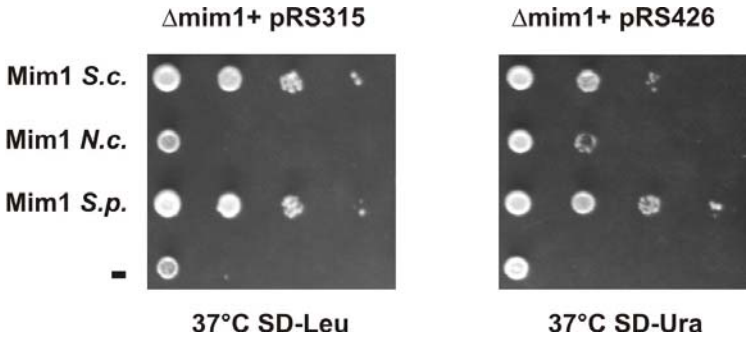


Figure 3.3. Mim1 from *S. pombe* and *N. crassa* can complement the deletion of Mim1 in S. cerevisiae. Cells carrying plasmid-encoded Mim1 variants were tested by dilution in 10-fold increments for their ability to grow at 37°C on glucose-containing medium. The Mim1 variants were expressed under the control of *S. cerevisiae MIM1* promoter (pRS315) or overexpressed (pRS426).

Mim1 from *S. pombe* fully complemented the deletion of Mim1 under all conditions tested, whereas *N. crassa* Mim1 complemented rather partially and only if it was overexpressed (Fig. 3.3). The partial complementation of *N. crassa* Mim1 was not due to lower expression levels since expression was confirmed by western blotting (data not shown).

3.2.2. The N- and the C-terminal domains of Mim1 are not crucial for its function.

To investigate in more detail the importance of different domains of Mim1 three different truncated Mim1 variants where constructed. In the first two truncated versions amino acid residues 1-34 (Mim1ΔN) or 76-113 (Mim1ΔC) were deleted. The third variant was constructed by deleting both the N- and the C- terminal domains, leaving only the TM segment of the protein (Mim1(TM)) (Fig. 3.4A).

These truncated versions of Mim1 were tested by drop dilution assay for their capacity to complement the deletion of native Mim1. Under all tested conditions, Mim1 lacking N- or C- terminus did not show any growth phenotype suggesting that Mim1 lacking either of these domains alone was fully functional. The complementation of function by these truncated versions of Mim1 suggests that they were properly inserted into the outer membrane. The Mim1 variant containing only the TM domain could not complement the function when expressed under the endogenous promoter but could do so when overexpressed (Fig. 3.4B).

Taken together it seems that neither the N- nor the C-terminal domains of Mim1 are crucial for the function of the protein and for its targeting to and insertion into the mitochondrial outer membrane.

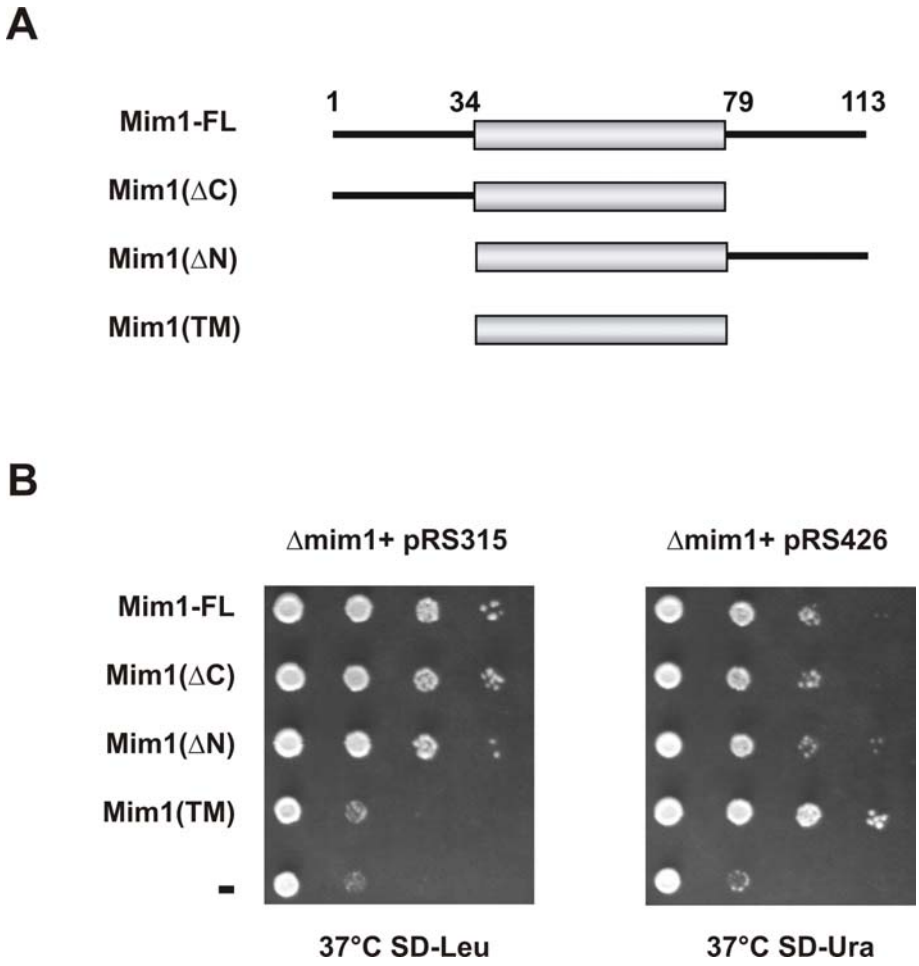


Figure 3.4. The N- and the C-terminal domains of Mim1 are not crucial for its function. A) Schematic representation of Mim1 variants. The putative TMS is represented by a box. **B)** Cells harbouring plasmid-encoded Mim1 variants were tested by dilution in 10-fold increments for their ability to grow at 37°C on glucose-containing medium. The Mim1 variants were expressed under the control of *S. cerevisiae MIM1* promoter (pRS315) or overexpressed (pRS426).

3.2.3. The truncated versions of Mim1 can support assembly of the TOM complex.

To investigate whether the truncated versions of Mim1 can support the assembly of the TOM complex, mitochondria from the wild type (wt) strain or strains carrying the truncated variants of Mim1 were isolated and analysed by BN-PAGE (Fig. 3.5A).

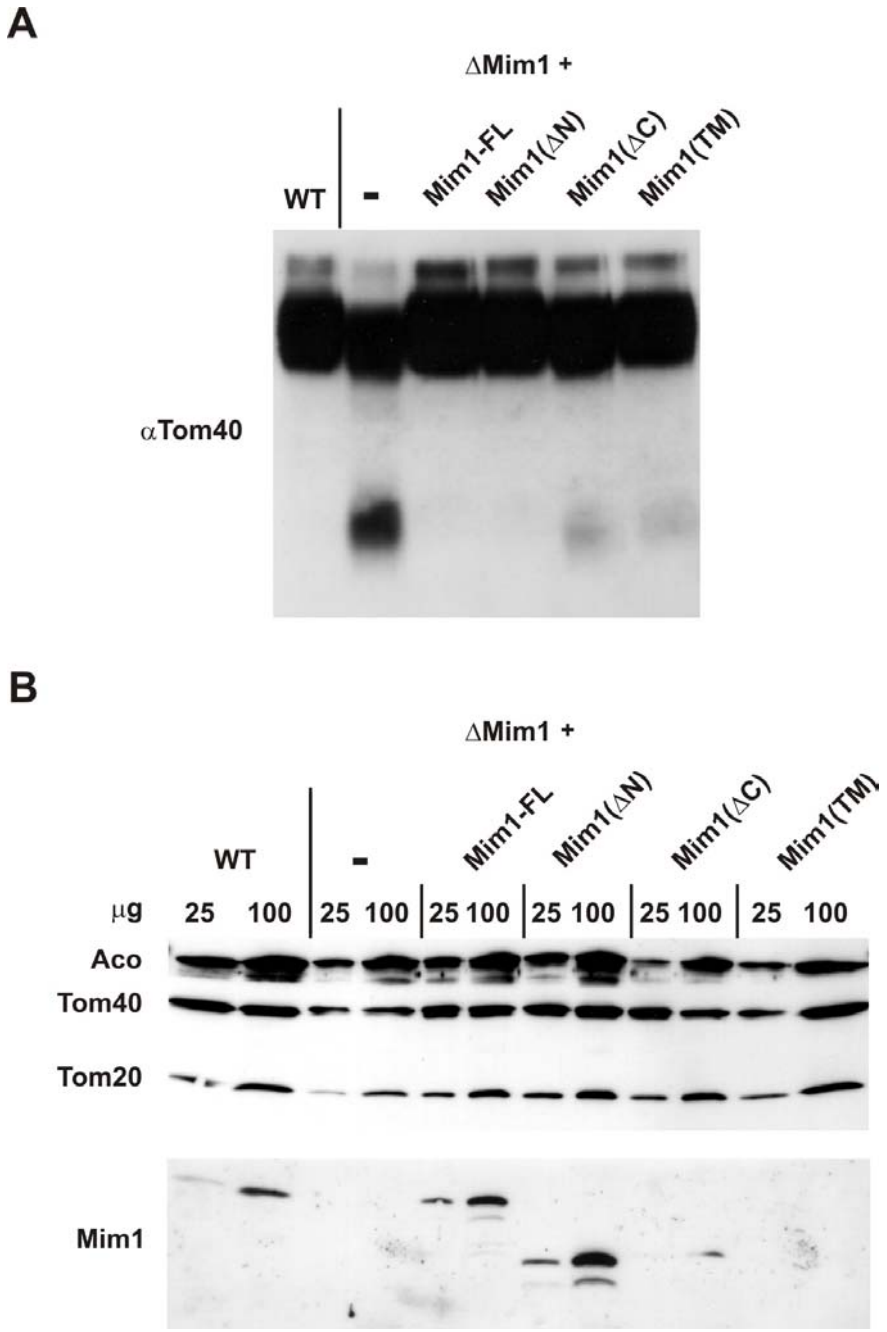


Figure 3.5. Truncated versions of Mim1 support assembly of the TOM complex. A) Mitochondria isolated from either wild type cells or from Δ*mim1* cells expressing the indicated plasmid-encoded Mim1 variants were lysed with 1% digitonin and subjected to BNGE and immunoblotting with antibodies against Tom40. **B)** Mitochondria were isolated from either wild type cells or from Δ*mim1* cells expressing empty plasmid (-) or the indicated plasmid-encoded Mim1 variants. The indicated amounts of mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies against various mitochondrial proteins. Aco, aconitase.

In mitochondria lacking Mim1, Tom40 species of lower molecular weight were observed as published previously (Waizenegger et al., 2005). In contrast, upon overexpression of the Mim1 truncated variants unassembled species of Tom40 were observed at very low levels or not at all, suggesting that expression of shorter Mim1 forms is sufficient for the correct assembly of the TOM complex. This result was in agreement with the fact that shorter forms of Mim1 could support growth. Similarly, the steady state levels of Tom20 and Tom40 in mitochondria harbouring the truncated version were similar to those in wild type mitochondria (Fig. 3.5B).

Although antibodies were raised against full-length Mim1, most of the epitopes reside in the C-terminal domain of Mim1 (data not shown). The transmembrane domain of Mim1 is very hydrophobic and probably does not contain any epitopes. Thus, a detection of Mim1(ΔC) and Mim1(TM) in this study was very limited.

3.3. Mim1 forms dimers or homooligomers in the mitochondrial outer membrane.

It was already shown by BN-PAGE and gel-filtration, that Mim1 is neither a subunit of the TOM complex nor of the TOB complex but rather of new complex with an approximate molecular mass of 200 kDa (Waizenegger et al., 2005). To study the composition of this complex a chemical crosslinking approach was applied. Intact mitochondria were treated with the crosslinking reagents disuccinimidyl glutarate (DSG), disuccinimidyl suberate (DSS), or 1,5-difluoro-2,4-dinitrobenzene (DFDNB). After the treatment with crosslinkers mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies against Mim1. One major crosslinking adduct band of apparent molecular mass of 28 kDa was detected after the treatment of mitochondria with all tested crosslinkers (Fig. 3.6A). The mass of 28 kDa suggested that this band corresponds to a homodimer of Mim1 since the predicted mass of Mim1 is 12.8 kDa. Another detected band of approximate mass of 40 kDa might reflect a trimer of Mim1.

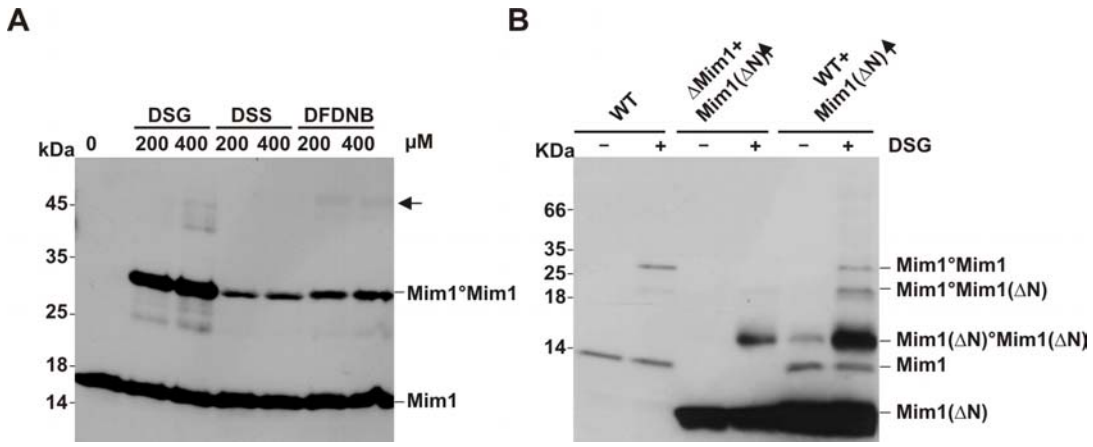


Figure 3.6. Mim1 forms dimers in the mitochondrial outer membrane. A) The indicated crosslinking reagents were added to intact mitochondria for 30 min at 0°C before the crosslinkers were quenched with an excess of glycine. Proteins were analysed by SDS-PAGE and immunostaining with antibodies against Mim1. The bands corresponding to Mim1 dimer (Mim1°Mim1) and putative Mim1 trimer (arrowhead) are indicated. **B)** Mitochondria were isolated from wild type cells or from wild type cells overexpressing Mim1(ΔN) or from Δ*mim1* cells over-expressing Mim1(ΔN). DSG was added to the mitochondria and proteins were analysed by SDS-PAGE and immunostaining with antibodies against Mim1. Bands corresponding to the various Mim1-containing adducts are indicated.

To confirm the hypothesis that Mim1 forms in the outer membrane dimers or even higher homooligomers, and to exclude the possibility that the 28 kDa adduct corresponds to Mim1 crosslinked to some other protein of a similar size, the crosslinking experiments were repeated with mitochondria isolated from the cells lacking the full length Mim1 and over-expressing Mim1(ΔN). Incubation of these mitochondria with the chemical crosslinker DSG resulted in a disappearance of the 28 kDa band and in appearance of a crosslinking adduct of an approximate mass of 16 kDa. Since the predicted molecular mass of Mim1(ΔN) is 8.9 kDa, the 16 kDa band corresponds probably to the homodimer Mim1(ΔN)-Mim1(ΔN). In addition, the crosslinking pattern was analyzed in a strain carrying both full-length Mim1 and Mim1(ΔN). As expected, besides homodimers of Mim1-Mim1 and Mim1(ΔN)-Mim1(ΔN), an additional band of 24 kDa was detected. This band represents a heterodimer of Mim1-Mim1(ΔN). Taken together, these crosslinking experiments further suggest that the N-terminal part of Mim1 is not required for the dimerization of the protein (Fig. 3.6B).

To confirm the homophilic interactions between two or more Mim1 molecules, pull-down experiments were performed with mitochondria from yeast strains expressing two different versions of Mim1, a heptahistidinyl-tagged version Mim1-His_7 and an HA-tagged version Mim1-HA_3. Mitochondria were also isolated from a control strain carrying only Mim1-HA_3. These cells grew as well as the wild type cells (Waizenegger et al., 2005 and data not shown), therefore the proteins are fully functional. Mitochondria were lysed and pull-down experiments with Ni-NTA beads were performed. Mim1-HA_3 was co-purified with the His tagged version of Mim1 while such interaction was not observed for two other outer membrane proteins, Tom40 and Tom70. To exclude the possibility that Mim1-HA_3 could interact with Ni-NTA beads in the absence of Mim1-His_7 in a non specific manner, this experiment was also performed using yeast strain expressing only Mim1-HA_3 (Fig. 3.7).

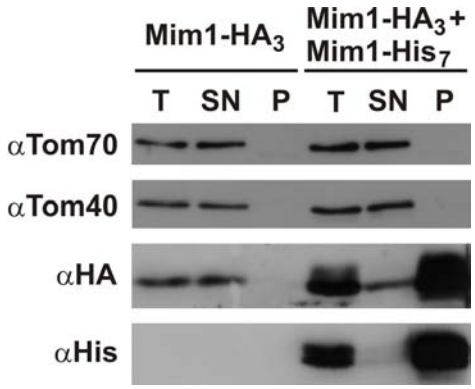


Figure 3.7. Two or more Mim1 molecules interact with each other in the outer mitochondrial membrane. Mitochondria were isolated from yeast strains expressing an HA-tagged Mim1 (Mim1-HA_3) or co-expressing Mim1-HA_3 and heptahistidinyl-tagged version of Mim1 (Mim1-His_7). The organelles were solubilized in buffer containing 1% digitonin. The total extract (T) was applied to a Ni-NTA affinity resin. Bound (pellet - P) and unbound (supernatant - SN) material were analysed by immunostaining using antibodies against Tom70, Tom20, HA and the His tags.

To summarize, results of crosslinking and pull-down experiments show that Mim1 complex in the outer mitochondrial membrane harbors two or more Mim1 molecules.

3.3.1. Two dimerization motifs in the transmembrane segment of Mim1 are crucial for homooligomerization and function.

Detailed sequence analysis revealed two GXXXG/A dimerization motifs in the TM domain of Mim1. The first motif includes residues from 57 to 61 and the second residues from 63 to 67. GXXXG and G/AXXXG/A motifs are known to mediate TM helix–helix interactions within biological membranes (Russ and Engelman, 2000; Senes et al., 2004). Considering the observation that Mim1 has the tendency to dimerize or homooligomerize, these sequences were tested for their role in the above dimerization. For that goal, mutations were introduced in these GXXXG motifs and the resulting strains were analyzed (Fig. 3.8).

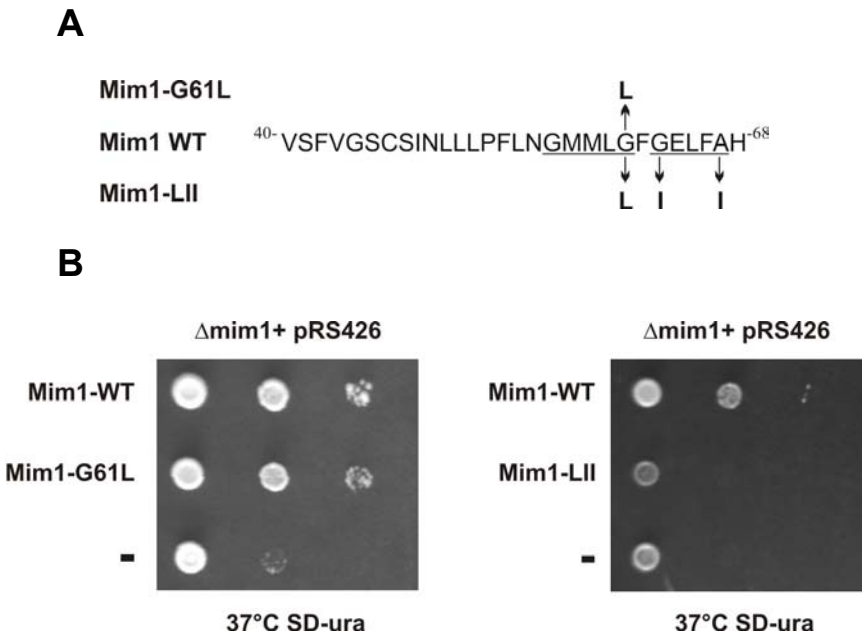


Figure 3.8. The double GXXXG/A motifs within the TMS are crucial for growth. **A)** Schematic representation of the mutations performed in the TMS of Mim1. **B)** Cells harbouring the indicated plasmid-encoded Mim1 variants or empty ("-") plasmid were tested by dilution in 10-fold increments for their ability to grow at 37°C on glucose-containing medium.

In the first mutant, only one amino acid (Gly) in position 61 was replaced by the bulky residue leucine (Mim1 G61L). This change did not show any effect on the yeast growth and did not affect the ability of Mim1 to dimerize. This can be explained by another dimerization motif sufficient to support

homooligomerization. To exclude this possibility, in addition to Mim1 G61L, two more amino acid residues Gly63 and Ala67 were replaced with Ile residues (Mim1-LII). This Mim1 mutant was not able to complement deletion of wt Mim1 under all conditions tested.

Mitochondria isolated from yeast carrying Mim1-LII instead of native Mim1 had significantly reduced steady state levels of Tom20 and moderately reduced amounts of Tom40 and porin (Fig. 3.9). The reduction levels were similar to the ones previously observed in the *Δmim1* strain. This result together with the fact that Mim1-LII could not complement the deletion phenotype of *Δmim1,* suggested that this mutatant Mim1 had an impaired function. To investigate whether this reduced function could be correlated to the altered oligomeric structure of Mim1, crosslinking experiments were performed. Mitochondria was isolated from the strain expressing Mim1-LII instead of wt Mim1 and DSG was added. The 28 kDa crosslinking adduct which corresponds to Mim1-Mim1 dimer completely disappeared suggesting that either Mim1-LII is not able to dimerize, or that two Mim1-LII molecules are positioned in a manner that they cannot be crosslinked.

To investigate whether the Mim1-LII variant was able to build oligomeric structures, mitochondria carrying this variant were analyzed by BN-PAGE. This analysis revealed that mutating the dimerization motifs resulted in the absence of the Mim1-containing complexes (Fig. 3.10). Thus, these motifs are indeed crucial for the ability of Mim1 to form oligomeric structures. In these mitochondria Tom40 lower molecular species were also observed by BN-PAGE as it was already shown for ΔMim1 mitochondria (see above). Thus, homooligomerization is essential for the function of Mim1 in the biogenesis of the TOM complex.

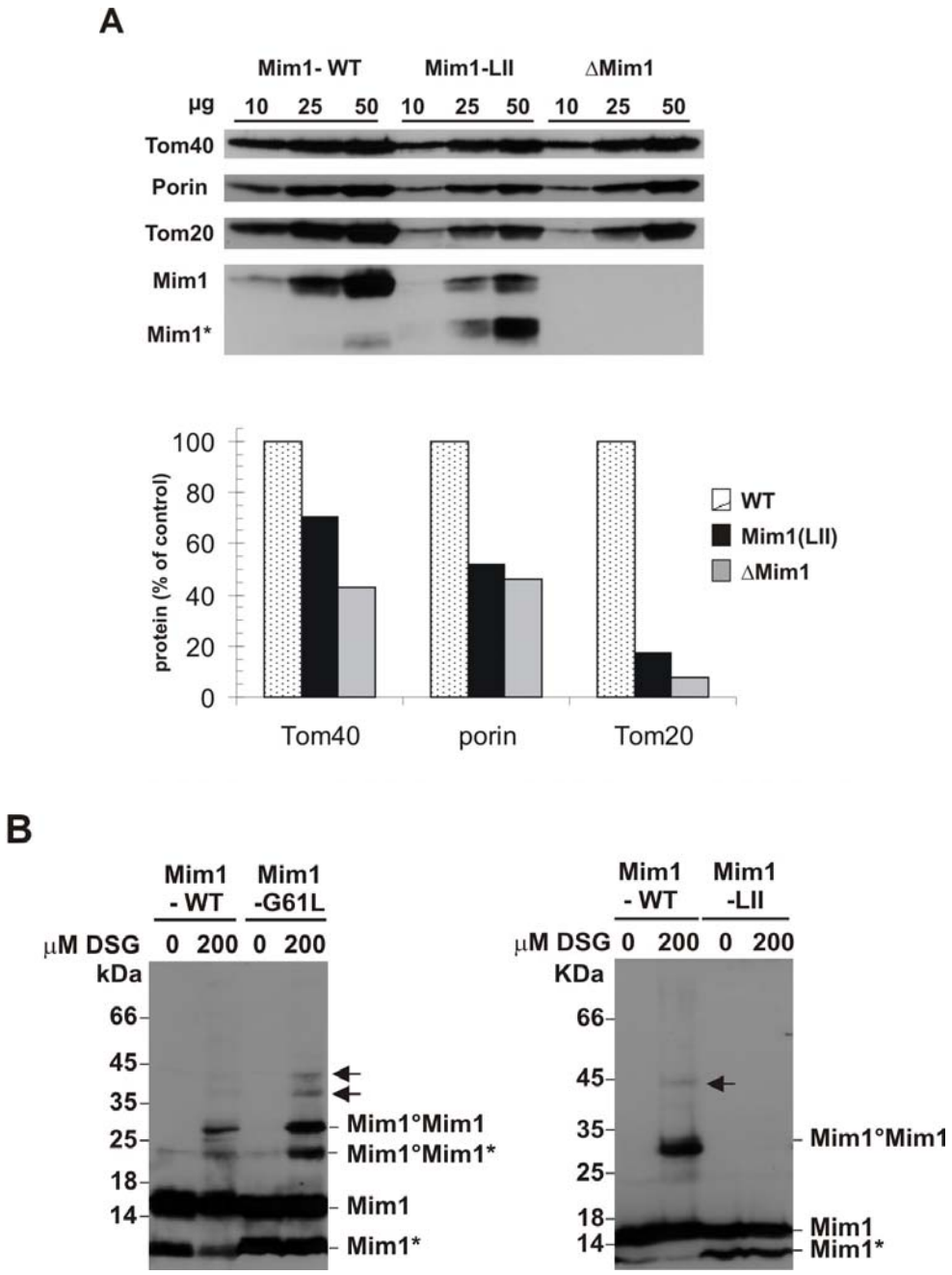


Figure 3.9. The double GXXXG/A motifs are essential for dimerization and function of Mim1. A) Mitochondria were isolated from Δ*mim1* cells carrying empty plasmid or expressing Mim1-WT or Mim1-LII. Mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins. Mim1*, proteolytic fragment of Mim1. Lower panel: the bands from the loading of 10 µg mitochondria were quantified and the amount of each protein is presented as percentage of the amount in wild type mitochondria. **B)** Mitochondria were isolated from Δ*mim1* cells overexpressing wt Mim1 or the indicated Mim1 variant. DSG was added to the mitochondria and proteins were analysed by SDS-PAGE and immunodecoration using antibodies against Mim1.

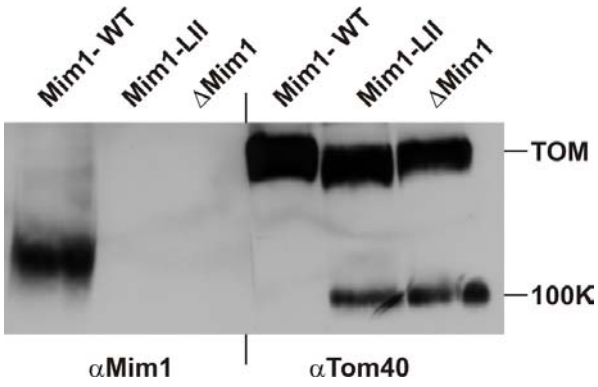


Figure 3.10. Dimerization motifs GXXXG/A are crucial for oligomerization of Mim1. Mitochondria were isolated from *Δmim1* cells carrying empty plasmid or expressing Mim1-WT or Mim1-LII and then analysed by BN-PAGE and immunodecoration.

3.3.2. Mim1 forms punctuate structures in the outer mitochondrial membrane.

To analyze the distribution of Mim1 along the mitochondrial outer membrane, the wt and ΔMim1 mitochondria were analyzed by immunofluorescence microscopy. Using affinity purified antibodies against Mim1, it was observed that Mim1 localizes to punctuate structures along the mitochondria tubuli. Very weak background signal was observed with cells lacking Mim1 (data not shown), demonstrating the specificity of antibodies (Fig. 3.11). This result, along with the pull down and crosslinking experiments showing a tendency of Mim1 to form homooligomers, suggested a possibility that Mim1 was organized in even larger homooligomeric structures in outer mitochondrial membrane.

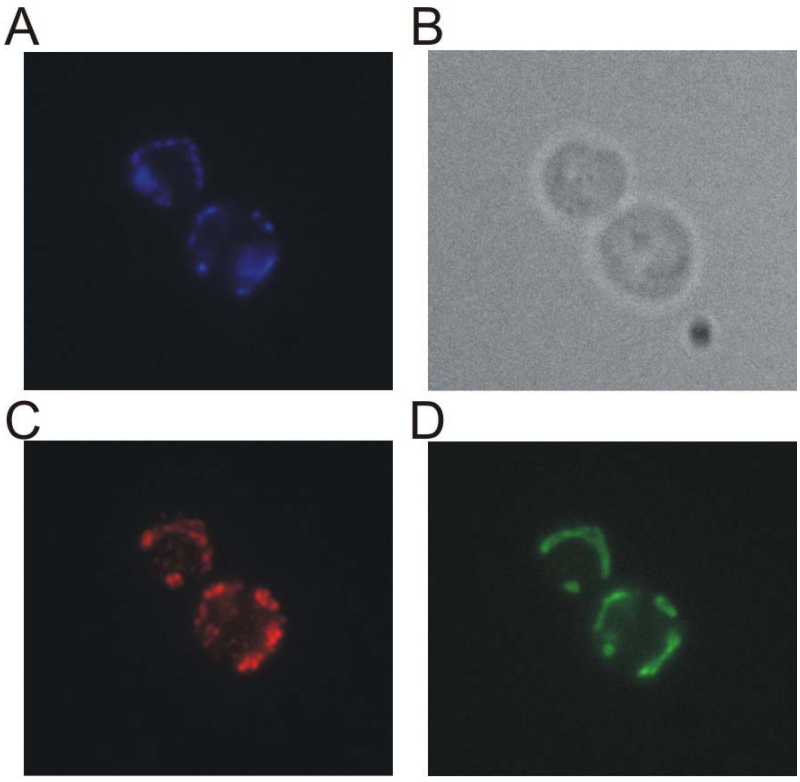


Figure 3.11. Mim1 resides in punctuate structures in mitochondria. Wild type cells containing mitochondria-targeted GFP were analyzed by fluorescence microscopy. The cells were stained by DAPI or by anti-Mim1 antibodies. Phase contrast image is also shown. **A)** DAPI **B)** Phase contrast **C)** antibodies against Mim1 **D)** mt-GFP

3.5. Deletion of Mim1 leads to a defective assembly of the TOM complex

Mim1 appeared to be crucial for the biogenesis of the TOM complex, since its depletion/deletion abrogated the assembly of the TOM complex and resulted in accumulation of Tom40 in a low molecular mass species. One of the goals of this study was to examine wheather Mim1 was involved in import and assembly of other components of the TOM complex. Since it was noticed that Tom20 steady state levels were severely reduced upon deletion of Mim1, whereas other outer membrane proteins like Tom40 and Tom70 were only moderately reduced, we investigated whether there was a difference in Tom20 levels in the strain depleted of Mim1 (Mim1↓) and the Mim1 deletion strain (*Δmim1*). The idea was to test whether some residual

amount of Mim1 in down-regulated mitochondria was sufficient for import and assembly of Tom20 into the TOM complex. Mitochondria from wt strain and Mim1-depleted/deleted mitochondria were analysed by SDS-PAGE. As shown in Figure 3.12A, Mim1 was depleted to a level almost undetectable by immunodecoration and the other mitochondrial proteins were only slightly affected or not at all. Steady state levels of Tom 20 in the strain lacking Mim1 were further reduced in comparison to Mim1↓ mitochondria.

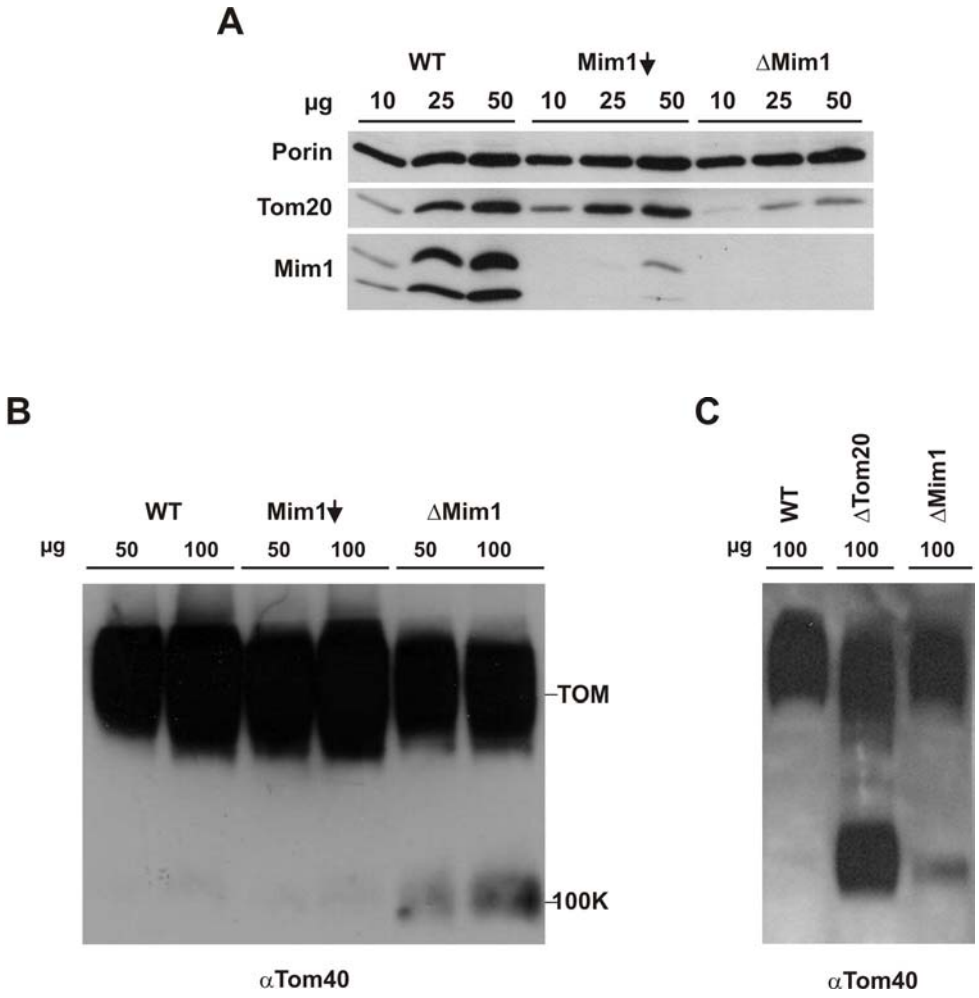


Figure 3.12. Main involvement of Mim1 in the biogenesis of the TOM complex is via its role in mediating the insertion of Tom20. A) Mitochondria were isolated from wt strain, from strain where Mim1 was depleted for 15h and from Δ*mim1.* Mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins. **B)** and **C)** Mitochondria isolated from the indicated strains were lysed with 1% digitonin and subjected to BN-PAGE and immunoblotting with antibodies against Tom40.

To analyze the assembly of the TOM complex in these strains, mitochondria were analyzed by BN-PAGE and immunodecoration using antibodies against Tom40. The difference in the assembly of the TOM complex in wt and Mim1↓ mitochondria was not significant and the TOM complex was fully assembled. In contrast, accumulation of non-assembled Tom40 species was observed in mitochondria lacking Mim1 suggesting that residual Mim1 in mitochondria depleted of Mim1 was sufficient for the full assembly of the TOM complex.

It has been shown (Model et al., 2002) that Tom20 is crucial for stable organization of Tom40 channel units into larger assemblies. Thus, BN-PAGE analysis of mitochondria lacking Tom20 was compared to ΔMim1 mitochondria. Figure 3.12.C shows that a larger portion of the Tom40 molecules migrated in a low molecular mass species in the absence of Tom20 as compared to the absence of Mim1.

It seems that the main involvement of Mim1 in the biogenesis of the TOM complex is via its role in mediating the insertion of Tom20 into the outer membrane and/or facilitating the assembly of the latter into the TOM complex.

3.6. Mim1 is required for optimal import but not assembly of Tom20

To test whether Mim1 was involved only in import of the receptor protein Tom20 or also in its assembly into the mature TOM complex, the import and co-immunoprecipitation experiments were performed. *In vitro* insertion of the Tom20 into the outer membrane of mitochondria depleted of Mim1 and lacking Mim1 was tested. To monitor the insertion of Tom20, a specific proteolytic assay was used. For this assay a fusion protein was constructed, with the first 38 amino acids of Tom70 from *N. crassa* fused in front of yeast Tom20, so that a protease protected fragment of properly inserted protein was 7-8 kDa which is easily detectable by SDS-PAGE (Ahting et al., 2005). As seen in Fig. 3.13, both the depletion and deletion of Mim1 resulted in decrease of the insertion of Tom20, but in the Mim1-deficient mitochondria, the reduction was more significant. In contrast, only minor reduction in the

import of the matrix-destined precursor, pSu9-DHFR, was observed suggesting that the reduction in the import efficiency of Tom20 did not result from a major defect in the TOM complex. These results correlate with the observation that the steady-state levels of Tom20 are highly reduced in ΔMim1 mitochondria and moderately reduced in Mim1-depleted mitochondria.

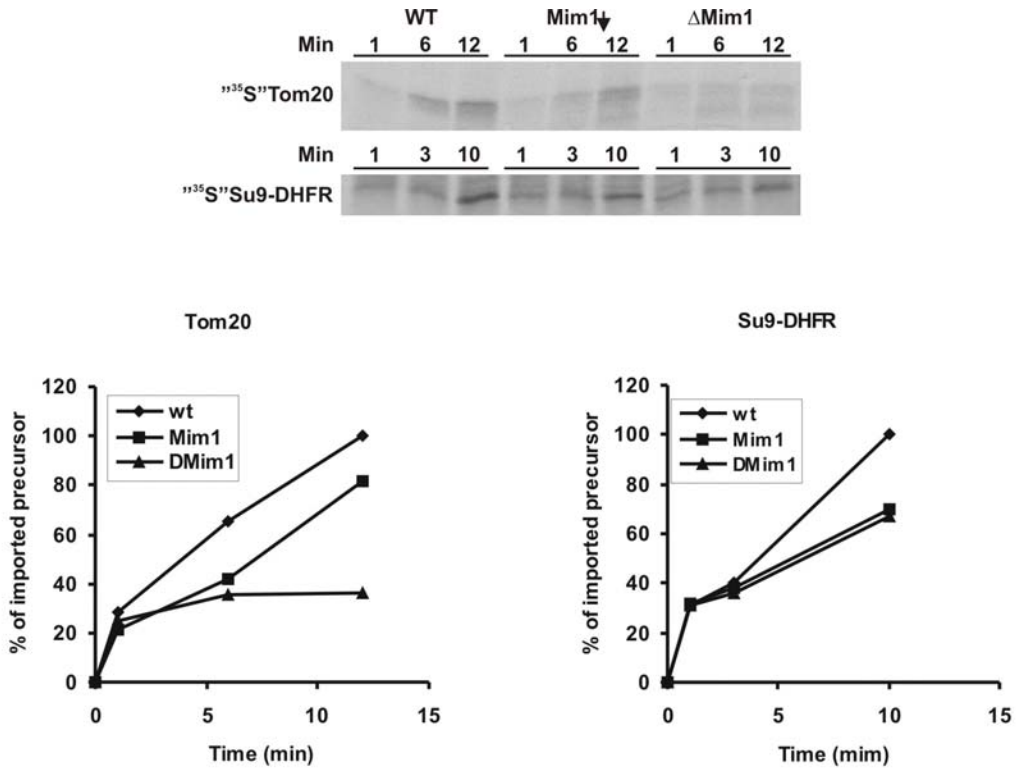


Figure 3.13. Mim1 is needed for import of Tom20. Mitochondria were incubated at 15°C with radiolabeled precursors of Tom20ext. and Su9-DHFR for various time periods. Mitochondrial pellets were resuspended in sample buffer, shaken at 95°C and then analyzed by SDS-PAGE and autoradiography.

Co-immunoprecipitation experiment was performed in wt and ΔMim1 mitochondria. Mitochondria were solubilized with 1% digitonin, and the efficiencies of antibodies against Tom40 to precipitate Tom20, and of antibodies against Tom20 to co-precipitate Tom40, were analysed. As expected, the total amounts of both components of the TOM complex were reduced in ΔMim1 mitochondria. Despite that, in both wild type and ΔMim1 mitochondria similar co-immunoprecipitation efficiency for Tom20 and Tom40 was observed suggesting that Tom20 is assembled in the TOM

complex even if Mim1 is absent (Fig.3.14.). Since Tom20 is only loosely attached to the TOM core complex (Dekker et al., 1998; Model et al., 2002) the efficiency of the co-immunoprecipitation was rather low in all samples.

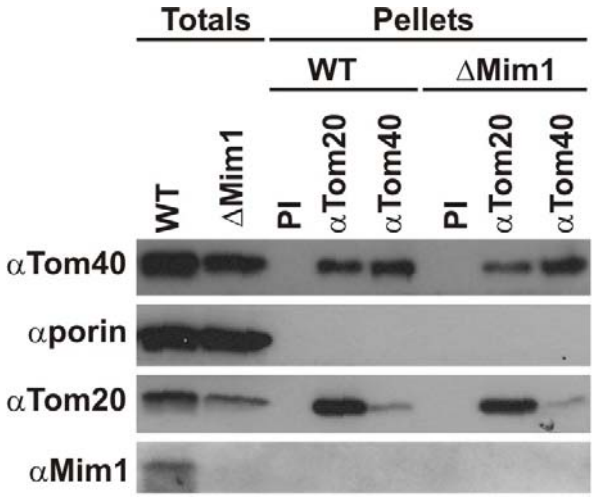


Figure 3.14. Mim1 does not play a crucial role in the assembly of Tom20 into the TOM complex. Mitochondria isolated from wt and *Δmim1* yeast strains were solubilized in buffer containing 1% digitonin. The extract was added to antibodies prebound to Protein A Sepharose. Bound material (Pellets) was analysed by immunostaining using antibodies against Tom40, porin, Tom20 and Mim1. A 5% fraction of total extract was loaded on the gel (Totals).

To summarize, these results suggest that Mim1 is playing a crucial role in the biogenesis of Tom20 but it is not absolutely required for the assembly step of the latter into the TOM complex. Thus, the main involvement of Mim1 in the biogenesis of the TOM complex is via its role in the insertion of Tom20 into the outer mitochondrial membrane.

3.7. Mim1 requires import receptors but not the Tom40 channel for its import into outer mitochondrial membrane

To study the insertion pathway of Mim1 into the outer mitochondrial membrane, *in vitro* import experiments were performed. Mitochondria were incubated with radiolabeled precursors of Mim1 and porin as a control, for various time periods at 15°C. After treatment with protease K a 7 kDa Mim1-specific protease protected fragment was observed.

First, import experiments were performed with wt mitochondria pre-treated with trypsin to remove all mitochondrial receptor proteins. According to the result shown in Figure 3.15 the import of Mim1 is highly dependent on exposed domains of outer membrane proteins.

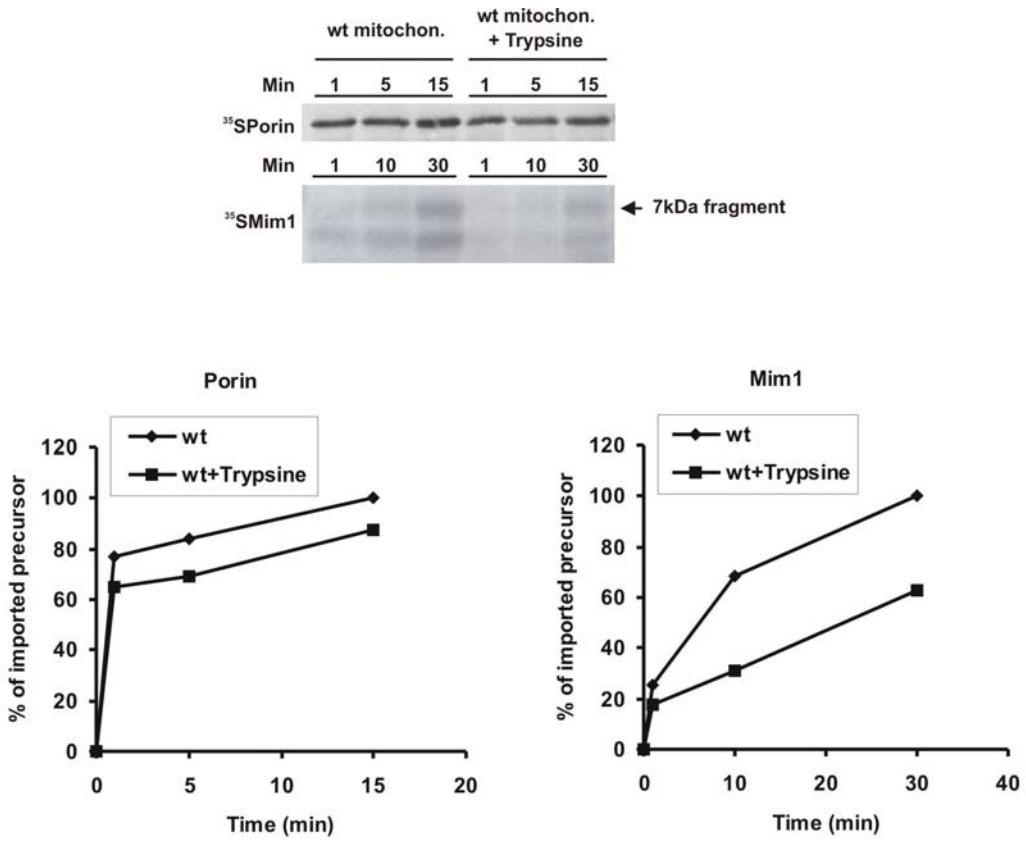


Figure 3.15. Mim1 requires receptors for its import into the outer mitochondrial membrane. Trypsin pre-treated wt mitochondria were incubated at 15°C with radiolabeled porin and Mim1 precursors for various periods of time. Mitochondrial pellets were resuspended in sample buffer, shaken at 95°C and then analyzed by SDS-PAGE and autoradiography.

To investigate whether the precursor of Mim1 utilizes the general import pore, an import experiment was performed with mitochondria that were pre-treated with excess amount of Su9-DHFR. Such a treatment with Su9-DHFR is known to occupy most of the TOM channels (Ahting et al., 2005). According to the results shown below the import channel is not required for insertion of Mim1 into the outer mitochondrial membrane. In contrast, the

import of porin was strongly affected by blocking Tom40 channels, as expected (Fig. 3.16).

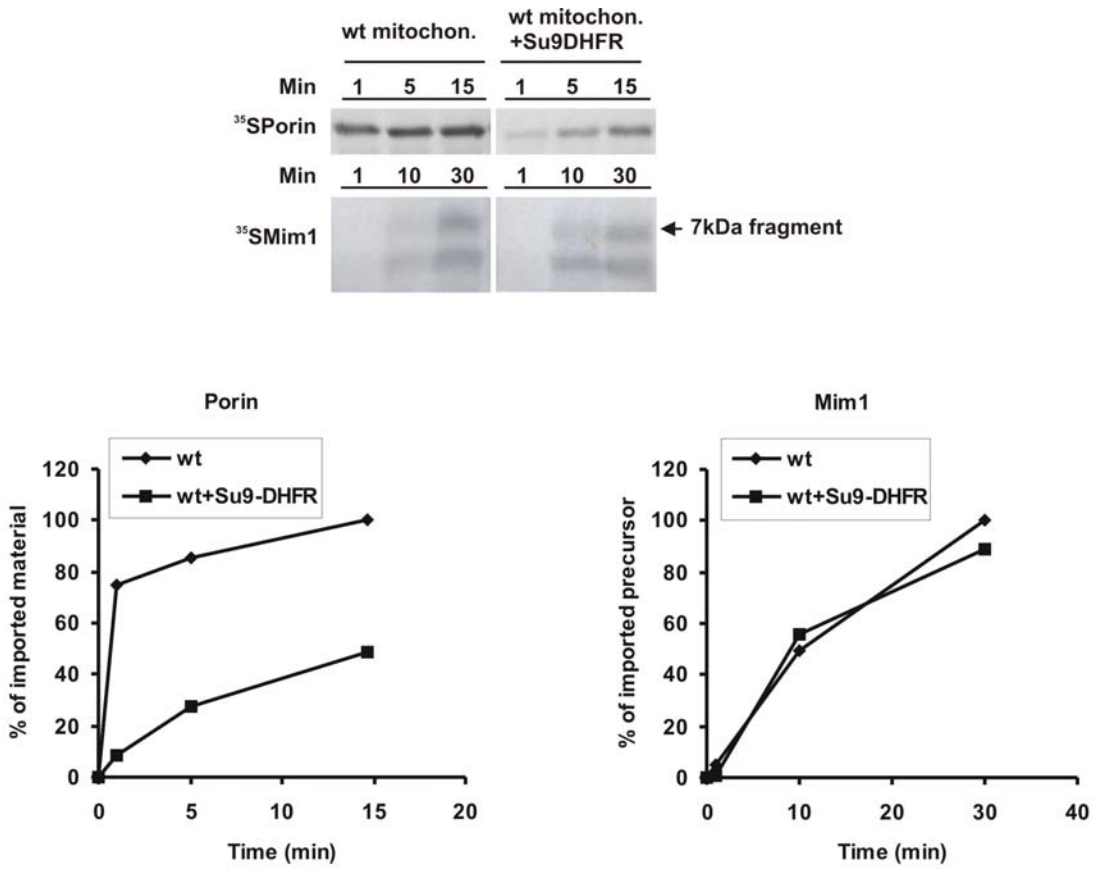


Figure 3.16. Mim1 does not need Tom40 channel for successful import into the membrane. Mitochondria were incubated at 15°C with radiolabeled porin and Mim1 precursors in the absence or presence of excess amount of Su9-DHFR. Mitochondrial pellets were resuspended in sample buffer, shaken at 95°C and then analyzed by SDS-PAGE and autoradiography.

To conclude, import of Mim1 into the outer mitochondrial membrane is dependent on the receptors exposed on mitochondrial surface but Tom40 channel is not required for this process.

4. DISCUSSION

Proteins residing in the outer mitochondrial membrane can be classified into several categories based on their topological organizations: the “signal-anchored” proteins with a C-terminal hydrophilic domain facing the cytosol, the “tail-anchored” proteins with N-terminal domains exposed on the mitochondrial surface, β-barrel proteins, and the proteins that span the outer membrane with two or more helices. Tom22 and Mim1 represent yet another small group of proteins with a single transmembrane domain and two hydrophilic domains, one present in the cytosol and another in the intermembrane space. Mim1 is the most recently identified protein of the latter group. It was first identified in a high throughput analysis of 200 proteins annotated in the yeast data base as essential for yeast viability (Mnaimneh et al., 2004). The authors of this study named the protein Mim1 for its involvment in mitochondrial import, and provided the initial evidence that Mim1 was essential for yeast cell viability. Shortly afterwards, Mim1 was characterized in more detail by our group. Simultanously, Mim1 was studied by Endo and co-workers who introduced the alternative name for it, Tom13 (Ishikawa et al., 2004; Waizenegger et al., 2005). Both groups characterized Mim1 as a protein with an undefined function in the biogenesis of the TOM complex. Interestingly, these studies also revealed that Mim1 was not essential for yeast cell viability even though the yeast cells lacking Mim1 exhibited severely impaired growth.

4.1. The transmembrane segment of Mim1 is the functional domain of the protein

Mim1 is composed of three domains: N-terminal domain present in the cytosol, C-terminal domain facing the intermembrane space (IMS), and a single putative α-helical transmembrane domain (TM) spanning the outer mitochondrial membrane. Until the identification of Mim1, the only known

protein in the outer mitochondrial membrane with such a topology was Tom22 (Kiebler et al., 1993; Lithgow et al., 1994; Nakai and Endo, 1995). However, Mim1 and Tom22 do not seem to have functional similarities. Tom22 is a component of the TOM core complex and it functions as a receptor for mitochondrial precursor proteins. Both the cytosolic and the IMS domains of Tom22 are important for the receptor function of Tom22 as it interacts with precursor proteins not only on the *cis*, but also on the *trans* side of the TOM complex (Court et al., 1996; Moczko et al., 1997). In addition, Tom22 was also reported to be involved in regulation of gating of the general import pore (GIP) (van Wilpe et al., 1999). In contrast, Mim1 is not a component of any known complex in the outer mitochondrial membrane (Waizenegger et al., 2005 and data shown here).

To better understand the function of Mim1, we investigated the structural characteristics of the protein. The results presented here demonstrate that the TM domain is the only functional part of the protein, while the exposed domains of Mim1 on both sides of the outer membrane are not necessary for its function. Additionally, these exposed domains appear to be crucial neither for the targeting of Mim1 to mitochondria nor for its insertion into the outer membrane. The truncated versions of Mim1 could complement the growth phenotype of *Δmim1* cells indicating that the protein lacking either of the hydrophilic domains is functional and thus properly inserted in the outer membrane. Furthermore, the truncated versions of Mim1 were able to support a full assembly of Tom40 into the TOM complex, suggesting that only the TM domain is crucial for the role of Mim1 in the biogenesis of the TOM complex. The experimental data showing that the TM domain alone, when over-expressed, was sufficient to complement the function of the full-length protein further confirmed that the membrane embedded segment of Mim1 is the functional part of the protein. Taken together, as the TM domain of Mim1 is the sole functional part of the protein. Mim1 does not act as a classical receptor for precursors of the TOM components, but rather performs its function within the membrane.

Mim1 proteins from *S.pombe* (*Sp*) and *N. crassa* (*Nc*) share sequence similarity to *S. cerevisiae* (*Sc*) Mim1 only in the transmembrane domain, but not in the N- and the C-terminal parts of the protein. These homologs could

complement deletion of Mim1 in yeast *S. cerevisiae* but not to the same extent. The S. *cerevisiae* yeast strain carrying *S. pombe* Mim1 grew as well as the wild type (wt) strain both when it was under the regulation of yeast *MIM1* promoter and when it was overexpressed. In contrast, *N. crassa* Mim1 could not provide full, but only partial complementation, and only when it was overexpressed. Since the expression of *N. crassa* Mim1 protein in yeast was confirmed by western blot analysis, the reason for the different complementation capacities of the two fungal homologs probably lies in the difference in size and/or charge of their pertinent hydrophilic domains. The exposed N- and C-terminal domains of *N. crassa* Mim1 (56 and 47 amino acid residues, respectively) are larger then those in both *S. pombe* (22 and 22 residues) and *S. cerevisiae* (43 and 41 residues) homologs. One could assume that despite clear sequence similarity in the TM domains of *N. crassa* and *S. cerevisiae* Mim1, the *Nc* version of the protein when expressed in yeast is either not properly inserted in the outer membrane or is not able to form functional structures within the membrane due to the larger size of the N- and the C- terminal domains. In addition, the C-terminal domain of *N. crassa* Mim1 contains significantly more both positively and negatively charged residues than the pertinent corresponding domain of yeast *S. cerevisiae* Mim1 and that may be yet another reason why *N. crassa* Mim1 could not fully complement deletion of Mim1 in *S. cerevisiae*. On the other hand, the exposed domains of *S. pombe* Mim1 are smaller and less charged than the pertinent equivalent domains of both *S. cerevisiae* and *N. crassa* homologs. Hence, the repulsive steric forces between the hydrophilic domains in a putative dimer or oligomer of Mim1 (see below) are less likely to occur in the case of *S. pombe* Mim1, than in the case of *N. crassa* version.

4.2. Structural organization of Mim1 molecules

Initial gel filtration and BN-PAGE analyses suggested that Mim1 is a component of a novel, yet unidentified, complex of approximate size of 200 kDa (Waizenegger et al., 2005). Pull-down and crosslinking experiments presented in this study demonstrated that Mim1 molecules interact with

each other in the outer membrane forming homodimers and possibly even homooligomers. The experiments performed with various chemical crosslinkers gave similar crosslinking pattern of Mim1 indicating that this protein is present in the membrane as a homodimer. The most efficient crosslinking was observed with the DSG crosslinker. In this case, it was possible to detect a band corresponding to Mim1 homotrimer. Since it is very difficult to detect crosslinking adducts bigger than a trimer, one may speculate that the basic structural unit of Mim1 is a homodimer that may congregate and form a higher structural assembly.

Next, the mechanism of Mim1 dimerization/oligomerization was tested. In addition to being the functional domain of Mim1, its TM domain also mediates the molecular organization of the protein, namely Mim1 forms dimers via its TM domains. The oligomerization element of Mim1 within the TM domain was identified. *In silico* analysis revealed two sequential GXXXG/A motifs, which are essential for the oligomerization. GXXXG is known to be a strong transmembrane helix-packing motif. This motif is found in various cellular membranes where it is frequently involved in high-affinity homo-oligomerization (Russ and Engelman, 2000). For example, GXXXG motifs within the TM segments of subunits e and g are involved in the dimerization/oligomerization of the yeast mitochondrial ATP synthase complex (Arselin et al., 2003; Bustos and Velours, 2005; Saddar et al., 2005), and such motifs in the TM domain of the amyloid precursor protein are critical for the etiology of the Aβ42 peptide (Munter et al., 2007). These two motifs are highly conserved among Mim1 homologs. Mim1 provides the first example of such a motif in a protein from the outer mitochondrial membrane and offers a special example of two sequential motifs where both appear to be important for dimerization. Moreover, Mim1 TM domain contains highly conserved Phe, which is known to be frequently associated with GXXXG motifs in position -3 resulting in FXXGXXXG domain. Phe in this position upstream of the dimerization motif was shown to stabilize membrane-spanning GXXXG motifs *(*Unterreitmeier et al., 2007). Mutation in only one dimerization motif did not affect the dimerization or the activity of Mim1. However, replacing Ala and Gly residues by the bulky Leu or Ile residues in both motifs rendered Mim1 inactive and prevented its oligomerization. Crosslinking experiments clearly demonstrated that upon

inserting point mutations in both GXXXG/A domains the crosslinking adducts corresponding to homodimer and homotrimer disappeared. Likewise, it was shown by BN-PAGE that the 200 kDa Mim1 complex was disassembled in the strain containing mutational alterations in both dimerization motifs (LII mutant). Moreover, in cells expressing Mim1-LII, Tom40 was dissociated from the TOM complex to the same extent as observed in cells lacking Mim1 (*Δmim1* strain). In addition, drop dilution assay showed that LII cells harbouring the Mim1-LII variant exhibited the same growth phenotype as the cells lacking Mim1. Taken together, these experimental evidence suggest that dimerization/homooligomerization of Mim1 are crucial for the function of the protein.

It remains unclear if the complex containing Mim1 harbours some additional proteins. Crosslinking and pull-down experiments showed no direct protein-protein interaction between Mim1 and any other outer membrane protein. In addition, genetic screens designed to identify interacting partners of Mim1 (data not shown) suggest that these complexes may contain solely Mim1 molecules (data not shown). In a screen for multicopy suppressors of the *Δmim1* phenotype, a genomic library of the yeast genome on a multicopy plasmid was transformed into *Δmim1* cells. More specifically, when genetic library was transformed into *Δmim1* strain all 17 positive clones carried a plasmid encoding Mim1. This suggests that Mim1 is nonredundant since its deletion could be complemented only by Mim1 itself.

4.3. The function of Mim1

Next, I asked what the exact role of Mim1 in the biogenesis of the TOM complex was. We previously observed that the deletion of Mim1 led to a significant reduction of the steady state levels of Tom20, whereas only a moderate reduction of steady state levels of Tom40 and Tom70 was observed (Waizenegger et al., 2005). It is also known that although Tom20 is not a subunit of the TOM core complex (Dekker et al., 1998), the structural arrangement of the latter depends on the presence of Tom20 (Model et al., 2002). Furthermore, it has been previously reported that Tom20 is required for efficient biogenesis of the TOM core components,

Tom40 and Tom22 (Harkness et al., 1994; Lithgow et al., 1994; Rapaport and Neupert, 1999). In this study, I propose that the observed phenotypes of *Δmim1* cells like altered mitochondrial morphology, import inhibition of precursor proteins, and hampered assembly of newly synthesized subunits of the TOM complex, are secondary to the primary effect on the integration of Tom20 into the outer membrane. It is shown here that even low, almost undetectable by immunodecoration levels of Mim1 in the outer membrane, were sufficient to maintain wt levels of Tom20 and Tom 40 that were fully assembled in the TOM complex. Despite extensive efforts, I was unable to detect any stable interaction between Mim1 and Tom20 (data not shown). Therefore, Mim1 appears to exert its role via an indirect interaction with precursor molecules of Tom20.

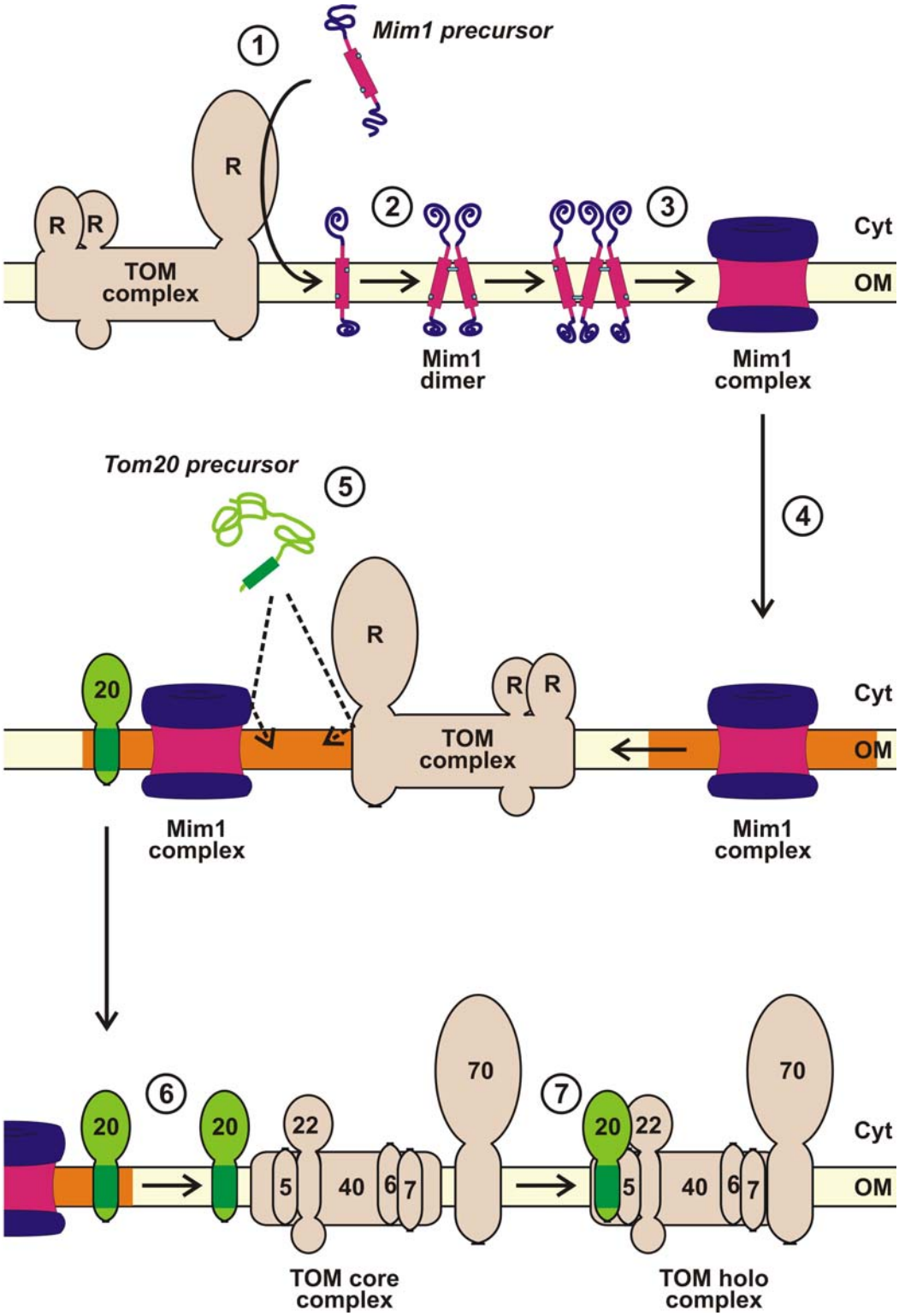
A successful integration of Tom20 into the outer membrane requires initial recognition, insertion into the membrane, and assembly into the TOM complex. As Mim1 does not function as a classical receptor for precursor proteins but rather within the membrane, its involvement in the initial recognition at the cytosolic surface of the organelle can be excluded. The current results demonstrate that the insertion of Tom20 molecules into the outer membrane is hampered by the absence of Mim1. However, the assembly of Tom20 into the TOM complex was not affected in mitochondria lacking Mim1. Therefore, Mim1 is involved mainly in the membrane integration of Tom20 precursors, but not in its assembly into the mature TOM complex.

How does Mim1 perform its function? Mim1 homologs are found in fungal mitochondria but not in other eukaryotes like plant or mammalian mitochondria. Thus, Mim1 appears to be involved in a pathway specific for fungi. Tom20 counterpart in mammalian mitochondria may, therefore, have different import and assembly pathway which does not require a protein with a Mim1-like function. As Mim1 was found to form punctuate structures in the outer membrane and biochemical studies revealed its propensity to form homooligomers, I propose that this structural organization creates a micro-environment in the outer membrane which facilitates the import of Tom20. A specific characteristic of the outer mitochondrial membrane in fungi is the presence of ergosterol. It is tempting to speculate that Mim1, in an unknown manner, organizes lipid environment needed for a successful import of

Tom20. In this case, Mim1 affects the lipid organization via the interplay of its highly conserved TM domain with ergosterol in the membrane. One cannot exclude the possibility that there is a protein in plants or mammals with a similar function, which does not share sequence similarity with Mim1 found in fungi.

The mechanism by which Mim1 is imported and embedded in the outer membrane has not been described. According to preliminary data presented in this study, it seems that Mim1 does not require Tom 40 channel to be successfully imported but it is highly dependent on receptors exposed on mitochondrial surface. This process remains to be analyzed and described in more detail.

On the basis of the results presented above, I suggest the following working model of structural organization and function of Mim1 (Figure 4.1). Mim1 precursor protein is recognized by the TOM complex receptors on mitochondrial surface and then inserted directly into the outer mitochondrial membrane without passing through the Tom40 channel (step 1). Upon its proper insertion in the membrane, Mim1 first forms homodimers via two GXXXG/A dimerization motifs (cyan) present in its transmembrane domain (magenta) (step 2). This domain is the sole element responsible for dimerization of Mim1. Homodimers, as basic structural units, are further organized into 200 kDa complex composed of several Mim1 molecules (step 3). This homooligomeric structure possibly organizes lipid environment (orange) (step 4) needed for one of the stages in import of Tom20 (step 5). Finally, upon its insertion in the outer membrane, Tom 20 is assembled into mature TOM complex in a process which is not dependent of Mim1 (steps 6 and 7).



4.1. Model of structural organization and function of Mim1. OM - outer membrane, Cyt - cytosol. See text for details.

5. Summary

The translocase of the outer mitochondrial membrane (TOM complex) is the general entry site for newly synthesized proteins into the organelle. The translocase is a multi-subunit complex composed of seven subunits: two receptor proteins, Tom70 and Tom20, and five components which form the core complex, Tom40, Tom22, Tom7, Tom6, and Tom5. All these subunits have to be imported from the cytosol into the outer membrane. The mechanisms by which the precursors of the Tom subunits are targeted to mitochondria, integrated into the outer membrane and assembled into a functional TOM complex is only partially understood.

Recently, an outer membrane protein named Mim1 was identified as an assembly factor of the TOM complex. Specifically, it was observed to play a role in the assembly of Tom40 into the TOM complex in the step after the interaction of the Tom40 precursor with the TOB complex. Mim1 is composed of an N-terminal cytosolic domain, a central putative transmembrane segment (TMS), and a C-terminal domain facing the intermembrane space. The contribution of the various domains to the overall function of the protein, as well as the molecular function of Mim1 remained unresolved.

In the present work I addressed some of these questions. In this thesis it is shown that Mim1 is required for the integration of the import receptor Tom20 into the outer membrane but not for its assembly into the TOM complex. Despite extensive efforts, a stable interaction between Mim1 and Tom20 was not detected. Thus, it is currently unclear whether Mim1 exerts its function via a direct or indirect interaction with Tom20 precursor. Tom20 is crucial for stable organization of Tom40 channel units into larger assemblies. Therefore, I propose that Mim1 can affect the assembly pathway of the TOM complex by acting at the Tom20 import level.

Structural characteristics of Mim1 required for its function were studied in detail. The N- and the C-terminal domains of Mim1 are crucial neither for the function of the protein nor for its biogenesis. Thus, the transmembrane segment of Mim1 seems to be the minimal functional domain of the protein. It is further shown that Mim1 forms homooligomeric structures via its TMS which contains two helix-dimerization GXXXG/A motifs. Mim1 variant mutated in both dimerization motifs is not able to form oligomeric structures and is inactive. Taken together, the homo-oligomerization is a prerogative for the function of Mim1 in mediating the integration of Tom20 into the mitochondrial outer membrane.

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Abbreviations

α	antibody
AAC	ADP/ATP carrier
Ab	antibody
ADP	adenosine diphosphate
Amp	ampicillin
APS	ammonium peroxodisulfate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BN-PAGE	blue native polyacrylamide gel electrophoresis
BSA	bovine serum albumin
C-	carboxy-
CBB	coomassie brilliant blue
cDNA	complementary DNA
CNBr	cyanogen bromide
CV	column volume
DFDNB	1,5-Difluoro-2,4-dinitrobenzene
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
DTT	dithiotreitol
dNTP	deoxyribonucleoside triphosphate
DSG	disuccinimidyl glutarate
DSS	disuccinimidyl suberate
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetate
gDNA	genomic DNA
GIP	general import pore
HA	Haemagglutinin
HEPES	N-2 hydroxyl piperazine-N´-2-ethane sulphonic acid
His	histidine
Hsp	heat shock protein
IgG	immunoglobuline G
IM	inner membrane
Imp	inner membrane peptidase
IMS	intermembrane space
IPTG	isopropyl-β,D-thiogalactopyranoside
KAN	kanamycin
kDa	kilodalton
LB	Luria Bertani
MBP	maltose binding protein
MOPS	N-morpholinopropane sulphonic acid
MPP	mitochondrial processing peptidase
MTS	matrix targeting signal
N-	amino-
N. crassa	*Neurospora crassa*
NADH	nicotine amide adenine dinucleotide
Ni-NTA	nickel-nitrilo triacetic acid
NMR	nuclear magnetic resonance

OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PAS	protein A-Sepharose
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	preimmune serum
PK	proteinase K
PMSF	phenylmethylsulfonyfluoride
Preprotein	precursor protein
ProtA	Protein A
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNasin	ribonuclease inhibitor
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SDS	sodium dodecyl sulfate
TBS	TRIS buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N‘,N‘-tetramethylene diamine
TIM	translocase of the inner mitochondrial membrane
TMD	transmembrane domain
TOB	translocase of outer membrane β-barrel proteins
TOM	translocase of the outer mitochondrial membrane
TPR	tetratricopeptide repeat motifs
Tris	tris-(hydroxymethyl)-aminomethane
TX-100	Triton X-100
UTR	3´ untranslated region
v/v	volume per volume
w/v	weight per volume
WT	wild type

Publications resulting from this thesis

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Curriculum Vitae

Jelena Popov-Čeleketić

23.06.1976	Born in Jagodina, Serbia
1983-1991	Elementary school "Rada Miljkovic" Jagodina
1991-1995	Gimnasium “Svetozar Markovic”, Jagodina
1995-2002	Studies of Biochemistry at the Faculty of Chemistry, University of Belgrade
2003-2004	Research Assistant in the Institute of Nuclear Sciences "Vinča", Belgrade
2004-2008	PhD research in the laboratory of Prof. Dr. Dr. Walter Neupert at the Adolf-Butenandt-Institut für Physiologische Chemie, Ludwig- Maximilians-Universität München

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