Identification of cytosolic factors interacting with mitochondrial preproteins

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1. Introduction

1.1 Mitochondria (origin, structure and function)

Mitochondria are eukaryotic organelles that play a crucial role in many cellular functions like respiration, energy production, metabolism of lipids and amino acids, apoptosis and aging (Scheffler, 2001). It is thought that this organelle evolved from free living bacteria, most probably α-proteobacteria, via symbiosis within a eukaryotes host cell (Margulis, 1970, Yang et al., 1985, Gray, 1993). As a result of the symbiotic relationship between these two organisms the proto-mitochondria converted to an organelle and during evolutionary processes the mitochondrial genome got reduced and has almost completely been transferred to the host genome (Adams and Palmer, 2003). An import machinery that imports proteins from the cytosol to mitochondria developed co-evolutionary (Adams and Palmer, 2003, Dyall et al., 2004).

Thus the mitochondrial proteome mainly consists of two set of proteins; those related to prokaryotic origin which have bacterial homologues and those that occur exclusively in eukaryotes (Gray et al., 2001). Based on proteomic analysis it has been estimated that mammalian or yeast mitochondria harbor about 1500 or 1000 different proteins, respectively. Based on their endosymbiosis origin, mitochondria encoded a limited amount of proteins that are mostly subunits of respiratory complexes. Thus nearly all mitochondrial proteins are encoded in the nuclear genome and translated as precursor

mitochondrial proteins by cytosolic ribosomes. Translated proteins are targeted to the mitochondria and subsequently imported into the organelle.

Mitochondria, like the nucleus and chloroplasts in plants, possess two membranes. Within the organelle, these two membranes define four structurally and functionally different compartments; the mitochondrial outer membrane (MOM), the mitochondrial inner membrane (MIM), the mitochondrial intermembrane space (IMS) and the matrix (Palade, 1953). Whereas the MOM has relatively low ratio of proteins to lipids, this ratio is high in the MIM. Another unique feature of the MIM is the high content of the diphosphatidylglycerol lipid cardiolipin, which is also an abundant lipid in bacterial membranes (Comte et al., 1976, Scheffler, 2001). Each of the sub-compartments contains a diverse subset of proteins with different functions, which are encoded in the nucleus and are post-translationally imported into mitochondria by specialized protein machineries such as the TOM (translocate of outer membrane) and TIM (translocate of inner membrane) complexes.

Since mitochondria constitute an intra-organeller environment, which is isolated from the cytosol, and nuclear encoded proteins have to be imported post-translationally, protein homeostasis need to be strictly maintained and controlled by regulated import machinery. These complexes guide mitochondrial precursors proteins to the corresponding mitochondrial sub-compartment and a set of molecular chaperones act to assist the import.

1.2 Import pathways of mitochondrial proteins

Most of mitochondrial proteins, which are encoded in nucleus and synthesized as precursor proteins in the cytosol, must be imported to different sub-compartments of the organelle with the help of dedicated protein translocases. For targeting to distinct mitochondrial sub-compartments precursor proteins contain an import signal within their sequences. The most frequent signal is the matrix targeting signal (MTS), a stretch of 15-55 amphipathic amino acid with α-helical structure enriched in positively-charged residues (Vogtle et al., 2009). Different kind of targeting and sorting signals direct mitochondrial precursor proteins to other destinations within the organelle (Fig. 1.1).

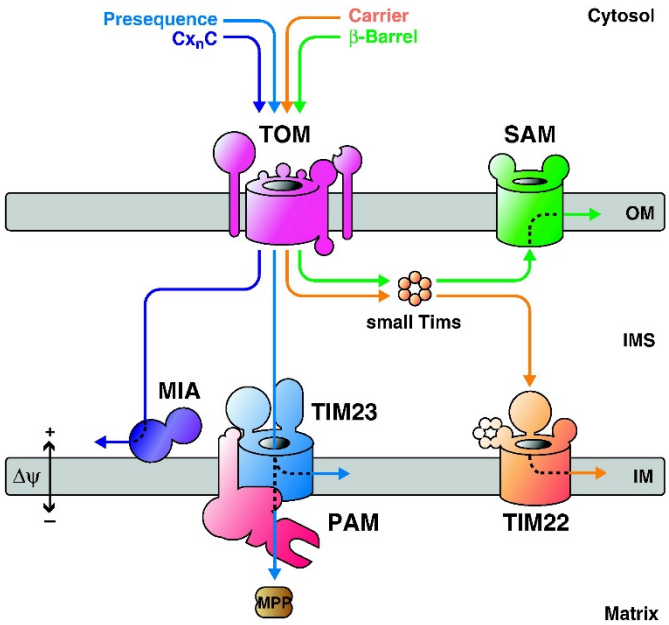


Fig. 1.1: Different targeting signals direct mitochondrial precursor proteins to specific translocation routes; After translocation of precursors through the general import pore (TOM complex), they are sorted to their correct location. Preproteins carrying an N-terminal signal interact with the TIM23 complex that either insert them into the inner membrane or translocate them to the matrix. Matrix proteins require the activity of the presequence translocase-associated import motor (PAM) for translocation. Inside the matrix the presequences are finally proteolitcally removed by mitochondrial processing peptidase (MPP). Carrier proteins of the inner membrane are inserted into the lipid bilayer by the TIM22 complex. β-barrel proteins are inserted to the OM with the help of small TIM chaperones and TOB/SAM machinery. Proteins of the IMS that contain cysteine-rich signal (Cx_nC) are imported via the MIA pathway (Figure adopted from: Dudek et al. 2013).

1.2.1 Translocase of the outer membrane (TOM complex)

The TOM complex as the translocase of the mitochondrial outer membrane provides the central entry gate that mediates the transport of various mitochondrial precursor proteins. Upon synthesis of mitochondrial precursor proteins in the cytosol, they are associated with cytosolic chaperones to prevent misfolding and aggregation (Zara et al., 2009, Bhangoo et al., 2007, Young et al., 2004). The outer membrane receptors Tom20 and Tom70 function as a docking site for mitochondrial precursor proteins. In addition, they act as a quality control machinery by allowing only those mitochondrial proteins which harbor a proper targeting signal to cross the mitochondrial membrane (Dudek et al., 2013). Except for some α-helical outer membrane proteins all mitochondrial precursor proteins cross the outer membrane via the TOM complex. Tom40 is the

central unit of the TOM complex and it forms an aqueous channel in a β-barrel conformation (Hill et al., 1998, Model et al., 2008). Additional subunit like Tom20, Tom22, Tom70/71 and the small Tom proteins Tom5, Tom6 and Tom7 are also part of the TOM complex. These subunits support the quaternary structure and function of the complex (Dudek et al. 2013).

Tom20 and Tom70/71 selectively bind to different mitochondrial precursor proteins (Brix et al., 1997). Tom20 can mainly interact with mitochondrial precursor proteins containing an N-terminal targeting signal via the hydrophobic face of the α-helical segment (Abe et al., 2000, Saitoh et al., 2007). Tom70/71 preferentially recognizes mitochondrial precursor proteins containing internal signals by binding hydrophobic segments of these proteins (Wiedemann et al., 2001). Tom22 has double function, it is needed for integrity of the TOM complex and it also interacts with precursor proteins (Moczko et al. 1997; Shiota et al. 2011).

Tom5 is thought to help in precursor protein transfer from Tom22 to the Tom40 channel and also in the biogenesis of Tom40 (Dietmeier et al., 1997, Becker et al., 2010). Tom6 and Tom7 work antagonistically, while Tom6 regulates the biogenesis of the TOM complex by association with an early intermediate, Tom7 destabilizes the TOM complex to facilitate assembly of newly imported subunits (Dekker et al., 1998, Becker et al., 2011b).

After initial recognition of precursor proteins via the Tom20 and Tom70 receptors they will be transferred with the help of the cytosolic domain of Tom22 and Tom5 through the Tom40 pore and bind to the IMS domains of Tom40, Tom7 and Tom22 (Moczko et al., 1997, Komiya et al., 1998, Rapaport et al., 1998, Gessmann et al., 2011). After passing the TOM complex, precursor proteins are targeted to different sub-compartment in mitochondria (MOM, IMS, MIM and matrix) (Fig. 1.1).

1.2.2 Biogenesis of β-barrel proteins (SAM/TOB complex)

After passing the TOM complex, mitochondrial outer membrane proteins like Tom40, Porin, Tob55/Sam50 and Mdm10, which have a β-barrel structure, are handed over to the soluble chaperone complexes in the IMS that are formed by small Tim chaperons. The two complexes are Tim9-Tim10 and Tim8-Tim13. The Tim9-Tim10 complex plays a crucial role in translocation of β-barrel proteins to the MOM and metabolite carrier translocation to the MIM (Curran et al., 2002, Webb et al., 2006), whereas the Tim8-

Tim13 complex is mainly involved in Tim23 biogenesis (Hoppins and Nargang, 2004, Paschen et al., 2000).

Translocation of β-barrel proteins from the IMS to the MOM is initiated by a signal in the last β strand of the precursor protein that is termed β-signal. This signal consists of polar amino acid like lysine or glutamine, glycine and two hydrophobic amino acids (Kutik et al., 2008). Hence, β-barrel proteins are delivered with the help of the β-signal from the small Tim chaperone complex to the TOB complex (Wiedemann et al., 2003, Kutik et al., 2008, Gentle et al., 2004).

The TOB complex consists of different subunits; the β-barrel protein Tob55/Sam50 which is embedded in the outer membrane and two peripheral membrane protein, named Tob38/Sam35 and Mas37/Sam37 that are exposed on the cytosolic side of the TOB complex (Dolezal et al., 2006, Wiedemann et al., 2003, Paschen et al., 2003, Waizenegger et al., 2004). Tob55 is embedded in the membrane by a β-barrel domain and forms an aqueous pore across the membrane (Kutik et al., 2008, Paschen et al., 2003). Tob38 imposes into this pore on the cytosolic side of the TOB complex and it is thought to form a binding site for precursor proteins containing the β-signal (Kutik et al., 2008). Finally with the help of its N-terminal polypeptide transport associated (POTRA) domain, Tob55 translocates the protein into the lipid bilayer (Chan and Lithgow, 2008, Stroud et al., 2011, Habib et al., 2007).

The biogenesis of Tom40 requires also involvement of Mdm10 (subunit of ERMES complex) and Tom22 (Thornton et al., 2010). The distribution of Mdm10 between the TOB and ERMES complexes is arranged by Tom7 (Becker et al., 2011b, Meisinger et al., 2006, Yamano et al., 2010)(Fig. 1.2). The ERMES complex consists of Mdm10, Mdm34, and Mmm1 that integrated in ER membrane, and Mdm12 as an adaptor protein that forms a direct connection between mitochondria and ER (Meisinger et al., 2007, Boldogh et al., 2003, Kornmann et al., 2011).

1.2.3 Biogenesis of α-helical proteins

Different pathways for the biogenesis of OM (outer membrane) α-helical proteins have been identified. Tom70 and Tom20 are signal-anchored proteins that are integrated to the MOM by single transmembrane segment at their N-terminal domain (Dudek et al., 2013). Mim1 supports the insertion of these proteins into the outer membrane (Popov-Celeketic et al., 2008)(Hulett et al., 2008, Becker et al., 2008). Furthermore Mim1 plays

an important role in the integration of multi-span outer membrane proteins (Papic et al., 2011, Becker et al., 2011a). So far, no insertion machinery has been identified for the insertion of tail-anchored membrane proteins such as Fis1 (Setoguchi et al., 2006, Kemper et al., 2008). It seems that the lipid composition of the target membrane may play a crucial role in assuring optimal targeting and membrane integration (Kemper et al., 2008, Krumpe et al., 2012). Both tail-signal-anchored proteins have relatively short transmembrane segment with mild hydrophobicity and flanking positively charged residues. This characteristics are thought to act as a mitochondrial import signal (Dukanovic and Rapaport, 2011). Tom22 as another α-helical outer membrane protein is inserted with the help of TOB-Mdm10 complex. In contrast to the assembly of β-barrel proteins, no factors in the IMS are involved in the biogenesis of outer membrane α-helical proteins, suggesting that they are directly targeted to the cytosolic side of the outer membrane (Fig. 1.2)

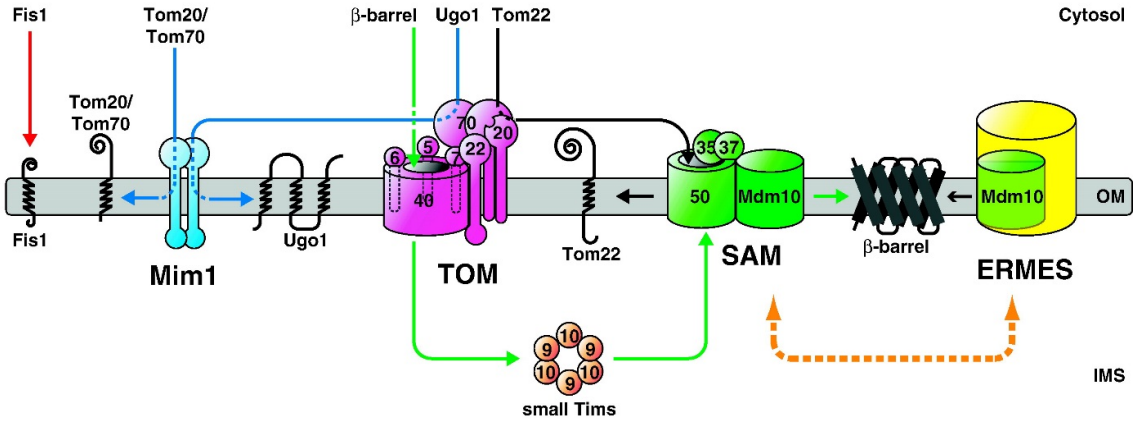


Fig. 1.2: Multiple mechanisms for inserting mitochondrial proteins into the outer membrane; some α-helical proteins like Fis1 seem to integrate into the outer membrane without any help of the translocases. Other proteins with an N-terminal α-helical membrane anchor like Tom20, Tom70 and multi-spanning α-helical proteins like ugo1 are dependent on Mim1 for membrane integration. Tom22 is recognized by receptors of the TOM complex and inserted into the membrane with the help of the TOB complex. The TOB complex plays also a crucial role in inserting β-barrel proteins into the outer membrane. These proteins are handed over from the TOM complex to the TOB complex via small Tim chaperones. Furthermore the ERMES complex is also involved in the biogenesis of β-barrel proteins. (Adopted from: Dudek et al. 2013).

1.2.4 Biogenesis of intermembrane space proteins (MIA pathway)

Some mitochondrial IMS proteins, like Tim9, Tim10 and Cox17, contain multiple cysteine residues that are responsible for the formation of disulfide bridge or binding to metal ions (Dudek et al., 2013). The mitochondrial IMS sorting signal (MISS) around the cysteine-rich motif targets these proteins to the IMS via the MIA pathway (Milenkovic et al., 2009, Sideris et al., 2009). Mia40 as an import receptor and Erv1 as a sulfhydryl oxidase are the essential core components of this pathway (Chacinska et al., 2004, Milenkovic et al., 2007). In the IMS, Mia40 makes a transient disulfide bond with precursor proteins as soon as they emerge from the TOM complex, thereby trapping client protein in the IMS (von der Malsburg et al., 2011, Chacinska et al., 2004, Muller et al., 2008). The initial binding of precursors occurs at a hydrophobic motif of Mia40 with a characteristic cysteine-proline-cysteine motif (Banci et al., 2011, Banci et al., 2009). In yeast, it has recently been shown that early substrate interactions with Mia40 are facilitated by Fcj1 (mitofilin), which helps to position Mia40 close to the TOM complex for binding to the precursors (von der Malsburg et al., 2011).

In the next step Mia40 catalyzes the interamolecular disulfide bridge in precursor proteins and leads to the reduction of Mia40 and the release of substrates to the IMS (Grumbt et al., 2007, Chacinska et al., 2004, Muller et al., 2008). Erv1 mediates re-oxidation of Mia40 by shuttling electrons to cytochrome c. These electrons are then transferred to cytochrome c oxidase and utilized in the respiratory chain (Mesecke et al., 2005, Banci et al., 2011, Bihlmaier et al., 2007, Stojanovski et al., 2008).

1.2.5 Biogenesis of inner membrane metabolite carrier (TIM22 complex)

Mitochondrial inner membrane proteins are integrated into the lipid bilayer in a α-helical conformation and can be distinguished by the presence or absence of an N-terminal signal. Multispan mitochondrial inner membrane proteins contain several internal import signals, which correspond to the transmembrane domains and also act as a targeting signal to the organelle. The vast majority of such proteins belongs to the carriers family like ADP/ATP carrier and phosphate carrier (PiC) (Dudek et al., 2013).

The TIM22 complex consists of the central pore, Tim22, and other subunits like Tim54, Tim18 and Sdh3 (Rehling et al., 2003, Sirrenberg et al., 1996, Gebert et al., 2011). A large domain of Tim54 is exposed to the IMS and provides a binding site for the Tim9-Tim10-Tim12 complex (Hwang et al., 2007, Wagner et al., 2008). Tim18 supports the

assembly of Tim54 into the Tim22 complex and Sdh3 interacts with Tim18 (Hwang et al., 2007).

In this pathway five stages have been identified; I: Hsp70 and Hsp90 bind to the precursor proteins to prevent them from aggregation and also to target them to the MOM (Young et al., 2003, Zara et al., 2009, Rehling et al., 2003). II: Upon binding to the Tom70 receptor, precursor proteins are handed over to the TOM complex. III: After crossing the MOM, proteins bind to the small Tim chaperones, Tim9 and Tim10 in the IMS by specific hydrophobic segments in order to prevent their aggregation (Koehler et al., 1998, Truscott et al., 2002). Targeting of precursor proteins to the inner membrane is initiated by association of further small Tim proteins such as Tim12, which works with the Tim9-Tim10 complex (Sirrenberg et al., 1998, Gebert et al., 2008). IV: docking of substrate loaded Tim9-Tim10-Tim12 complex to the Tim54 receptor and insertion of the precursor into the TIM22 complex that is composed of two pores whereas each pore has enough space for insertion of two α-helices (Rehling et al., 2003). V: in the final step the precursors are released into the lipid bilayer and assembled to their mature and functional form.

1.2.6 Biogenesis of proteins with N-terminal signals (TIM23 complex)

Most mitochondrial protein carry an N-terminal presequence that targets them to the mitochondrial matrix or the inner membrane (Vogtle et al., 2009). For mitochondrial proteins with a presequence, the import through the TOM complex is tightly coupled with translocation across the TIM23 complex in the MIM (Dekker et al., 1998, Chacinska et al., 2003). After passage through the TOM complex, precursor proteins associate with IMS segments of the Tom22 receptor (Moczko et al., 1997, Komiya et al., 1998). At this early stage of import, precursor proteins are in the vicinity of the TIM23 complex, so transfer of proteins occurs while their N-terminal part passes the TIM23 complex and the rest of the precursor is still bound to the TOM complex (Schleyer and Neupert, 1985, Schulke et al., 1997). Upon passing the TIM23 complex, precursor proteins are either targeted to the mitochondrial matrix or will be assembled into the MIM. The latter ones contain a hydrophobic signal downstream of their N-terminal presequence, which induces translocation arrest and consequently their lateral release in to the lipid bilayer (Glick et al., 1992, Bohnert et al., 2010). This pathway was therefore named the “stop-transfer” mechanism.

The TIM23 complex is composed of three integral membrane subunits: Tim23, Tim17, and Tim50. Tim23 forms a membrane potential dependent pore (van der Laan et al., 2007, Truscott et al., 2001, Alder et al., 2008). The N-terminal domain of TIM23 faces the IMS and it functions as a preprotein receptor domain (Komiya et al., 1998, Bauer et al., 1996). Tim17 plays an important role in stabilizing and regulating the Tim23 channel and in sorting of preproteins to different destinations (Chacinska et al., 2010, Chacinska et al., 2005, Martinez-Caballero et al., 2007). However the exact function of each component has not yet been understood at the molecular level. Tim50 has previously been proposed to keep the Tim23 channel in its closed state to prevent leakage of ions and potential dissipation (Meinecke et al., 2006). Previous studies also showed that the Tim50 interacts with precursor proteins at an early stage of import (Geissler et al., 2002, Yamamoto et al., 2002, Mokranjac et al., 2003). The fact that it is located in the vicinity of Tom22 in the IMS points to this direction (Shiota et al., 2011). Tim23/Tim50 together are involved in the transfer of precursor proteins from the MOM to the protein-conduction channel of the MIM membrane. This transfer reaction is facilitated by Tim21 that is an additional membrane-integral component of the TIM23 machinery that dynamically associates with the TIM23 core components (Chacinska et al., 2005, Mokranjac et al., 2005). The insertion of preproteins, which are $\Delta\psi$-dependent, is supported by the recruitment of the proton-pumping respiratory chain complexes III (cytochrome bc1) and IV (cytochrome c oxidase)(van der Laan et al., 2006, Yamamoto et al., 2002, Saddar et al., 2008). Binding of this super complex to Tim23 is carried out with the help of Tim21 (Fig. 1.3)(van der Laan et al., 2006, Wiedemann et al., 2007).

1.2.7 Translocation of preprotiens into the mitochondrial matrix

For full translocation of soluble precursor proteins into the matrix, the $\Delta\psi$-dependent activity of TIM23 alone is not enough. Additional driving-force, which is provided from ATP hydrolysis in the matrix, is required. This energy is utilized by the presequence translocase-associated import motor (PAM) consisting of Pam16, Pam17, Pam18 and the mitochondrial Hsp70 protein. mtHsp70 assists in mitochondrial protein import by its ATPase activity (Kang et al., 1990, Mapa et al., 2010). In this composition, the TIM complex is also called $TIM23_{PAM}$ or $TIM23_{MOTOR}$ (van der Laan et al., 2010). Additional factors like Mge1, Tim44 and MPP have crucial role in protein import into the mitochondrial matrix. Mge1 mediates ATP-ADP exchange of mtHsp70 (Voos et al.,

1994, Schneider et al., 1996). Tim44 interacts with mtHsp70 in an ATP-dependent manner to help the association of mtHsp70 with the TIM23 complex (D'Silva et al., 2004, Schneider et al., 1994). There are two different models that explain the function of $Tim23_{PAM}$ and the action of mtHsp70: (I) trapping of preproteins by a Brownian ratchet mechanism that prevents back-sliding of the polypeptide chain, or (II) active pulling (force-generation) (Sato et al., 2005, Shariff et al., 2004, Geissler et al., 2002, Okamoto et al., 2002). When the precursors reach to the matrix, MPP cleaves the N-terminal signal (targeting sequence) of almost all entering precursors (Hawlitschek et al., 1988)(Fig. 1.3).

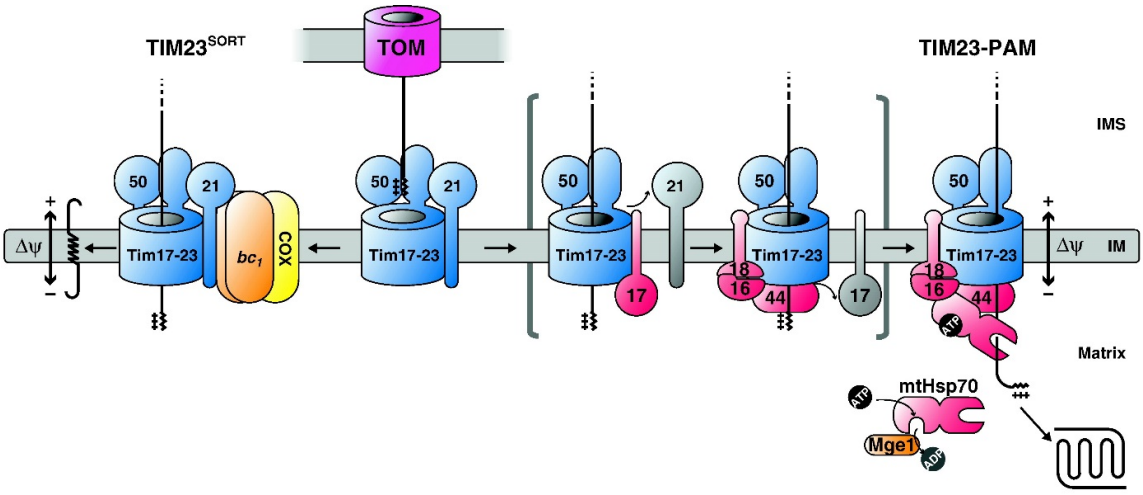


Fig. 1.3: TIM 23 acts as the translocase of the inner membrane for preprotiens carrying a presequence; The inner membrane potential (Δψ) drives positively charged presequence proteins from the TOM complex to the TIM23 complex with the help of Tim21. For membrane integration of presequnce proteins, the Tim23-Tim21 complex in association with respiratory chain complexes such as cytochrome bc_1 complexes and cytochrome *c* oxidase (COX) are implicated. Import of presequence proteins to the matrix requires the recruitment and activation of the PAM module. In this process, Pam17 displaces Tim21 and in cooperation with Tim44 triggers the subsequent binding of Pam16 and Pam18. Pam17 is released during the assembly steps of PAM. mtHsp70 cooperates with Pam16–Pam18, Tim44 and Mge1 to mediate ATP hydrolysis for the import of preproteins into the mitochondrial matrix. TIM23 complex: (Adopted from: Dudek et al. 2013).

1.3 Involvement of cytosolic factors in mitochondrial protein import

During their synthesis on cytosolic chaperones nascent chains should be kept unfolded until sufficient structural information is available for folding. Thus, cytosolic

chaperones are required to keep the proteins in a native state. Especially in the molecular crowded environment of the cytoplasm, unspecific intermolecular interactions can lead to aggregation of nascent polypeptides. Thus newly synthesized proteins are dependent on the co-translational interaction with chaperones to prevent protein degradation, aggregation and to keep them in an import competent state (Young et al., 2004).

1.3.1. Molecular chaperones

Molecular chaperones or heat shock proteins (HSPs) have been observed in all organisms and they are crucial for the survival of the cell. Initially, HSPs were identified by induction of heat stress or other stress condition in the cell. Later it turned out that they are involved in or required for many cellular functions also under normal growth conditions. The cellular activities of molecular chaperones in both housekeeping tasks and stress conditions depend on their ability to interact with hydrophobic segment of unfolded proteins or proteins that have not yet acquired the native conformation. It has been shown that HSPs interact with proteins to stabilize and protect them from aggregation or degradation (Bukau 1998). Ribosomal translation and protein transfer in the cell are reactions that are prone to expose unfolded segments of proteins to the surrounding aqueous environment or require posttranslational folding/unfolding events (Hartl, 1996a, Netzer and Hartl, 1998). Hence, the essential function of molecular chaperones is connected to proteins and organelle biogenesis. Molecular chaperones are classified into four groups according to their molecular size, Hsp70s, Hsp90s, chaperonins and small heat shock protein (sHsp) Although their basic functions are similar they display completely different structures and participate in diverse cellular process.

1.3.1.1 The Hsp70 family

The Hsp70 proteins are a family of highly conserved ATPases with a molecular weight of approximately 70 kDa. They can be found in prokaryotes and in most compartments of eukaryotic cells (Hartl, 1996). In *S. cerevisiae,* the Hsp70 proteins are divided into four subfamilies; Ssa, Ssb, Sse, and Ssz (stress seventy related). The most important Hsp70 family in the cytosol comprises four members (Ssa1–Ssa4) and the expression of at least one of them is essential for viability (Werner-Washburne et al., 1987, Wegele et al., 2003). At physiological temperature Ssa1 is expressed at high level. At elevated

temperature (37°C), however, its expression is further simulated by a factor of about 10. In contrast, Ssa2 is expressed at the same level at all temperatures (Werner-Washburne et al., 1987). Ssa3 and Ssa4 are expressed at extremely low levels at physiological temperature, but at higher temperatures, the expression is increased (Craig et al. 1989). While the Ssa family members are soluble in the cytosol, Ssb1 and Ssb2 are associated with nascent polypeptides that emerge from translating ribosomes (Nelson et al., 1992, Pfund et al., 1998, Craig and Jacobsen, 1985). Ssz acts similarly. It forms a ribosome-associated complex with Hsp40 and stimulates the mitochondrial translocation of ribosome-nascent chain complexes (Gautschi et al., 2001, Gautschi et al., 2002, Hundley et al., 2002). Finally, at physiological temperatures, Sse1 and Sse2 are expressed at moderate or very low levels, respectively. During heat stress conditions their expression is increased by several fold (Hideyuki et al., 1993).

Hsp70 proteins consist of an N-terminal ATPase domain of about 45 kDa that is followed by a C-terminal peptide or substrate binding domain (SBD) of approximately 18 kDa and a more variable segment of around 10 kDa, which ends in mammalian cells with conserved EEVD motif (Flaherty et al., 1991, Zhu et al., 1996, Hartl, 1996). The Hsp70 proteins assist in a wide range of cellular activities. They have essential roles in the folding of newly synthesized protein, in protein translocation across membranes, in preventing protein aggregation and misfolding, and in the control of the activity of regulatory proteins (Hartl, 1996b, Bukau et al., 2006). This versatility results from i) evolutionary amplification and diversification of the *hsp70* gene which has produced special Hsp70 chaperones, ii) co-chaperones like J proteins and nucleotide exchange factor (NEFs) which are recruited by Hsp70 proteins to fulfill specific functions in the cell, and iii) cooperation of Hsp70 proteins with other chaperones (Bukau et al., 2006, Mayer and Bukau, 2005).

The basic function of Hsp70 proteins is to bind and release hydrophobic segments of unfolded polypeptide chains by an ATP–hydrolytic reaction cycle. ATP binds to the N-terminal domain of Hsp70, induces conformational changes in the SBD that lead to an opening of the substrate-binding pocket. Substrate binding, together with the action of J proteins, results in ATP hydrolysis and closing of the SBD. This results in trapping the substrate proteins (Bukau et al., 2006).

Several findings hint that co-chaperones are required for binding of Hsp70 proteins to substrates or to other chaperones (Mayer and Bukau, 2005). The co-chaperones of

Hsp70 can be subdivided into different functional or structural classes. Members of the first group of co-chaperones have a conserved J-domain that mediates the interaction with their respective Hsp70 partner protein. Examples for these co-chaperones are Hsp40 in eukaryotes and DnaJ in bacteria (Mayer and Bukau, 2005, Hartl, 1996). The J-domain of DnaJ binds to the polypeptide binding domain of DnaK, a bacterial Hsp70. Recruitment of DnaK to DnaJ provides a combined function of two chaperones and of ATP cycling (Hartl, 1996).

Nucleotide exchange factors (NEFs) form the second group of Hsp70 co-chaperones and play a critical role in the functional cycle of Hsp70. They can promote the release of ADP from and binding of ATP to Hsp70, which results in substrate release. GrpE and BAG are two important NEFs. GrpE facilitates nucleotide disassociation from DnaK whereas BAG proteins interact with the eukaryotic, cytosolic Hsp70 (Bukau et al., 2006, Takayama and Reed, 2001).

Another group of Hsp70 co-chaperones contains tetratricopeptide repeat (TPR) dicarboxylate clamp domains that are involved in the regulation of heat shock proteins. This group entails co-chaperones like Hip which interacts with the ATPase domain of human Hsc70 (Höfeld et al., 1995). Hop (Sti1 in yeast) has been identified as a regulator of heat shock proteins and also as a component of the progesterone receptor complex (Nicolet and Craig, 1989, Smith et al., 1993). Hop interacts with the EEVD motif of the C-terminal domains of Hsp70 and Hsp90 (Scheufler et al., 2000, Odunuga et al., 2003). Additionally, studies showed that Sti1 in yeast stimulates the ATPase activity of yeast Hsp70, Ssa1 (Wegele et al., 2003). CHIP was discovered as a TPR motif-containing co-chaperone of eukaryotes (Ballinger et al., 1999). It competes with Hop for binding to the C-terminal domain of Hsp70 and Hsp90 (Nikolay et al., 2004). It also acts as E3-ubiquitin ligase ubiquitinating the Hsc70 substrate *in vitro* and *in vivo* and promoting the degradation of substrates by the proteasome (Meacham et al., 2001, Hohfeld et al., 2001, Connell et al., 2001).

It has also been shown that Hsp70 proteins can dock onto a special TPR domain in Tom70 receptors at the mitochondrial outer membrane. In other words, Tom70 functions like as a membrane-localized co-chaperone (Young et al., 2003).

1.3.1.2 The Hsp90 family

Hsp90 is one of the highly conserved and abundant heat shock proteins in eukaryotes. It is involved in the activation and stabilization of a wide variety of client proteins and in the folding of a defined set of signaling molecules like steroid-hormone receptors and kinases (Welch and Feramisco, 1982, Li and Buchner, 2013). Unlike Hsp70, Hsp90 is not required for *de novo* folding of proteins but it facilitates the final maturation of its target proteins (Nathan et al., 1997). The yeast *S. cerevisiae* contains two sets of related genes of the *HSP82* family: i) *HSC82* which is expressed permanently at a very high level and is moderately stimulated by heat stress, and ii) *HSP82* which is expressed at low levels and is induced more strongly by heat (Borkovich et al., 1989). Both *HSP82* and *HSC82* are essential for viability of yeast cells (Borkovich et al., 1989).

Hsp90 is a flexible protein composed of three different domains (Minami et al., 1994, Ali et al., 2006, Hawle et al., 2006, Chacinska et al., 2005). The N-terminal domain contains an ATP binding site, which has interesting features. For instance, it entails several conserved amino acid residues, which form a lid. This lid is closed in the ATP bound state and is open in the ADP bound state (Ali et al., 2006). The C-terminal domain is required for the permanent homodimerization of Hsp90 and a conserved MEEVD motif in the C-terminal domain functions as a docking site for TPR domain containing co-chaperones (Scheufler et al., 2000). The middle domain (M-domain) is involved in ATP hydrolysis and in the interaction with client proteins and with some chaperones (Meyer et al., 2004). Hsp90 has a highly charged linker segment located between the N-terminal and the M-domain and the dimer association site has been placed at the extreme C-terminal region of the protein (Palmer et al., 1995). These findings show that the Hsp90 monomers are associate via their C-terminal domains and the middle and N-terminal domains bend in opposite direction (Maruya et al., 1999).

The Hsp90 clients include protein kinases, steroid hormone receptors (SHRs) and transcription factors such as p53 (Zhao et al., 2005, McClellan et al., 2007, Picard, 2002). Therefore, Hsp90 is important not only for protein folding but also for other cellular process including signal transduction, intracellular transport and protein degradation (Li and Buchner, 2013).

The different cellular functions of Hsp90 are regulated by the help of co-chaperones and this regulation is a conserved feature of the eukaryotic Hsp90 system. Until now, 20 co-chaperones of Hsp90 have been identified (Li et al., 2012). The co-chaperones regulate

the Hsp90 functions in different ways. Examples for this include inhibition or activation of the Hsp90 ATPase activity or recruitment of specific client proteins. The maturation of Hsp90 also requires different co-chaperones which work together in a cycle (Smith, 1993). The most important co-chaperones of Hsp90, Hop/Sti1 contain a TPR motif and bind to the open conformation of Hsp90 inhibiting its ATPase activity (Onuoha et al., 2008, Richter et al., 2003b). Other co-chaperones such as p23/Sba1 inhibit the Hsp90 ATPase activity by binding preferentially to its N-terminal domain in the closed, nucleotide-bound state (Ali et al., 2006, McLaughlin et al., 2006). Aha1 is another Hsp90 co-chaperone, which has been identified as the only activator of the Hsp90 ATPase (Nathan et al., 1999). Likewise, proteins from the peptidyl-prolyl cis-trans isomerase (PPIase) family, which are also termed immunophilins, can function as co-chaperones of Hsp90 (Li et al., 2012).

Hop/Sti1 binds on two sites on Hsp90 to prevent the N-terminal domains of the two monomers to close, thereby inhibiting ATP hydrolysis completely in a non-competitive manner (Richter et al., 2003b). Furthermore, the presence of three TPR domains in Hop/Sti1 allows simultaneous binding and modulation of Hsp70 and Hsp90, which facilitates client protein transfer between these chaperones (Chen and Smith, 1998, Wegele et al., 2006, Wegele et al., 2003). In the co-chaperone cycle of the Hsp90 system, Hsp40, Hsp70 and client proteins form an early complex which then binds to Hsp90 via Hop/Sti1 to from an intermediate complex (Smith, 1993). Hop/Sti1 serves as an adaptor between Hsp70 and Hsp90 (Chen and Smith, 1998, Johnson et al., 1998, Wegele et al., 2006). Biochemical studies showed that the TPR1 and TPR2 domains bind to the EEVD-motif of Hsp70 and Hsp90, respectively (Scheufler et al., 2000, Brinker et al., 2002). In addition to Hop/Sti1, the third complex contains a protein of the PPIase family such as Cyp40/Cpr6 or Cpr7 and the co-chaperone P23/Sba1 has also been found in the chaperone cycle (Smith, 1993, McLaughlin et al., 2006). Hop/Sti1 binds to the open conformation of Hsp90 and inhibits its ATPase activity. The other EEVD motif of Hsp90 is occupied by a PPIase protein. Hsp90 then adopts a closed conformation after binding of ATP. Binding of P53/Sba1 stabilizes the closed state of Hsp90 and promotes exit of Hop/Sti1 from the complex. Finally, in the last step another PPIase protein associates with Hsp90 to form the late complex. After hydrolysis of ATP, P23/Sba1 and the folded client are released from Hsp90 (Li et al., 2011).

1.3.1.3 The chaperonin system

Chaperonins have an essential function in ATP-dependent folding of a subset of proteins under both normal and stress conditions. There are two distinct groups of chaperonins: i) members of the GroEL (or Hsp60) family and ii) TRic family chaperonins (Hartl, 1996). The GroEL type chaperonins in eubacteria, mitochondria and chloroplasts consist of two stacked seven-membered rings that functionally cooperate with small co-factors like Hsp10/GroES. GroEL/GroES proteins of *E. coli* are the best studied members of the Hsp60/Hsp10 chaperonin system. In contrast, the second group of chaperonins, the TRiC family, occurs in archaebacteria and the cytoplasm of eukaryotic cells. Chaperones of this family consist of eight or nine membered double rings that act independently of Hsp10 (Harlt, 1996; Horwich et al., 2007; Vabulas et al., 2010).

1.3.1.4 Small heat shock proteins

Small heat shock proteins (sHSPs) are molecular chaperones, which are distributed, in different species from bacteria to humans. So far 10 proteins (HSB1-HSB10) have been assigned to the superfamily of sHSPs (Zeng et al., 2013). These proteins have a conserved C-terminal, α-crystallin domain that forms a β-sheet sandwich with a molecular mass of 18-24 kDa (Mymrikov et al., 2011). sHSPs act under stress conditions such as heat shock or protein overexpression. They form an oligomeric complex with 12-24 subunits that forms a hollow sphere with an opening to the inside (Kim et al., 1998). This complex binds specifically to non-native proteins and cooperates with other ATP-dependent chaperones like Hsp70 to prevent protein aggregation (Jakob and Buchner, 1994, Horwitz, 1992, Walter and Buchner, 2002). The sHSP system in *S. cerevisiae* consists of two proteins, Hsp26 and Hsp42. Hsp42 is active under all conditions tested *in vitro* and *in vivo,* whereas Hsp26 is active only during heat stress (Haslbeck et al., 2004, Walter and Buchner, 2002).

1.4 Chaperones involved in delivery of nascent polypeptides to the mitochondrial surface

There are two processes to minimize or prevent aggregation and misfolding of mitochondrial precursor proteins during their translocation in an unfolded conformation across mitochondrial membranes. The first is coupling of translation and translocation,

and the second involves recruitment of molecular chaperone complexes and cytosolic factors to stabilize mitochondrial precursor proteins (Beddoe and Lithgow, 2002).

Previous studies indicated that translationally active ribosomes are present on the surface of mitochondria (Ades and Butow, 1980, Suissa and Schatz, 1982). It seems that sequences within the mRNA might act as binding sites for factors that i) target the mRNA to the vicinity of the mitochondria, or ii) stabilize the mRNA to increase their chances to reach the mitochondrial surface before being degraded (Gratzer et al., 2000). In this way many polysomes are targeted to the mitochondrial surface and precursor proteins are directly translated in the vicinity of the TOM complex without being exposed to the cytosol where they could aggregate (Beddoe and Lithgow, 2002). In a few cases, mRNAs encoding mitochondrial precursor proteins were suggested to contain *cis* sequences which target the precursor to the mitochondrial surface (Lithgow et al., 1997). For example, the mRNA coding for the Atm1 transporter of *S. cerevisiae* is targeted to the mitochondria (Corral-Debrinski et al., 2000). Interestingly, the β-subunit of the F_1F_0-ATPase is translated close to the mitochondria, whereas the mRNA coding for the α-subunit of this complex is uniformly distributed in the cytosol (Egea et al., 1997). Since there is no absolute coupling of translation and translocation of mitochondrial precursor proteins, many of the mitochondrial precursor proteins are translated on cytosolic ribosomes that are distant from mitochondria. These precursor proteins can be prone to aggregation and misfolding, but different chaperones are known that bind and stabilize them and hence prevent their aggregation. In other words, by the help of these molecular chaperones, the precursor proteins are kept in an import competent state.

Hsp70 is one of the important molecular chaperones, which are involved in this process. In Mammalian cells, Hsc70 acts co-translationally to prevent aggregation or misfolding. It has been shown that it interacts with nascent chains of mitochondrial and non-mitochondrial proteins (Beckmann et al., 1990, Frydman et al., 1994). In yeast, different isoforms of Hsp70 work in this way. Ssb1 associates with ribosome and then interacts with newly translated mitochondrial protein to stimulate their import into mitochondria. Ssz1 can promote import of mitochondrial precursor protein that are bound to ribosomes (Gautschi et al., 2001). Ssa1 also stimulates the import of precursor proteins into isolated mitochondria.

Another molecular chaperone, Hsp40/Ydj1 seems to act in the import of mitochondrial precursor proteins by cooperating with Hsp70/Ssa1 (Becker et al., 1996, Cyr et al., 1992). A previous study has suggested that Ydj1 has a farnesyl moiety attached to its C-terminal domain, allowing the protein to be localized to intracellular membranes (Caplan et al., 1992).

The nascent-chain associated complex (NAC) also plays an important role in mitochondrial protein import (Funfschilling and Rospert, 1999). NAC is a heterodimeric protein complex that associates with ribosomes and can be cross-linked to different nascent chains including mitochondrial precursor proteins (Wiedmann et al., 1994). Similarly, the ribosome-associated complex (RAC) is a heterodimeric protein complex consisting of Ssz1 and Zuo1 that keeps client proteins in an import component state (Gautschi et al., 2001).

Additionally, the mitochondrial import-stimulating factor (MSF) was shown in mammalian cells to specifically recognize aggregated mitochondrial precursor proteins and to restore their solubility and import capability (Hachiya et al., 1993, Komiya et al., 1997). Additionally, the analysis of the *in vitro* import of mitochondrial precursor protein suggested that MSF binds to the Tom70 receptor. The precursor proteins were then transferred to the Tom20 receptor upon ATP hydrolysis (Komiya et al., 1996, Hachiya et al., 1995). Human Tom20 consists of five segments: an N-terminal membrane anchor, a linker segment with charged amino acid, a TPR motif, a glutamine rich segment and a C-terminal segment (Goping et al., 1995). In mammalian cells, the arylhydrocarbon receptor-interacting protein (AIP) was identified as a Tom20-interacting protein which plays an important role in mitochondrial import of Tom20-dependent preproteins (Yano et al., 2003). It belongs to the PPIase family that is ubiquitous in prokaryotes and eukaryotes (Galat and Metcalfe, 1995). AIP contains three TPR motifs that facilitate binding to Hsp90 (Young et al., 1998, Bell and Poland, 2000). A previous study indicates that AIP binds specifically to mitochondrial presequence containing proteins and also to the Tom20 receptor. It has been suggested that AIP, with the help of Hsc70, might keep presequence containing proteins in an unfolded state. This complex is then targeted to Tom20 and finally forms a ternary complex comprised of Tom20, AIP, Hsc70 and the precursor protein (Yano et al., 2003). Moreover other studies identified Djp1 as a cytosolic co-chaperone mediates the

import of Mim1 in mitochondrial outer membrane by the help of Tom70 receptor (Papic et al., 2013).

Until now, only a few cytosolic factors involved in mitochondrial protein import were identified and functionally characterized. It is still unclear whether cytosolic factors interact with all precursor proteins to protect them from aggregation and misfolding or whether they are specific for just a small subset of mitochondrial precursor proteins. So far, the direct interaction between molecular chaperones and the mitochondrial import machinery has only been shown in the import of metabolite carrier proteins of the inner mitochondrial membrane.

The molecular chaperones that is involved in the import of metabolite carrier proteins of the inner membrane seems to have a passive function in keeping the precursor proteins in an unfolded state and an active role in targeting the precursor to the mitochondrial receptors. Cytosolic Hsp70 is generally involved in the folding of proteins and in preventing the proteins from aggregation. It could be that the role of Hsp70 in mitochondrial protein import is an extension of this activity. The cytosolic domain of Tom70 contains seven TPR motifs. The C-terminal part of this domain is responsible for the recognition of internal sequences within precursor proteins (Brix et al., 2000). Interestingly, the N-terminal segment of Tom70 has a similar function as the TPR domain-containing co-chaperones (Hop/sti1) of Hsp70 and Hsp90 (Scheufler et al., 2000). This means that Tom70 is a membrane-localized co-chaperone recognizing Tom70-dependent precursor proteins in the cytosol that are associated with a multi-chaperone complex including Hsp90 and Hsp70 in mammals or only Hsp70 in yeast. Docking of the chaperone-precursor complex to Tom70 is necessary for the recognition of internal targeting signals within the precursor protein by the core portion of Tom70. Subsequently, multiple Tom70 dimers are recruited to form a high molecular weight complex. The Hsp70 and Hsp90 chaperones remain bound to help Tom70 in preventing the aggregation. Finally ATPase cycling by the chaperone complex induces the transfer of precursor proteins from Tom70 to the import pore. ATPase cycling, which is mediated by the chaperone complex, induces the transfer of preproteins from Tom70 to the import pore (Fig. 1.4).

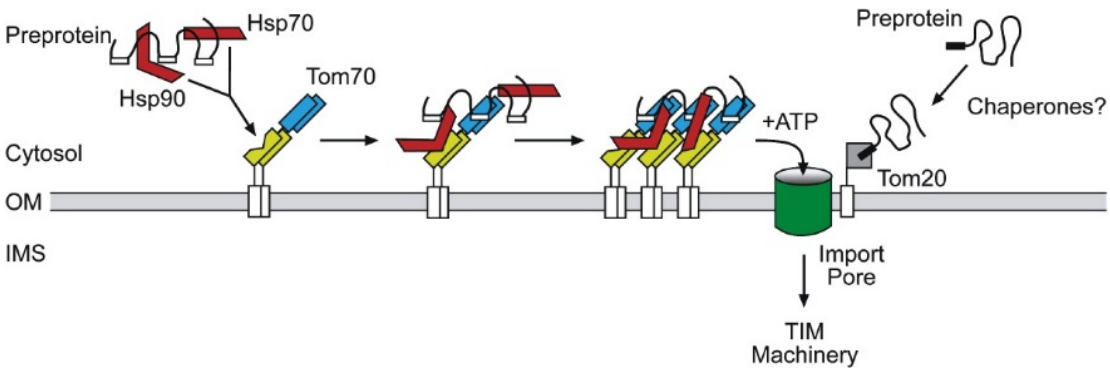


Fig. 1.4: Role of Hsp70/Hsp90 complex in import of mitochondrial metabolite carrier precursor proteins in mammalian cells; Tom70 receptor acts as a membrane co-chaperone and recognizes the internal signal of metabolite carrier precursor proteins, while the latter is associated with a multi-chaperone complex including Hsp90 and Hsp70 in mammals or only Hsp70 in yeast. Docking of the chaperone complex to Tom70 is necessary for recognition of internal targeting signals by the core portion of Tom70. Subsequently, multiple Tom70 dimers are recruited to form a high molecular weight complex. Hsp70 and Hsp90 remain bound to help Tom70 in preventing the aggregation and ATPase cycling. Finally ATPase cycling induces the transfer of preproteins from Tom70 to the import pore (Adopted from: Young et al., 2003).

1.5 Aim of this study

The involvement of the cytosolic factors Hsp70 and Hsp90 in the targeting of mitochondrial proteins was demonstrated so far only for precursors with internal signals. However, it is still unclear which cytosolic factors are involved in targeting presequence-containing mitochondrial proteins to the Tom20 receptor. The general aim of this study was to identify such cytosolic factors, to characterize their involvement in stabilization and targeting of precursor proteins, and to study the physiological role they play in mitochondrial biogenesis.

2. Materials and Methods

2.1 Materials

2.1.1 *E. coli* and yeast strains

E. coli cells of the strain XL1-Blue and BL-21(DE3) were used for cloning and expression of recombinant proteins, respectively. Yeast strains used in this study are listed in Table 1.

Table 2.1. List of yeast strains

Strain	Genotype	Reference
W303α	*MATα; ade2-1, can1-100, his3-11, leu2-3, leu2-112, trp1-1, ura3-1*	Rothstein, 1989
YPH449	*MATa; ade2-10, his3-200, leu2-1, trp1-63, ura3-52, lys2-801*	Sikorski and Hieter, 1989
JSY7452	*MATα; ade2-1, leu2-3, his3-11.15, trp1-1, ura3-1, can1-100*	Kondo-Okamoto, 2006
sti1Δ	W303α, *sti1::HIS3*	Strain collection, Rapaport group
sti1Δ	YPH499, *sti1::HIS3*	Strain collection, Rapaport group
*tom20*Δ	W303α, *sti1::Kan*MX4	Müller et al., 2011
*tom20*Δ*sti1Δ*	W303α, *sti1::HIS3, tom20::Kan*	This study
*tom70/71*Δ	JSY7452, *tom70::TRP1,tom71::HIS3*	Kondo-Okamoto et al., 2006
*mim1*Δ	W303α, *mim1::Kan*MX4	Dimmer et al.
*mim1*Δ	YPH499, *mim1::HIS3*	Popov-Celektic et al. 2008
*mim2*Δ	W303α, *mim2::HIS3*	Dimmer et al.
*mim1*Δ*sti1*Δ	YPH499, *sti1::Kan, mim1::HIS3*	Strain collection, Rapaport group

2.1.2 Media

The most frequently used media in this study for *E. coli* and yeast strains are listed in Table 2.2 and Table 2.3, respectively. All media were autoclaved before use and antibiotics were filtered before adding to the media.

Table 2.2. Media for *E. coli*

Media	Composition
LB media	1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0
LB solid media	Liquid LB medium supplemented with 1.5 % (w/v) agar.
LB + Amp	LB media (liquid or solid) supplemented with Ampicillin (100 μg/ml)

Table 2.3. Media for yeast

Media	Composition
YP media	2% (w/v) bacto peptone, 1% (w/v) yeast extract, pH 5.5. Carbon sources were added from separately autoclaved stock solutions to a final concentration of 2% (w/v) glucose (YPD) or 3% (w/v) glycerol (YPG).
YP agar	YP liquid medium supplemented with 1.5% (w/v) agar.
S media (Synthetic medium)	0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 0.3 μM adenine, 0.5 μM uracil (uracil is not added for S-Ura media), 1% (v/v) amino acid solution, carbon sources (2% (w/v) glucose (SD) or 3% (w/v) glycerol (SG)).
S agar media	S liquid media supplemented with 1.5 (w/v) agar
D-Glucose stock solution	40% (w/v) D-glucose
Glycerol stock solution	100% glycerol
100 × stock amino acid	0.2% (w/v) arginine, 0.4% (w/v) tryptophan, 1% (w/v) leucine, 0.4% (w/v) lysine, 0.2% (w/v) histidine, 0.6% (w/v) phenylalanine, 0.2% (w/v) methionine.
2.4 M sorbitol	437.2 g/l sorbitol.
Sporulation medium	1% (w/v) potassium acetate, 0.1% (w/v) bacto-yeast extract, 0.05% (w/v) glucose, 2% (w/v) bacto agar supplemented with; Leu, Trp, Ura, Ade

2.1.3 Buffers

2.1.3.1 Buffers for molecular biology

Table 2.4. Buffers for agarose-gel electrophoresis.

Buffer	Composition
TAE buffer	40 mM Tris-Base, 1.14 ml/l acetic acid, 1 mM EDTA, pH 8.0
DNA loading buffer	6% (v/v) glycerol, 0.05% (w/v) bromophenol-blue, 0.05% (w/v) xylene cyanol.

Table 2.5. Buffers for small scale plasmid isolation from *E. coli.*

Buffer	Composition
E1	50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A.
E2	200 mM NaOH, 1% (w/v) SDS in water.
E3	3 M potassium acetate, pH 5.5, adjusted with acetic acid.

Table 2.6. Buffers for PCR.

Buffer	Composition
10 × Pfu buffer with $MgSO_4$	100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% (v/v) Triton X-100, 1 mg/ml BSA, 20 mM $MgSO_4$, 200 mM Tris, pH 8.8
10 × Taq buffer with $(NH_4)_2SO_4$	200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 750 mM Tris-Hcl, pH 8.8

Table 2.7. Buffers for preparation of chemical competent *E. coli* cells.

Buffer	Composition
TfbI buffer	30 mM potassium acetate, 100 mM RbCl, 100 mM $CaCl_2$, 50 mM, $MnCl_2$, 15% (v/v) glycerol, pH adjusted to 5.8 with acetic acid.
TfbII buffer	100 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) glycerol, pH adjusted to 6.5 with NaOH.

2.1.3.2 Buffers for isolation of mitochondria

Table 2.8. Buffers used for mitochondrial isolation. All buffers were freshly prepared.

Buffer	Composition
Resuspension buffer	100 mM Tris, 10 mM DTT, without pH adjusting.
Spheroblasting Buffer	1.2 M Sorbitol, 20 mM potassium phosphate buffer, pH 7.2.
Homogenization buffer	0.6 M sorbitol, 1 mM EDTA, 1 mM PMSF, 0.2% (w/v) fatty acid free BSA, 10 mM Tris adjusted to pH 7.4 with HCl.
SEM buffer	250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH adjusted to 7.4 with KOH.

2.1.3.3 Buffers for protein analysis

Table 2.9. Buffers for SDS-PAGE.

Buffer	Composition
2 × Laemmli loading buffer	4% (w/v) SDS, 20% (v/v) glycerol, 0.02% (w/v) bromophenol-blue, 5% (v/v) 2-mercaptoethanol, 160 mM Tris, pH adjusted to 6.8 with HCl.
Running buffer	50 mM Tris, 380 mM glycine, 0.1 % (w/v) SDS.
1.0 M Tris-HCl, pH 6.8	
1.0 M Tris-HCl, pH 8.8	

Table 2.10. Buffer for Western blotting.

Buffer	Composition
Blotting buffer	20 mM Tris, 150 mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol.

Table 2.11. Buffers for immunodetection.

Buffer	Composition
Ponceau staining buffer	0.4 g Ponceau S, 8.5 ml 72% (w/v) TCA in 200 ml cold H_2O
TBS buffer	5 mM Tris, 150 mM NaCl, pH adjusted to 7.5 with HCl.
TBS buffer + 0.05% (v/v) Triton X-100	TBS buffer + 0.05% (v/v) Triton X-100.
Blocking buffer	5% (w/v) dry skim milk in TBS buffer.
ECL	0.2 mM p-coumaric acid, 1.25 mM Luminol, 100 mM Tris, pH adjusted to 8.5 with HCl; H_2O_2 30% (w/v) was mixed before usage with ECL solution in ratio 1:1000.

Table 2.12. Buffers for *in vitro* import of proteins into isolated mitochondria.

Buffer	Composition
F5-import buffer	250 mM sucrose, 10 mM MOPS, 80 mM KCl, 5 mM $MgCl_2 \times 6H_2O$, 3% (w/v) fatty acid free BSA, pH adjusted to 7.2 with KOH.
SEM-K80 buffer	SEM buffer + 80 mM KCl

Table 2.13. Buffers For *in vivo* pulse-chase experiment

Buffer	Composition
4 × TENN	200 mM Tris, pH 8, 20 mM EDTA, 2% (v/v) NP40 600 mM NaCl
TENNS	5% (w/v) sucrose, 1% (v/v) NP40, 0.5 M NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA

Table 2.14. Buffers for stripping of nitrocellulose and PVDF membranes.

Buffer	Composition
Stripping buffer	86 mM Tris-HCl (pH 6.8), 2.9 % (w/v) SDS, 1% (v/v) β-mercaptoethanol.
TBST buffer	TBS buffer supplemented with 0.05% (v/v) Tween-20.

2.1.3.4 Buffers for purification of proteins and pull-down experiments

Table 2.15. Buffers for purification of proteins with GST-tag.

Buffer	Composition
GST basic buffer	20 mM HEPES-NaOH (pH 7.25), 100 mM NaCl, 1.5 mM $MgCl_2$.
GST lysis buffer	0.2 mg/ml Lysozyme, 2 mM PMSF, 1 mM DTT, 3 mM EDTA, 1% Triton X-100, EDTA-free Complete protease inhibitors (Roche) in GST basic buffer.
GST elution buffer	15 mM reduced L-Glutathione in GST basic buffer, pH adjusted to 7.25 with NaOH.

Table 2.16. Buffers for pull-down with Ni-nitrilotriacetic acid (Ni-NTA) agarose beads.

Buffer	Composition
lysis buffer	20 mM sodium phosphate buffer, 300 mM NaCl, 2 mM PMSF, EDTA-free Complete protease inhibitors (Roche), 10 mM imidazole, pH 7.5.
Wash buffer	20 mM sodium phosphate buffer, 300 mM NaCl, 50 mM imidazole, pH 7.5.

2.1.4 Chemicals and stock solutions

Table 2.17. Chemicals and stock solutions.

Chemical	Concentration	Solvent	Company
Ampicillin	100 mg/ml	H_2O	Sigma
ATP	0.2 M	H_2O	Sigma
BSA	1 mg/ml	H_2O	Sigma
DTT			Gerbu

PEG	50% (w/v)	H_2O	Sigma
EDTA			Roth
Geneticindisulfate (G418)	100 mg/ml	1 mg/ml	Roth
L-Glutathione, reduced	15 mM	GST-buffer	Sigma
IPTG	1 M	H_2O	Gerbu
Luminol	4.4%	DMSO	Sigma
Protein ladder			Fermentas
DNA ladder			Thermo scientific
Midori green			Nippon genetic,
PMSF	0.2 M	Ethanol	Serva
Complete protease inhibitors		H_2O	Roche
RNase inhibitor	40 U/µl		Promega
SDS	10% (w/v)	H_2O	Roth
Salmon sperm DNA	10 mg/ml	H_2O	Roth
Triton X-100	20% (v/v)	H_2O	Roth
Cycloheximide	100 µg/ml	H_2O	Sigma
Bradford reagent		SEM buffer	Roth
Panceau		H_2O	Serva
p-Coumaric acid	1.5%	DMSO	Sigma

2.1.5 List of plasmids

Table 2.18. Plasmids used in this study. Selection in *E. coli* was resistance to ampicillin.

Name	Promotor	Marker (Yeast)	References
pES426×cyb2-DHFR-His-TAG4	ADH	TRP	This study
pES426×cyb2-DHFR-His-TAG8	ADH	TRP	This study
pES426×cyb2-DHFR-His-TAG10	ADH	TRP	This study
pES426×cyb2-DHFR-His-TAG13	ADH	TRP	This study
pES426×cyb2-DHFR-His-TAG16	ADH	TRP	This study
pYX132×pSu9-DHFR-His	TPI	TRP	This study
pYX132×DHFR-His	TPI	TRP	This study
pYX113×pSu9-DHFR-HA	GAL	URA	This study
pES426×Cyb2-DHFR-His_6	ADH	TRP	This study
pGEM4×pSu9-DHFR	SP6		Pfanner et al. 1987
pRS426×DHFR-His_6	TPI	URA	A. Schmitt
pRS426×pSu9-DHFR-His_9	TPI	URA	A. Schmitt
pYes2×Cyb2-DHFR-His_6	Gal	URA	A. Schmitt
pYX132×Mdm38-HA	TPI	TRP	K.S. Dimmer
pYX142-mt GFP	TPI	LEU	Westermann et al. 2000
pES426	ADH	TRP	Laboratory stock
pRS426	TPI	URA	Laboratory stock
pGEX-4T-1	Tac		J. Dukanovic
pGEX-4T-1-$Tom70_{cd}$	Tac		J. Dukanovic
pGEX-4T-1-$Tom20_{cd}$	Tac		J. Dukanovic

pPR1-PGK1+3SUP4-tRNACUA (= pBpa)	TPI	TRP	A. Schmitt

2.1.6 Oligonucleotides

Table 2.19. Oligonucleotides used in this study.

Name	Sequence
Cyb2-DHFR-His-TAG4 FW	5′ CCC GAA TTC ATG CTA AAA TAG AAA CCT TTA CTA AAA ATC TCG AAG 3′
Cyb2-DHFR-His-TAG8 FW	5′ CCC GAA TTC ATG CTA AAA TAC AAA CCT TTA TAG AAA ATC TCG AAG 3′
Cyb2-DHFR-His-TAG10 FW	5′ CCC GAA TTC ATG CTA AAA TAC AAA CCT TTA CTA AAA TAG TCG AAG 3′
Cyb2-DHFR-His-TAG13/16 FW	5′ CCC GAA TTC ATG CTA AAA TAC AAA CCT TTA CTA AAA ATC TCG AAG 3′
Cyb2-DHFR-His TAG Rev	5′ CCC AAG CTTTTA ATG GTG ATG GTG ATG GTG ATG GTG 3′
pSu9-DHFR Fwd	5′ AAA GAA TTC ATG GCC TCC ACT CGT GTC 3′
pSu9-DHFR Rev	5′ AAA AAG CTT GTC TTT CTT CTC GTA GAC 3′
Sti1 Fwd	5′GCC GCA ATT GAC CAA ACT ATT G 3′
Sti1 Rev	5′ ATT GTT GAC GTA AAG TTG TGC C 3′

2.1.7 Antibodies

All primary antibodies were diluted in TBS buffer and stored at -20°C except for α-His (Biomol) which was diluted in PBS buffer supplemented with 0.09 % (w/v) NaN_3 and stored at 4°C. All antibodies were raised in rabbit except for the antibody against DHFR (Bioscience), which was raised in mouse. The secondary antibodies goat anti-rabbit IgG (H+L)-HRP-conjugate and goat anti-mouse IgG (H+L)-HRP-conjugate (Biorad) were diluted in blocking buffer. All antibodies used in this study are listed in Table 2.20.

Table 2.20. Primary antibodies used in this study.

Primary antibody	Marker for	Dilution
α-DHFR		1:250
α-His6		1:4000
α-Tom20	MOM	1:2000

α-Tom22	MOM	1:500
α-Tom40	MOM	1:4000
α-Tom70	MOM	1:2000
α-Aco	Mitochondrial matrix	1:4000
α-Hep1	Mitochondrial matrix	1:500
α-Pic2	MIM	1:2000
α-Porin	MOM	1:4000
α-Fis1	MOM	1:500
α-Fum	Mitochondrial matrix	1:500
α-Hsp60	Mitochondrial matrix	1:10000
α-Yah1	Mitochondrial matrix	1:1000
α-Aha1	Cytosol	1:10000
α-Bmh1	Cytosol	1:1000
α-Hsp26	Cytosol	1:8000
α-Hsp82	Cytosol	1:20000
α-Hxk1	Cytosol	1:5000
α-Sba1	Cytosol	1:5000
α-Sti1	Cytosol	1:20000
α-Ssb2	Cytosol	1:200
α-Ydj1	Cytosol	1:50000
α-Ssa1	Cytosol	1:20000
α-Sis1	Cytosol	1:20000
α-pSu9		1:1000

2.1.8 Enzymes

Restriction enzymes were used in their corresponding buffer according to the manufacturer´s instructions.

Table 2.21. Enzymes used in this study.

Enzyme	Source
T4-DNA-Ligase	Fermentas
Shrimp Alkaline Phosphatase	Fermentas
Taq DNA polymerase	Fermentas
Pfu DNA polymerase	Fermentas
Proteinase K	Roche
Zymolyase 20T	Seikagaku Biobusiness
SP6 RNA polymerase	Promega
RNase A	Applichem
Restriction endonucleases (EcoRI, HindIII)	New England Biolabs
Lysozyme	Serva

2.2 Methods

2.2.1 Methods in molecular biology

2.2.1.1 Polymerase Chain Reaction (PCR)

PCR was used to amplify DNA fragments from yeast genomic or plasmid DNA (Saiki, 1985). The standard PCR reaction mix was performed as described in Table 2.22 and 2.23.

Table 2.22. Reaction mixture for PCR

Component	Volume
Plasmid DNA	1 µl (1 ng)
dNTP mix (10 mM)	2 µl
10 × Pfu Buffer with $MgSO_4$	10 µl
5′ primer (20 pmol)	1 µl
3′ primer (20 pmol)	1 µl
H_2O	83 µl
Pfu Polymerase (2.5 U/µl)	2 µl

Table 2.23. Thermocycling conditions for PCR

Step	Temperature	Time
1	95°C	5′
2	95°C	1′
3	55°C	1′
4	75°C	1′30′′ → Step 2: × 10
5	95°C	1′
6	65°C	1′
7	75°C	1′ 30′′ → Step 5: × 35
8	75°C	10′
9	4°C	Hold

2.2.1.2 DNA purification and analysis

Agarose gel electrophoresis was used to separate DNA fragments according to their size. For this purpose agarose gels were prepared with 0.5-2% (W/V) agarose in TAE buffer and 3% midori green. DNA samples were mixed with DNA Sample buffer and loaded on the gel. Electrophoretic separation was performed in TEA buffer at 70 or 120 V for small or big gels, respectively. For estimating the length of DNA fragments, DNA ladder mix (Thermo scientific) was applied in parallel. Visualization of DNA fragments was performed with UV light.

2.2.1.3 DNA extraction from agarose gel

To obtain purified DNA fragments, which can be used for further investigation, DNA fragments of interest were cut out of the agarose gel with a scalpel and subsequently extracted and purified using the DNA extraction kit (Fast Gene^{TM} or PEQLAB) according to the manufacturer´s instructions. Finally, DNA was eluted in 30 µl of sterile H_2O.

2.2.1.4 Restriction digestion of DNA

The digestion of inserts and host vectors was performed using high-fidelity restriction enzymes (New England Biolabs) in respective recommended buffer. For sub-cloning 3 µg of insert and vector DNA were digested by 1 µl (20 U) of each endonucleases in a total volume of 20 µl. For analytical reaction, 12.5 µl plasmid DNA, which obtained from "mini-preparation", was incubated with 0.25 µl (5 U) of each enzyme in final volume of 15 µl. The restriction digestion mixture was incubated at 37°C for one hour. Then, the restriction enzymes were inactivated by incubation at 65°C for 20 min. The resulting DNA fragments were analyzed by agarose gel electrophoresis.

2.2.1.5 Dephosphorylating of DNA fragments

The digested DNA typically possesses 5´-phosphate group that is required for ligation. To prevent self-ligation and to increase the following ligation efficiency, the phosphate group was removed by addition of one µl shrimp alkaline phosphates (SAP) and incubation for another one hour at 37°C. After inactivation of SAP for 20 min at 65°C both insert and vector were loaded on agarose gel electrophoresis for extraction and purification of the DNA fragment of interest.

2.2.1.6 Ligation of DNA

After purification, ligation of vector (100 ng) and insert (1.3 or 7 fold molar ratio to the vector) was performed using one µl T4 DNA ligase and the corresponding buffer in a final reaction volume of 15 µl. Reaction mixture was incubated at 4 °C overnight while stay afloat in the water bath. After inactivation of T4 DNA ligase for 15 min at 65°C, the reaction mixture was transformed into *E. coli* competent cells.

2.2.1.7 Preparation and transformation of *E. coli* competent cells

Preparation of chemically competent cells

Preparation of competent *E. coli* cells was performed using the strains XL1-Blue or BL21. An overnight pre-culture of cells was grown at 37°C in 50 ml LB medium without ampicillin. Then the pre-culture was diluted to an OD_{600}=0.1 in 400 ml LB media and cells were incubated at 37°C till they reached an OD_{600}=0.5. Afterward the culture was placed on ice to cool down and then cells were harvested by centrifugation (3000 g, 10 min, 4°C). The cells pellets were resuspended in 160 ml TfbI buffer and kept 15 min on ice. Subsequently, cells were harvested again by centrifugation (3000 g, 10 min, 4°C), resuspended in 16 ml TfbII buffer, and kept on ice for 15 min. The resulting competent cells were aliquoted in samples of 200 µl in precooled Eppendorf tubes, snap frozen in liquid nitrogen, and stored at -80 °C.

Tarnsformation of *E. coli* competent cells

Transformation of *E. coli* cells with plasmid DNA was performed using the strains XL1-Blue or BL21. For transformation, the ligation mixture (15 µl) was added to 200 µl of competent cells and incubated for 40 min at 4°C. Afterward, the cells were subjected to a heat shock at 42°C for 90 s and immediately transferred to ice for 15 min. Then one ml of LB medium was added to the mixture and cells were incubated for one hour at 37°C with slight shaking. Cells were collected by centrifugation (16000 g, 30 sec, RT), resuspended in 100 µl LB medium, plated on LB agar medium supplemented with ampicillin (100 µg/ml), and finally incubated overnight at 37°C. Single colony were picked and incubated in liquid medium for further analysis.

2.2.1.8 DNA isolation from *E. coli* cells

For isolation of plasmid DNA, single colonies containing plasmid DNA were picked from solid LB supplemented with Ampicillin and applied for liquid culture.

2.2.1.9 Small scale plasmid DNA preparation (“Mini prep”)

To isolate small amount of plasmid DNA, alkaline lysis method was applied (Birnboim and Doly, 1979). Single colonies were picked and cultured for 16 h at 37°C in five ml LB supplemented with ampicillin. Next, one ml of culture was collected by centrifugation (16000 g, 30 sec, RT) and cells were resuspended in 300 µl E1 buffer.

Alkaline lysis performed by adding 300 µl E2 buffer and subsequent incubation for 5 min at RT. After neutralization by adding 300 µl E3 buffer, plasmid DNA was separated from cell debris by centrifugation (15000 g, 15 min, RT). Subsequently supernatant were transferred for precipitation to a reaction tube containing 600 µl of 96% isopropanol. Sample were centrifuged (15000 g, 15 min, 4°C), the supernatant discarded and DNA pellet was washed with ice-cold 70% (V/V) ethanol and dried for 5-10 min at 50 °C. DNA plasmid was resuspended in 30 µl water and stored at -20°C.

2.2.1.10 Medium scale plasmid DNA preparation ("Midi prep")

For isolation of medium amounts of plasmid DNA the Pure Yield Plasmid Midiprep System (Promega) was used according to the manufacturer's instruction. After determination of DNA concentration, isolated plasmids were stored at -20 °C.

2.2.2 Methods in yeast genetics

2.2.2.1 Cultivation of *S. cerevisiae* cells

Yeast cells were grown on full or selective media, with fermentable (as glucose, galactose, sucrose) or non-fermentable (glycerol or lactate) carbon sources. Liquid cultures were grown at 30°C and moderate shaking (120 rpm). The growth of cells was monitored by determination of OD_{600}. Yeast cultures on solid media were stored for up to several weeks at 4°C. For long term storage, yeast cells were resuspended in 15% (v/v) glycerol and stored at -80°C.

2.2.2.2 Transformation of yeast cells

For the transformation of yeast cells, the lithium acetate method with slight modifications was used (Gietz, 1995). Cells were grown on solid YPD medium overnight at 37°C. A small amount of cells were scraped from the YPD plate and suspended in one ml H_2O. After centrifugation (16000 g, 30 sec, RT), the cells were resuspended in one ml of 100 mM sterile lithium acetate. Cells were then incubated at 30°C for 5 min with slight shaking (500 rpm). Afterwards, the cells were harvested again by centrifugation (16000 g, 30 sec, RT) and resuspended in a solution containing the following ingredients: 240 µl 50% (w/v) polyethylene glycol 3350, 55 µl water, 36 µl one M lithium-acetate, 10 µl of a solution with 5 mg/ml heat-denatured salmon sperm DNA, and 5 µl DNA (100-600 ng/µl). After thorough mixing, the mixture was incubated at 42°C for 30 min with shaking at 800 rpm. Subsequently, cells were

sedimented by centrifuge (16000 rpm, 30 sec, RT) and streaked on a plate of the appropriate selective medium. Plates were incubated at 30°C for 2-5 days until colonies appeared. Transformed colonies were streaked on fresh plates for further experiments. For transformation of DNA fragments for subsequent homologous recombination, yeast cells from logarithmic growing liquid cultures were used instead of cells from plate.

2.2.2.3 Mating of yeast strains and tetrad analysis

For creating double-deletion yeast strains, two types of cells harboring each a single deletion and having a different mating type (*MATa* or *MATα*) were streaked on separate YPD plates as parallel line and incubated overnight at 30°C. Replica plating was done with a velvet-covered disk onto new selective medium. After the first plate was replicated on new selective medium, the cell lines of the second plate were replicated on the same new plate perpendicular to the previous cells. The new plate was incubated at 30°C for a couple of days to allow growth of only mated diploid cells. After growing, the heterologous diploid knock out cells, were streaked on to a sporulation medium and incubated at room temperature to stimulate sporulation. The amount of sporulated cells was monitored by microscopy.

Subsequently, a small amount of cells was picked from the sporulation plate and resuspended in solution containing 1.2 M sorbitol supplemented with 100 µg/ml zymolyase. Digestion of the cell wall of the asci was performed by incubation for 10 min at 30°C. One drop of this mixture was spotted on a new YPD plate. Tetrad dissection was done with micromanipulator (ZEISS, Axioscope 40). Finally, single ascis were selected and divided into four spores and each spore was shifted by a tiny needle and located at a certain position on the plate. After incubation at 30°C for a few days the spores grew out to colonies which were then streaked on a new selective medium to analyze growth by auxotrophic markers. The mating type of single colonies was determined by mating-type-PCR (Huxley, 1990).

2.2.3 Methods in cell biology

2.2.3.1 Drop Dilution Assay

Drop dilution assays were performed to compare the growth phenotype of yeast strains. For this assay, yeast cells were inoculated in 20 ml liquid culture of YPD or synthetic medium. The culture was diluted to an OD_{600} of 0.2 and grown further to reach to an

OD_{600} of one. The cells were harvested by centrifugation (3000 g, 5 min, RT) washed once with water and resuspended in sterile water to an OD_{600} of 2.0. The OD_{600} of all strains was adjusted to the same value with sterile water. Cell suspensions of the strains to be analyzed were diluted with water in a fivefold series and five µl of each dilution was spotted on solid media. The plates were incubated at 15°C, 30°C or 37°C for up to 7 days.

2.2.3.2 Isolation of crude mitochondria from yeast cells

Yeast cells were harvested from culture at an OD_{600} of ca. 1.2 by centrifugation (3000 g, 5 min, RT) and washed once with water. The cell pellets were resuspended in SEM buffer supplemented with 2 mM PMSF to yield a total OD_{600} of 270 or higher. After adding 600 mg glass beads to the suspension, cells were lysed by five times extensive vortexing for 30 seconds with pauses on ice in between. After centrifugation (1000 g, 5 min, 4°C), the supernatant, which contains the whole cell lysate, was transferred to a new tube and protein concentration was determined by Bradford assay. Crude mitochondria were harvested from the whole cell lysate by centrifugation (13200 g, 10 min, 4°C). The pellet contains the crude mitochondria whereas the supernatant contains proteins from the cytosolic and ER fractions. The mitochondrial fraction was dissolved directly in 2×Laemmli buffer whereas the proteins of the supernatant were precipitated first with trichloroacetic acid (TCA) (cf. 2.2.4.2) before addition of 2×Laemmli buffer. All samples were subjected to SDS-PAGE.

2.2.3.3 Isolation of pure mitochondria from yeast cells

Isolation of pure mitochondria from *S. cerevisiae* cells was performed by enzymatic spheroblastation according to a previously described method (Daum et al., 1982). Yeast cells were grown at different temperatures of 30°C, 37°C, or 25°C in 0.5-1 liter of appropriate medium (YPD, YPG, SD, SG). The cells were harvested at an OD_{600} of 1.2-2.0 by centrifugation (3000 g, 5 min, 20°C), washed with water, and reisolated. Afterwards the pellet was weighted and resuspended in resuspension buffer (2 ml/g cells). After shaking for 10 min at 30°C, the cells were harvested by centrifugation (3000 g, 5 min, 20°C) and resuspended in 50 ml of 2.4 M sorbitol. Cells were harvested by centrifugation (3000 g, 5 min, 20°C), resuspended in spheroblasting buffer (6.6 ml/g cells) and incubated while shaking for 60 min at 30°C. The spheroblasted cells were harvested by centrifugation (2000 g, 5 min, 2°C) and then the pellet was resuspended in

homogenization buffer (6.6 ml/g cells). The cell suspension was homogenized with a glass dounce tissue grinder by 12 strokes in an ice-bath. The homogenized lysate was cleared from cell debris by two consecutive centrifugation steps (2000 g, 5 min, 4°C). The pellet was discarded and mitochondria were isolated from the lysate by centrifugation (18000 g, 15 min, 4°C). The supernatant from this step was kept if cytosolic proteins had to be analyzed.

The pellet was resuspended in 30 ml SEM buffer supplemented with 2 mM PMSF. The mitochondria were further purified by two low speed centrifugations (2000 g, 5 min, 4°C) and finally pure mitochondria were isolated by high speed centrifugation (18000 g, 15 min, 4°C) and resuspended in 250 µl SEM buffer. Protein concentration was determined by the Bradford assay. The mitochondria were then aliquoted, snap frozen and stored at -80°C. The supernatant which contains crude cytosolic proteins was either subjected to chloroform/methanol precipitation, followed by SDS-PAGE or clarified by another centrifuge step (18000 g, 15 min). Glycerol was added to the resulting supernatant to a final concentration of 10%. Aliquots of 2 ml were prepared, snap-frozen in liquid nitrogen and stored at -80°C.

2.2.3.4 Cycloheximide treatment of yeast cells

To study the stability of yeast proteins, cells were treated with the protein synthesis inhibitor cycloheximide. Yeast cells were grown at various temperatures in 400 ml of appropriated culture medium to reach an OD_{600} of 0.8-1.0. Next, 100 µg/ml cycloheximide was added to the culture (time=0) and then aliquots of 100 ml were removed at different time points (0, 1, 2 and 4 h after addition of cycloheximide). The cells were harvested by centrifugation (3000 g, 5 min, RT) and the pellets were subjected to further analysis such as crude mitochondrial isolation or whole cell lysate analysis by SDS-PAGE and western blotting.

2.2.3.5 Fluorescence microscopy of yeast cells

To study mitochondrial morphology, yeast cells were transformed with an expression vector harboring GFP fused to the mitochondrial presequence of subunit 9 of F_0-ATPase of *Neurospora crassa (*Westermann and Neupert, 2000, Mozdy et al., 2000). The transformed cells were grown overnight at 30 °C or 37 °C in 20 ml of selective medium. The culture was diluted to an OD_{600} of 0.1 and incubated to reach an OD_{600} of 0.6-0.8. One ml of the culture was harvested by centrifugation (8000 g, 30 sec, RT) and

resuspended in the fresh medium. Then, 5 µl of the cell solution sample was mixed with 5 µl of 1% low melting point agarose (Roth), placed on a microscope slides and covered with a coverslip. Fluorescence microscopy was done with a ZEISS Axioscope microscope. Pictures were taken with an Axio Cam MRm system (Zeiss). Fluorescence of GFP was excited at 488 nm and its emission peak was monitored at 509 nm.

2.2.3.6 *In vivo* site-directed crosslinking

For studying the interaction of a protein of interest with other cytosolic factors site-specific cross-linking was used according to a previously published protocol with slight modification (Chen S. and P.G., 2007, Carvalho P. et al., 2010, Tamura et al., 2009). First, yeast cells were transformed with two plasmids to introduce the photo-reactive cross linking residue, p-benzoyl-L-phenylalanine (Bpa, Bachem) into the protein of interest. One plasmid contains the coding sequence for the protein of interest which harbors an amber stop codon (TAG) in the desired position and the other plasmid contains the coding sequence of an amber suppressor tRNA and its cognate aminoacyl-tRNA synthetase that specifically charge the suppressor tRNA with Bpa (Chin et al., 2003b). Pre-cultures of yeast cells containing both plasmids were grown overnight at 30°C on selective medium (SD-trp-leu-phe medium supplemented with Bpa) and diluted to an OD_{600} of 0.3 in 200 ml of the same media. Bpa was dissolved in one M NaOH and freshly added to the culture to a final concentration of 0.2-0.6 mM. As a control, a culture without Bpa was prepared. The cells were incubated at 30°C with slight shaking to reach to an OD_{600} of 1.0-1.2 and then they were harvested by centrifugation (3000 g, 5 min, RT). For photo-crosslinking reactions, the cell pellets (from both cultures with and without Bpa) were divided into two samples of 800 µl water each (75 OD_{600} units of cells per sample) and transferred to a 12-well plate (Becton Dickinson). The suspensions were placed on ice and one sample was exposed to ultraviolet light (Blak-Ray® Ultraviolet Lamp, B-100 AP; UVP, USA, λ = 365 nm) for one hour at 4°C (+UV). The other half of the cells sample served as a control and was stored at 4°C without exposure to (-UV). Next, both irradiated cells (+UV) and non-irradiated cells (-UV) were transferred to 2 ml microcentrifuge tubes, washed once with water and resuspended in 200 µl SEM buffer supplemented with 2 mM PMSF. The samples were subjected to whole cell protein extraction by mechanical cell disruption (cf.2.2.3.2). After determination of the protein concentration in the whole cell lysates, the samples (+/- Bpa, +/- UV) were applied to SDS-PAGE analysis.

2.2.4 Methods in protein biochemistry

2.2.4.1 Determination of protein concentration

Protein samples were diluted (1:5 or 1:10) in one ml Bradford reagent (original reagent diluted fivefold in SEM buffer). As a standard calibration samples of 200-1000 µg/ml of BSA (bovine serum albumin) were used. Absorbance was measured at a wavelength of 595 nm by photometer (Eppendorf® BioPhotometer®).

2.2.4.2 Methanol/Chloroform protein precipitation

Soluble proteins were precipitated with chloroform and methanol precipitation method, as described previously (Wessel and Flügge, 1983). Protein samples were mixed by short vortexing with four volumes of methanol. Next, one volume of chloroform was added and vortexed and finally three volumes of water were added and vortexd extensively for 20 sec. Precipitated proteins were harvested by centrifugation (16000 g, 5 min, RT). The supernatant was removed as much as possible without disturbing the interface layer. One ml of methanol was added to the pellet and transferred to 1.5 ml Eppendorf tubes. After another extensive mixing, the sample was centrifuged again (16000 g, 2 min, RT). Supernatant was removed and the protein pellet was air-dried for 5 min and dissolved by boiling for 10 min in 2×Laemmli buffer.

2.2.4.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analyzed by discontinuous SDS-polyacrylamide electrophoresis as described before (Lämmli, 1970). Depending on the sizes of the proteins of interest, gels with polyacrylamide content between 8-15% were used. Polyacrylamide gels were casted between two glass plates using one mm spacers and were composed from a bottom gel to prevent leakage, a separating gel to separate proteins and a stacking gel on top. The composition of the acrylamide gels is specified in Table 2.24. Protein samples were dissolved in Laemmli buffer and cooked for 5 min at 95°C before their analysis by SDS-PAGE. Electrophoresis was performed at 25 mA in a vertical installation between two reservoirs of running buffer for about 2-5 hours according to the acrylamide percentage and the size of protein of interest. Protein ladder (PAGE Ruler™; Fermentas) was used as a molecular weight standard.

Table 2.24. Composition of polyacrylamide gels used for SDS-PAGE analysis of protein samples.

Component	Stacking gel	Running gel			Bottom gel
	-	8 %	10 %	12.5 %	15%
40% aa / bis-aa (29:1)	563 µl	2.5 ml	3.13 ml	3.91 ml	3.75 ml
1M Tris pH 8.8	-	4.69 ml	4.69 ml	4.69 ml	3.75 ml
1M Tris pH 6.8	625 µl	-	-	-	
water	3.76 ml	5.18 ml	4.55 ml	3.77 ml	2.39 ml
10% APS	50 µl	125 µl	125 µl	125 µl	100 µl
TEMED	4 µl	10 µl	10 µl	10 µl	8 µl

2.2.4.4 Detection of proteins in polyacrylamide gels by Coomassie staining

After SDS-PAGE, proteins in polyacrylamide gels were visualized by Coomassie staining. The gel was incubated for one hour in Coomassie staining solution. Subsequently was destain two times for 30 min with water and once again for 2 h in destaining solution (30 % (v/v) methanol, 10 % (v/v) acetic acid) to visualize the protein bands on a clear background.

2.2.4.5 Western Blotting

Transfer of proteins from polyacrylamide gels onto nitrocellulose membranes (Protran, Roth) was done using the semi-dry electroblotting method (Kyhse-Andersen, 1984). First two layer of filter paper (Whatman; Roth) were placed on the graphite electrode of a blotting chamber followed by a nitrocellulose membrane, the separating gel and finally additional two layers of the filter paper. Filter paper, nitrocellulose membrane and gel were shortly soaked in blotting buffer before assembly. Electroblotting was performed for one hour at 220 mA, which corresponds to approximately one mA per cm^2 of nitrocellulose membrane. Blotting efficiency was checked by Ponceau staining.

2.2.4.6 Immunodetection of proteins

Proteins were transferred from polyacrylamide gel onto nitrocellulose membrane as described above. The membrane was incubated in blocking buffer for one hour at RT to block unspecific binding sites. Afterwards, the membrane was incubated with primary antibodies for either one hour at RT or overnight at 4°C. Next, the membrane was washed three times for five min in TBS, then once in TBS-Tween 20 and finally once again in TBS. The membrane was then incubated for one hour at RT with the secondary

antibody followed by washes as described above. Finally, ECL solution was applied to the membrane and chemoluminescence signal was detected with X-ray films (Fuji Super RX films; Fujifilm) and developed using a Konica X-ray film developing machine SRX-101A (Konica Minolta).

2.2.4.7 *In vivo* pulse chase experiment

Pulse chase analysis was performed to examine processing of proteins over the time during import into mitochondria. First, cells were grown on selective medium overnight at 30°C and diluted to an OD_{600}=0.6 in 25 ml culture to reach an OD_{600} of 1.2. Cells were harvested by centrifugation (3000 g, 5 min, RT) and washed twice with 2.5 ml sterile water to get rid of methionine. Subsequently cells were diluted in 25 ml of medium without methionine to make sure that the cells use hot methionine once we add it. The cells were starved by incubation one hour at 30°C in a shaker and harvested by centrifugation (3000 g, 5 min, RT). Next, cells were dissolved in 2.5 ml of media without methionine and incubated again 10 min at 30°C. After incubation, 7µl of ^{35}S-Met was added to the culture and incubated for 5 min for pulse phase. Labeling or pulse phase was stopped by adding 25 µl of 0.1 % (w/v) cycloheximide and in parallel 25µl of 0.3% cold methionine was added to the culture to start the chase phase. Samples (500µl) were taken at different time point and added immediately to a previously prepared 1.5 ml Eppendorf tubes already containing 50 µl of sodium azide (100 mM) solution to kill the cells and subsequently transferred immediately on ice. Cells were harvested by centrifugation (4500g, 1 min, 4°C) and washed twice with 500 µl sodium azide (10mM) and then dissolved in 400 µl TE Buffer supplemented with one mM PMSF. Next, 300 µg of glass beads were added to the mixture and cells were broken by mechanical cell disruption. Cell debris and beads were harvested by centrifugation (1000 g, 3 min, 2°C) and supernatant which contain mitochondria and cytosolic protein transferred to the new Eppendorf tubes. To release proteins from mitochondrial protein, 100 µl of TENN×4 buffer was added to the 300 µl of supernatant. This suspension was added to the 20 µl HA which previously had been washed three times with one ml TENN×1 buffer. After overnight incubation of mixture in 4°C, beads were sedimented and washed three times with TENNS buffer. Finally, the beads resuspended in 50 µl 2×Laemmli buffer. All samples were applied to SDS-PAGE analysis and autoradiography.

2.2.4.8 *In vitro* synthesis of radiolabeled proteins

To synthesize ^{35}S-radiolabeled proteins, genes of interest were cloned in the plasmid pGEM4 (Promega) and the gene was transcribed into mRNA by SP6-polymerase (Melton D.A. et al., 1984). Transcription reaction was performed for 1 h at 37 °C and contained the ingredients indicated in Table 2.25.

Table 2.25. Composition of transcription reaction

Component	Volume
5 × Tr-puffer	10 µl
0.1 M DTT	5 µl
RNase inhibitor (Promega)	2 µl
2.5 mM rNTP-mix (GE Healthcare)	10 µl
7 methyl- G (5´) ppp (5´) G cap (Amersham)	2.6 µl
SP_6 Polymerase	1.5 µl
DNA Plasmid (1µg/ml)	5 µl
H_2O	13.9 µl
Total	50 µl

After incubation, synthesized mRNA was precipitated by adding 5 µl of 10 M LiCl and 150 µl ethanol p.A and the reaction mixture was incubated for 3 h at -20°C or overnight at -80°C. Afterwards the RNA was collected by centrifugation (37000 g, 20 min, 2°C) and washed once with 500 µl ice-cold 70% ethanol and was isolated by centrifugation again (37000 g, 5 min, 2°C). The pellet was resuspended in 37 µl H_2O supplemented with 2 µl RNase inhibitor. Aliquots (12.5 µl) were stored at -80°C.
For translation of mRNA into ^{35}S-labeled protein, the translation mixture was composed of the components indicated in Table 2.26

Table 2.26. Composition of translation reaction

Component	Volume
Transcribed RNA	12.5 µl
RNase inhibitor	0.5 µl
Mg-acetate 15 µm	3.5 µl
AA without met 1mM	1.75 µl
Reticulocyte lysate	50ml
^{35}S-Met	6µl

The reaction was incubated for one hour at 30°C. After incubation, 6 µl of methionine (58 mM) and 12 µl of Sucrose (1.5 M) were added to the reaction. To remove

ribosomes the reaction mixture was centrifuged (90000 g, 50 min, 4°C). The cleared supernatant was used for *in vitro* import experiments.

2.2.4.9 *In vitro* import of proteins into mitochondria

Isolated mitochondria were resuspended in import buffer (50 µg organelle/ 100 µl). Radiolabeled proteins (3-6 µl of lysate) were added at 25°C or 37°C to the import reaction and the mixture was incubated for various time periods. Import reactions were then inhibited by addition of 600 µl SEM-K80 buffer. Mitochondria were reisolated by centrifugation (13200 g, 10 min, 2°C) and subjected to PK treatment to remove unimported proteins. The pellets were resuspended in 2×Laemmli buffer and analysed by SDS-PAGE and autoradiography (c.f. 2.2.4.10).

2.2.4.10 Proteinase K treatment of mitochondria

Isolated mitochondria were resuspended in 100 µl SEM buffer supplemented with Proteinase K (200 µg/ml) and incubated on ice for 30 min. The reactions were stopped by adding PMSF (2 mM) and incubating the mixture for 5 min on ice. The organelles were re-isolated by centrifugation (13200 g, 10 min, 2°C). The pellets were resuspended in 2×Laemmli buffer and incubated for 5 min at 95°C before analysis by SDS-PAGE.

2.2.4.11 Autoradiography and quantification of bands

Radiolabeled proteins were transferred to nitrocellulose membrane and detected by X-ray film that was exposed to the membrane (Kodak Bio Max MM). Exposure time was dependent on the signal intensity and varied between two to seven days. For quantification of autoradiography and immunodetection films, AIDA Image Analyzer v.4.19 software was used.

2.2.4.12 Pull‑down with Ni-nitrilotriacetic acid (Ni-NTA) agarose beads

Whole cell lysate with or without UV treatment was resuspended in 500 µl lysis buffer (Table 2.15) and applied to the mechanical cell disruption with glass beads. After determination of protein concentration 9% (v/v) Triton X-100 was added to one mg protein of the lysate. The samples were incubated for 30 min at 4°C. To clarify the lysate from undissolved material, the mixture was centrifuged (30000 g, 30 min, 4°C). In parallel, 50 µl Ni-NTA agarose beads (1:1 slurry) were washed once with water and pre-equilibrated with lysis buffer for one hour at 4°C. The supernatant was applied to

the equilibrated beads and the mixture was incubated for one hour at 4°C on an overhead shaker. Afterward the beads were harvested by centrifugation (400 g, 2 min, 4°C) and washed twice with wash buffer for 5 min at 4°C. Finally, the beads were sedimented again and resuspended in 50 µl 2×Laemmli buffer. All samples were applied to SDS-PAGE analysis.

2.2.4.13 Over-expression and purification of GST-tagged proteins Expression and purification of recombinant proteins in *E. coli*

Plasmids encoding recombinant proteins pGEX-4T-1 (GST), pGEX-4T-1-Tom70cd (Tom70 cytosolic domain-GST), and pGEX-4T-1-Tom20cd (Tom20 cytosolic domain-GST) were expressed in *E. coli* BL-21 cells. The cells were initially cultivated in one liter of LB medium supplemented with 100 µg/ml ampicillin till an OD_{600} of 0.5-0.8. Then expression of the recombinant proteins was induced by adding one mM IPTG to the culture and cells were grown for further 4 h at 37°C. Subsequently the cells were harvested and stored at -20°C until further use. Expression of the desired protein was monitored by SDS-PAGE followed by Coomassie staining.

The harvested cells were resuspended in GST lysis buffer (1 gr cells/10 ml buffer) and incubated for 45 min at 4°C with shaking (120 rpm). The resuspended cells were applied to a French press (Emulsifelx-C3) machine. Cell debris and unbroken cells were separated by centrifugation (15000 g, 15 min, 4°C). Next, the supernatant was applied to two ml GSH-sepharose™ 4B beads (GE Healthcare), which previously washed with 15 ml water and equilibrated with 20 ml GST basic buffer, and incubated overnight at 4°C on an overhead shaker. Subsequently, the mixture of lysate and beads was transferred to a purification column and the column was washed with 20 ml of GST basic buffer. Finally elution was performed with 15 ml GST elution buffer. Fractions of one ml were collected and protein concentration was measured by Nanodrop photometer (PEQLAB-nanodrop-ND). Fractions containing more than 1 mg/ml proteins were dialyzed three times for one hour in two liter of GST basic buffer using Slide-a-Lyzer dialysis cassettes (Pierce/Perbio, Thermo Fisher Scientific,) and aliquoted into samples of 100 or 200 µg.

2.2.4.14 Pull-down of purified GST-tagged proteins and yeast cytosol with GSH-sepharose beads

The GST-tagged proteins and GST alone (as control), were incubated with 100 µl of GSH-sepharose™ 4B beads, which were previously washed 5 times with GST basic

buffer for 2 h at 4°C, in a final volume of 400 µl on an overhead shaker. As Tom70cd-GST, Tom20cd-GST, GST has a molecular weight of approximately 100, 50 and 25 kDa, respectively, four times more Tom70cd-GST (800 µg) and two times more Tom20cd-GST (400 µg) were utilized in order to employ the same molar amount (8 nmol) of all three proteins. Afterward GSH-sepharose™ beads coated proteins were treated with 3% TBS in cytosol lysis buffer (CLB, 0.6 M Sorbitol, 10 mM Tris-HCl, and pH 7.4) to block unspecific binding sites. In parallel GST-coated GSH-sepharose™ beads were incubated with 2 ml cytosol, (supplemented with 0.5 mM EDTA, 1 mM PMSF, protease inhibitor) for 2 h at 4°C. Finally the pre-cleared cytosol was incubated overnight at 4°C on an overhead shaker with protein-coated GSH-sepharose™ beads. After the incubation the mixture was centrifuged (13000 g, 1 min, 4°C) and the supernatant was discarded. The beads were washed 3×times with CLB supplemented with 100 mM NaCl and then resuspended in 200 µl of 2×Laemmli buffer, heated at 95°C for 5 min and 30 µl aliquot was analyzed by SDS-PAGE and immunodecoration.

3. Results

3.1 Identification of cytosolic factors that interact with presequence containing mitochondrial precursor proteins

3.1.1 Optimization of the integration of a photo-reactive amino acid for *in vivo* photo-crosslinking

So far, it is not clear which cytosolic factors assist the targeting of presequence-containing mitochondrial precursor proteins to the Tom20 receptor. In this study, I wanted to identify such cytosolic factors. To this end, I used *in vivo* site-directed photo-crosslinking of the model precursor protein pCyb2-DHFR-His_9 to search for such potential interaction partners. The model protein pCyb2-DHFR-His_9 consists of the N-terminal mitochondrial targeting sequence (aa 1-87) of Cytochrome b2 (L-lactate cytochrome-c oxidoreductase, Cyb2), a dihydrofolate reductase (DHFR) moiety and a C-terminal His-tag. Cyb2 resides in the mitochondrial intermembrane space. It is required for lactate utilization and its expression is repressed by glucose and anaerobic conditions (Lodi and Guiard, 1991, Guiard, 1985).

In vivo site-directed photo-crosslinking requires the efficient integration of the photo-reactive, unnatural amino acid p-benzoyl-L-phenylalanine (Bpa) at a specific site of the protein of interest. The incorporation of Bpa is accomplished using the so-called tRNA suppressor method (Chin et al., 2003a, Chen et al., 2007). For this a mutant version of

the protein of interest containing an internal amber stop codon (TAG) at a specific position is co-expressed with an amber suppressor tRNA which recognizes the amber stop codon and an aminoacyl-tRNA synthetase which charges the suppressor tRNA with Bpa. In this way, the photo-reactive Bpa is incorporated into the protein in response to an amber stop codon during translation. Upon UV irradiation (λ = 350 nm) Bpa reacts preferentially with C-H bonds in its vicinity, thereby forming of a new covalent C-C bond (Kauer et al., 1986).

Since in this study I was interested in interactions of the presequence part of the protein, Bpa was inserted into the N-terminal targeting sequence of pCyb2-DHFR-His_9 (Fig. 3.1).

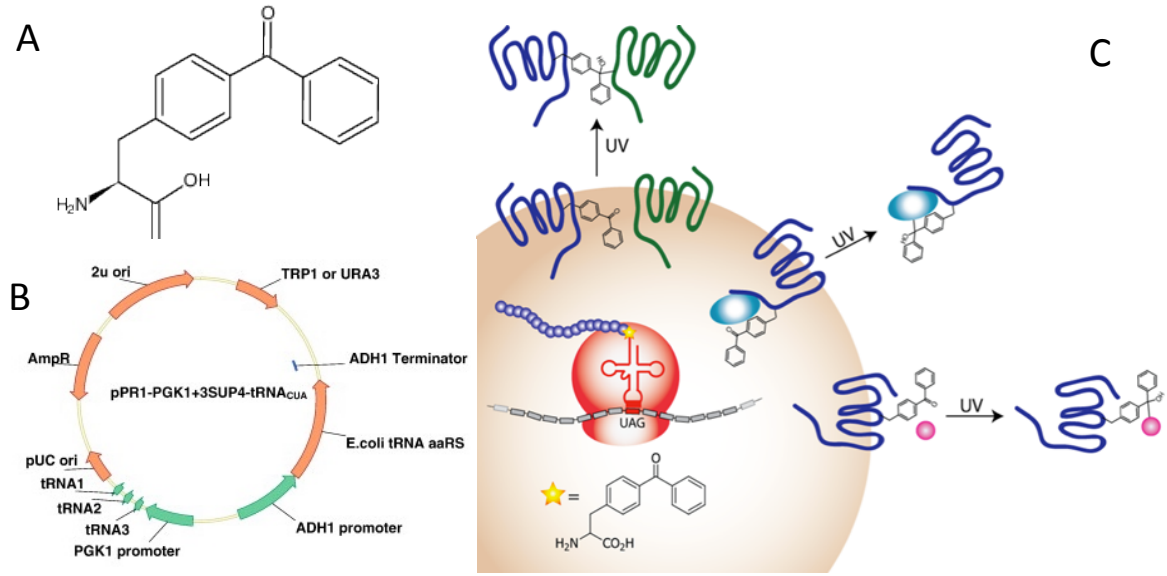


Fig. 3.1: Schematic representation of the photo-crosslinking method; A) Chemical structure of the photo-reactive diarylketone p-benzoyl-L-phenylalanine (Bpa). B) Map of the plasmid used for expression of the orthogonal amber suppressor tRNACUA/aaRS pair in yeast (Chen et al., 2007). C) Bpa is inserted into a polypeptide chain during translation using a suppressor tRNA system, which recognizes a specific codon. Upon UV irradiation, Bpa can react with molecules in its vicinity and forms a covalent bond (www.sakmarlab.org/reserach).

To search for the optimal conditions for a high crosslinking efficiency different constructs of pCyb2^{amber}-DHFR-His_9, which have the amber stop codon (TAG) at different positions (residue 4, 8,10, 13 or 16) were expressed in yeast cells that harbor the orthogonal amber suppressor tRNACUA/aaRS pair which charges the tRNA with Bpa. The cells were grown on selective medium supplemented with 0.6 mM Bpa or

without Bpa (as a control) and harvested in the logarithmic growth phase. After UV illumination, the cells were lysed by mechanical cell disruption and the whole cell extract was analyzed by SDS-PAGE and immunoblotting with antibodies against DHFR (Fig. 3.2). To increase the chances of the newly translated proteins to interact with cytosolic factors the cross-linking experiments were also performed in *mim1*Δ cells where import of mitochondrial proteins is hampered and slowed-down.

For cells expressing pCyb2^{amber}-DHFR-His$_9$, immunobloting with antibodies against DHFR revealed additional bands when the cells were exposed to UV irradiation. From all the constructs tested, replacement of codon 16 by TAG (pCyb2^{amber16}- DHFR-His$_9$ construct) gave rise to the highest number crosslinking bands (Fig. 3.2 E). This means that residue 16 is a good location for the *in vivo* photo-crosslinking probe. pCyb2-DHFR-His$_9$ alone has molecular weight of about 38 kDa. Thus, it can be assumed that cross-linked adducts of this protein should have a molecular mass higher than 40 kDa and indeed such bands were observed.

Of note, the same band pattern and also very similar amounts of the putative cross-linked adducts were observed in both WT and *mim1*Δ cells after UV treatment (Fig. 3.2). Thus, all further photo-crosslinking experiments were performed in WT cells.

After identifying pCyb2^{amber16}-DHFR-His$_9$ as the best construct for studying putative interaction partners of the mitochondrial presequence, this construct was used in all further experiments as a model precursor protein.

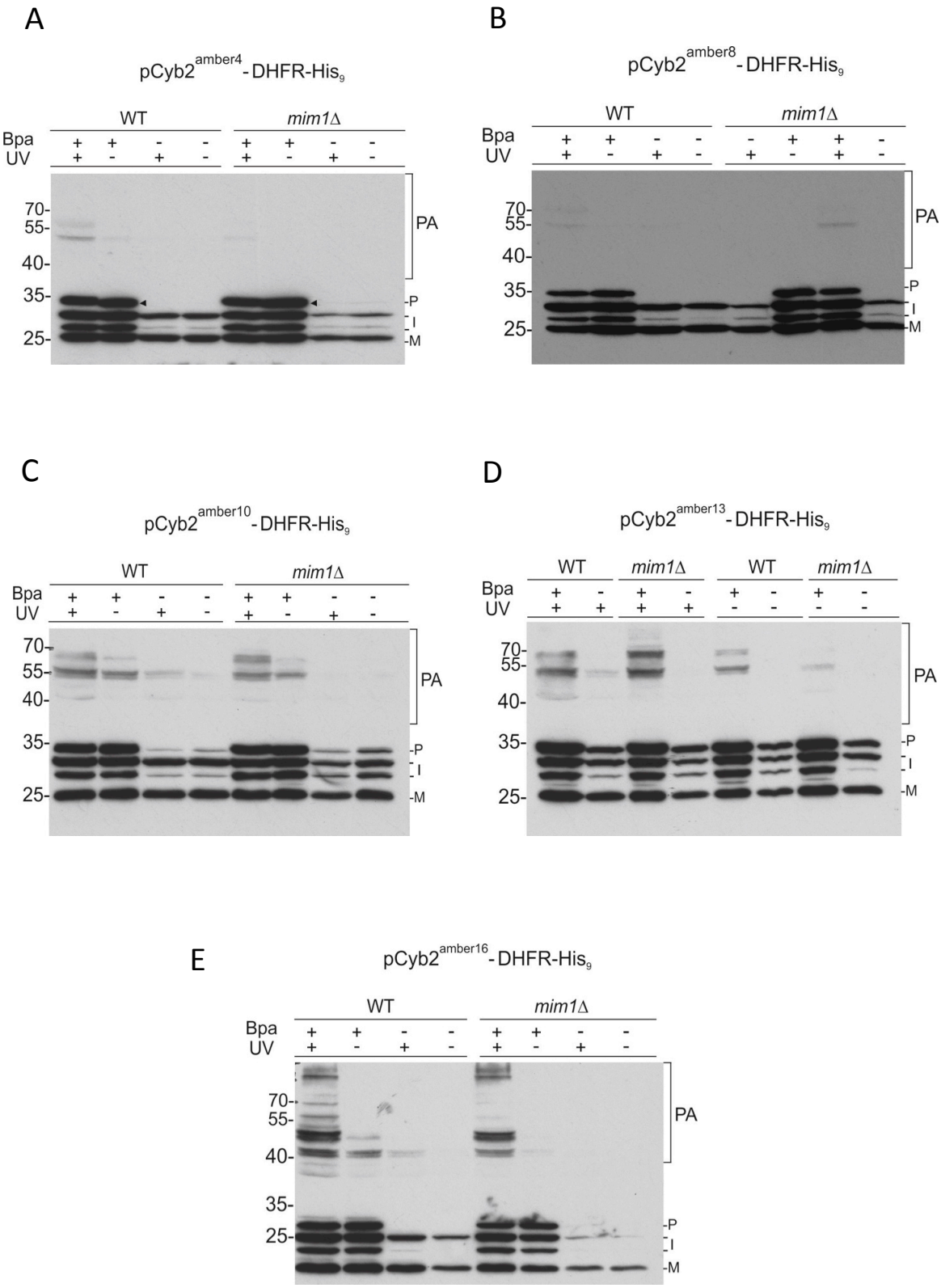


Fig. 3.2: pCyb2^{amber}-DHFR-His$_9$ constructs can be used for photo-crosslinking; The indicated pCyb2^{amber}-DHFR-His$_9$ constructs were expressed in WT and *mim1*Δ cells. The cells were grown in selective medium in the absence or presence of Bpa and harvested in the logarithmic growth phase for subsequent UV irradiation. As a control, half of the cells were not subjected to UV illumination. Finally, the cells were lysed and whole cell extracts were subjected to SDS-PAGE, western blotting, and immunodetection with antibodies against DHFR.

3.1.2 Identification of cytosolic factors that interact with Cyb2^{amber16}- DHFR-His$_9$

To identify the aforementioned cross-linking adducts the experiments were performed as described above and then, the cell lysate was subjected to affinity purification of the His-tagged protein via Ni-NTA beads. The rational was to enrich the cross-linked species and to separate them from the non-relevant proteins. Samples containing cross-linked proteins were then subjected to SDS-PAGE. Identification of the proteins that were cross-linked to pCyb2^{amber16}-DHFR-His$_9$ was achieved by immunodecroation using antibodies against known cytosolic factors and mitochondrial outer membrane proteins that are involved in the mitochondrial import process (Table 3.1)

Table 3.1: A list of cytosolic factors and mitochondrial import receptors that were examined for their incorporation into cross-linking adducts.

Cytosolic chaperones, co-chaperone and import receptors		References
Hsp82	Hsp90 chaperone in *S. cerevisiae*; its ATPase activity is regulated by various co-chaperones.	(Finkelstein and Strausberg, 1983, Prodromou et al., 1999)
Ssa1	Member of the Hsp70 family forms a chaperone complex with Ydj1; involved in protein folding and protein translocation	(Kim et al., 1998b, Ziegelhoffer et al., 1995, Deshaies et al., 1988).
Sti1	Co-chaperone of Hsp90; inhibits Hsp90 ATPase; interacts with the Ssa group of cytosolic Hsp70 chaperones and activates the ATPase activity of Ssa1; adapter protein connecting the Hsp70 and Hsp90 chaperone machinery; homologous to mammalian Hop	(Nicolet and Craig, 1989, Richter et al., 2003a, Wegele et al., 2003)
Ydj1	Co-chaperone of cytosolic Hsp70 proteins Ssa1 and Ssa2; belongs to Hsp40/DnaJ family; involved in protein translocation across membranes	(Caplan et al., 1992, Caplan and Douglas, 1991)
Sba1	Co-chaperone of Hsp90; homologous to mammalian p23 proteins	(Fang et al., 1998)
Aha1	Co-chaperone of Hsp90; activates ATPase activity of Hsp90; expression is regulated by stress	(Panaretou et al., 2002, Siligardi et al., 2004)
Hsp26	Member of small heat shock protein family; suppresses aggregation of unfolded proteins; activity only under stress conditions; not express	(Bentley et al., 1992, White et al., 2006)
Sis1	Hsp40 co-chaperone; interacts with Ssa1; has different substrate specificity than Ydj1	(Luke et al., 1991, Fan et al., 2004)
Tom40	MOM general import pore	(Pfanner et al.1996)
Tom22	MOM receptor	(Pfanner et al.1996)
Tom20	MOM receptor	(Pfanner et al.1996)

Specific cross-linked bands after UV irradiation were detected for the molecular chaperones Hsp82 and Ssa1 as well as for the co-chaperones Ydj1 and Sti1 (Fig. 3.3). Of note, despite the unspecific binding of these chaperones to the Ni-NTA beads, the cross-linking adduct is visible only upon UV irradiation. For Sti1, more than one specific band was detected. This might be due to different crosslinking configurations that migrate at different apparent molecular weights in SDS-PAGE. However, specific cross-linked bands were detected neither for the other cytosolic factors nor the import receptors. Collectively, it can be concluded that Hsp82, Ssa1, Ydj1 and Sti1 are potential interaction partner of pCyb2-DHFR-His_9. This indicates that these cytosolic factors might be involved in the import of presequence-containing mitochondrial proteins.

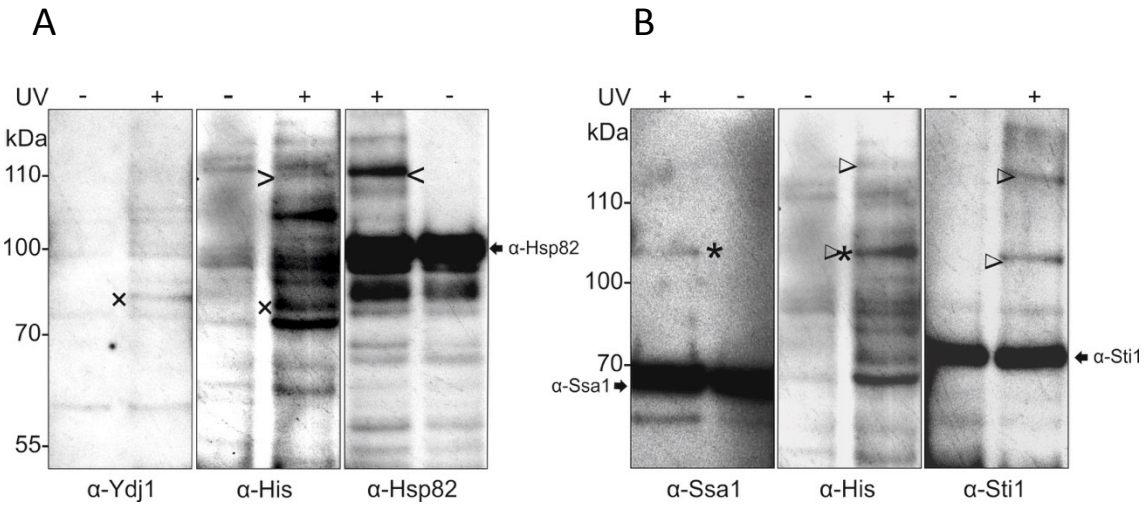


Fig. 3.3: Sti1, Ssa1, Hsp82 and Ydj1 are potential interaction partners of pCyb2-DHFR-His_9; A) Crosslinking experiments were performed and cells lysate was affinity-purified by Ni-NTA beads. Bound material was analyzed by SDS-PAGE and immunodecoration. The middle panel is immunodecorated with antibodies against the His-tag to detect all cross-linked products. Left panel was decorated with antibodies against Ydj1 and the right one with antibodies against Hsp82. Ydj1-containing photo-adduct is indicated with "x" and Hsp82-containing one with an arrowhead. B) Experiments were performed and analyzed as described in Panel A. Ssa1-containing photo-adduct is indicated with an asterisk and Sti1-containing one with a triangle.

3.2 The Role of Sti1 in the import of mitochondrial proteins

3.2.1 *In vivo* import of pCyb2-DHFR-His_9 and pSu9-DFHR-His_6 is affected upon deletion of *STI1*

Next, I wanted to study the involvement of the candidates in biogenesis of mitochondrial proteins. The most straightforward method is to study the phenotypes of strains deleted for the candidate proteins or where these are down-regulated. However, since Hsp82 is an essential chaperone in yeast and acts in different cellular pathway its deletion strain is not viable. For Ssa1 and Ydj1, it has been already demonstrated that they are involved in import of mitochondrial preproteins to their organelle (Caplan. et al. 1992, Deshaies et al. 1988). In contrast, so far, nothing is known about interaction of Sti1 with mitochondrial proteins and its involvement in their targeting to mitochondria. To better understand the role of Sti1, we tested whether the import efficiency of mitochondrial preproteins is affected *in vivo* by the absence of Sti1. To this end, two *sti1Δ* strains and their corresponding wild types (W303α and YPH499) were transformed with the presequence-containing model proteins pCyb2-DHFR-His_9 or pSu9-DHFR-His_6. Cells were grown on selective medium, harvested and the mitochondrial and cytosolic fractions were isolated, analyzed by SDS-PAGE and immunoblotting. In this way the steady-state levels of the two different constructs in the different backgrounds can be examined (Fig.3. 4).

The results indicate that the amount of pCyb2-DHFR-His_9 detected in mitochondria of both Sti1 deletion strains is reduced in comparison to those in organelles from WT cells (Fig. 3.4 A). Of note, both the precursor and mature forms of the protein were affected. Interestingly, the quantity of the precursor form in the cytosol was hardly affected by the absence of Sti1 in the YPH499 background but was dramatically altered in the W303 background (Fig. 3.4 A). These results indicate that in the absence of Sti1 less molecules of pCyb2-DHFR-His_9 are imported into the organelle and the non-imported ones are probably degraded.

In contrast, the import of pSu9-DHFR-His_6 into mitochondria was not affected by the absence of Sti1 in the YPH499 background. Surprisingly, in the W303α background, the precursor form of the model protein was reduced whereas the mature form had elevated levels in the mitochondrial fraction of *sti1Δ* cells when compared to control organelles (Fig. 3.4 B). Collectively, these observations suggest that the effect of the deletion of Sti1 on the *in vivo* import of the model proteins depends on the genetic

background and varies between the two proteins. Hence, to further analyze *in vivo* the effect of Sti1 on the import of mitochondrial proteins all further experiments and analysis were performed in the W303α background.

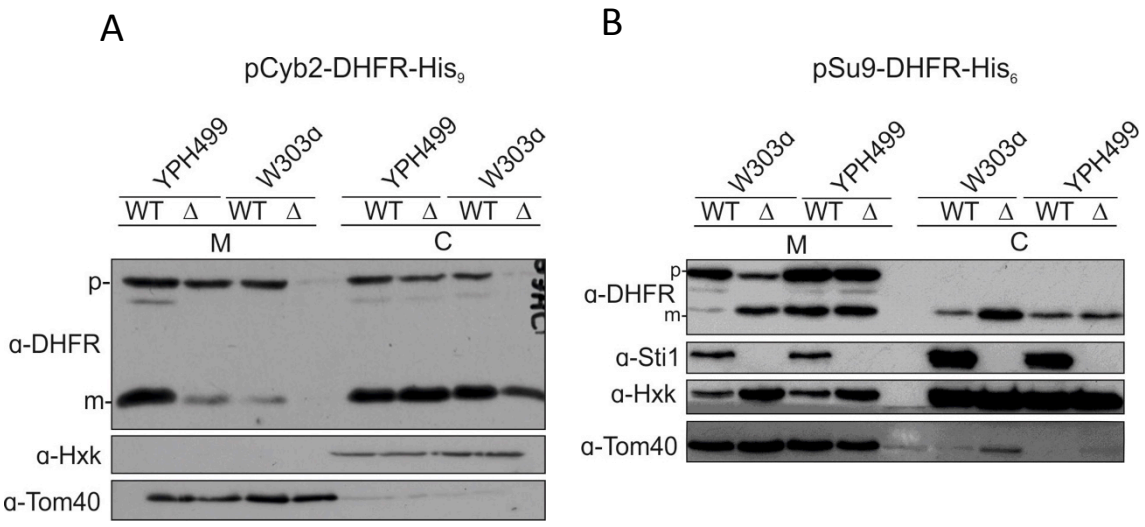


Fig. 3.4: Steady-state levels of pCyb2-DHFR-His$_9$ and pSu9-DHFR-His$_6$ in *sti1Δ* cells are altered; Wild type (WT) and *sti1Δ* (Δ) cells from W303α and YPH499 backgrounds harboring a plasmid encoding pCyb2-DHFR-His$_9$ or pSu9-DHFR-His$_6$, were grown on selective medium. The mitochondrial (M) and cytosolic (C) fractions were isolated and analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. The precursor and mature forms of the preproteins are indicated with p and m, respectively. Hexokinase (Hxk) and Tom40 are used as marker proteins for the cytosol and mitochondria, respectively.

3.2.2 Steady-state levels of mitochondrial presequence-containing proteins are not affected in *sti1Δ* cells

The previous experiment revealed that the biogenesis of both pCyb2-DHFR-His$_9$ and pSu9-DHFR-His$_6$ was affected in a *STI1* deletion strain in the W303α background. Next, we tested whether the reduced amount of imported proteins is related to the absence of Sti1 or to the expression system. To this end, we investigated the steady-state levels of the model proteins when they are encoded by another vector, pYX132 instead of pES426 or pRS426. DHFR alone, pSu9-DHFR and pSu9-GFP were expressed in the *sti1*Δ and the corresponding wild type strain. Samples were prepared as before, analyzed by SDS-PAGE and immunodecoration (Fig. 3.5).

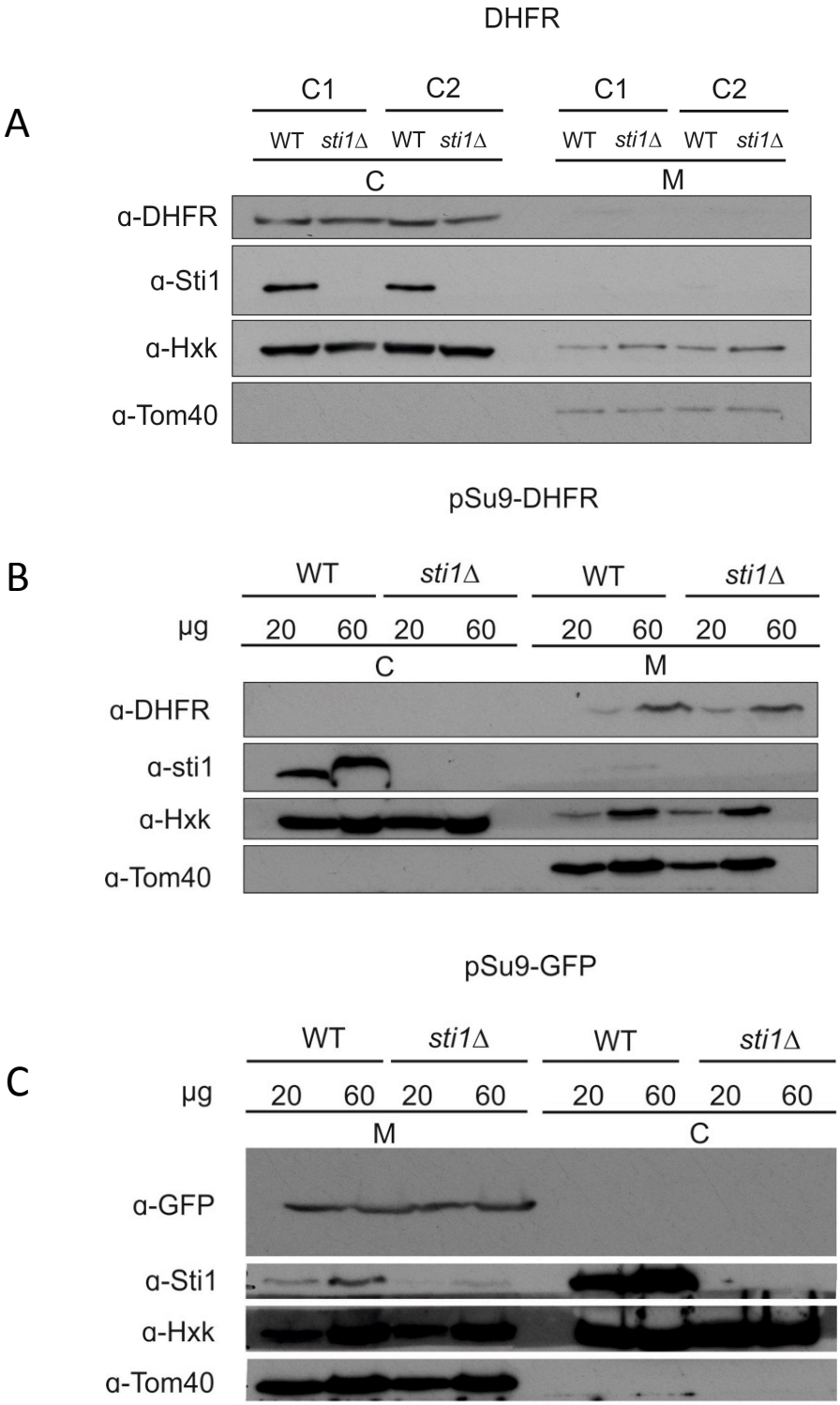


Fig. 3.5: The import of the model preproteins is not affected in *sti1*Δ cells; WT and *sti1*Δ cells were transformed with the pYX132 vector encoding pSu9-DHFR or pSu9-GFP or with pYX142-DHFR. The cells were grown on YPG medium, harvested and lysed to isolate mitochondrial (M) and cytosolic (C) fractions. Both fractions were subjected to SDS-PAGE and immunodecoration with the indicated antibodies. C1: colony 1, C2: colony 2.

The results show that the steady-state levels of proteins expressed from the pYX132 vector is similar in WT and *sti1Δ* cells. Therefore, the previously reduced amount of protein in *sti1Δ* cells is probably related to the expression system rather than actual import deficiency in the deletion strain.

3.2.3 The steady-state levels of presequence-containing proteins expressed from various vectors are not affected in *sti1Δ* cells

To further investigate the effect of the expression vector on the steady-state levels of the expressed proteins, other pYX vectors and proteins were checked in the WT and *sti1*Δ strains. Since pYX132 is a centromeric plasmid and pRS426 or pEs426 are 2µ plasmid, we tested whether the previously observed effects on the steady-state levels of the expressed protein were due to the type of plasmid. WT and *sti1Δ* cells were transformed with pRS426-DHFR, pRS426-Fis1, pYX232-Oep37, pYX142-Mim1, and pYX132-Mcr1, and whole cell lysate samples were prepared as before and analyzed by SDS-PAGE and immunodecoration (Fig. 3.6).

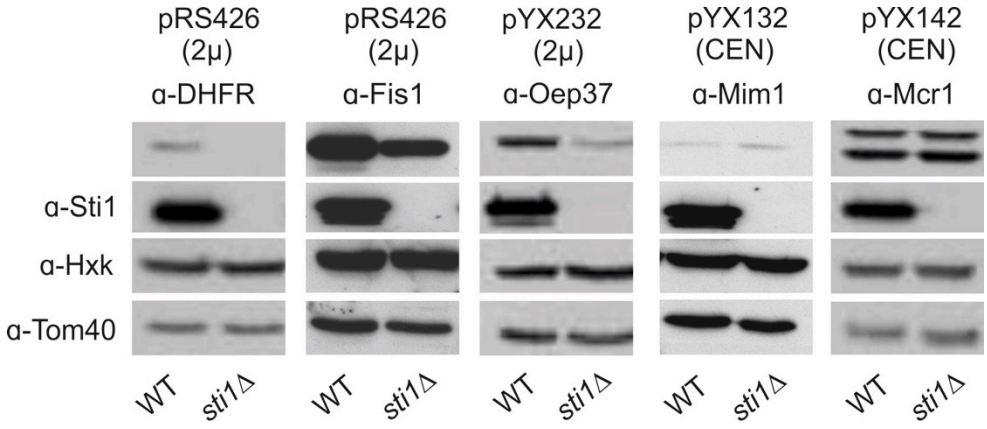


Fig. 3.6: Comparison of protein expression from vectors with different replication elements; The indicated constructs were expressed in WT and *sti1Δ* cells. Whole cell lysates were subjected to SDS-PAGE and decorated with corresponding antibodies.

The Results indicated that the amount of DHFR, Fis1, and Oep37 which were expressed from the 2µ plasmids were reduced in the *sti1*Δ strain when compared to the WT while, the steady state levels of Mcr1 and Mim1 which were expressed from the centromeric vectors were similar in both strains. Hence, we can conclude that the reduced amounts of proteins in the *sti1*Δ strain is related to their higher expression level from the

multicopy plasmids. Since DHFR, Fis1 and Oep37 reside in the cytosol or the MOM this effect is not related to the presence of a presequence.

3.3 Deletion of *STI1* does not influence the levels of pSu9-DHFR-HA

Next, we intended to analyze the influence of Sti1 on the import *in vivo* of precursor proteins by pulse-chase experiments. To this end, pYX113-pSu9-DHFR-HA was expressed in WT and *sti1Δ* cells and radiolabeled methionine (S^{35}-Met) was added for a brief period. The radiolabeled methionine was then washed away and non-radiolabeled methionine was added to the cultures. The cells were lysed and subjected to a pull-down with HA beads and samples were subjected to SDS-PAGE and autoradiography. The experiments were performed at either 30°C or 37°C (Fig. 3.7 A and B).

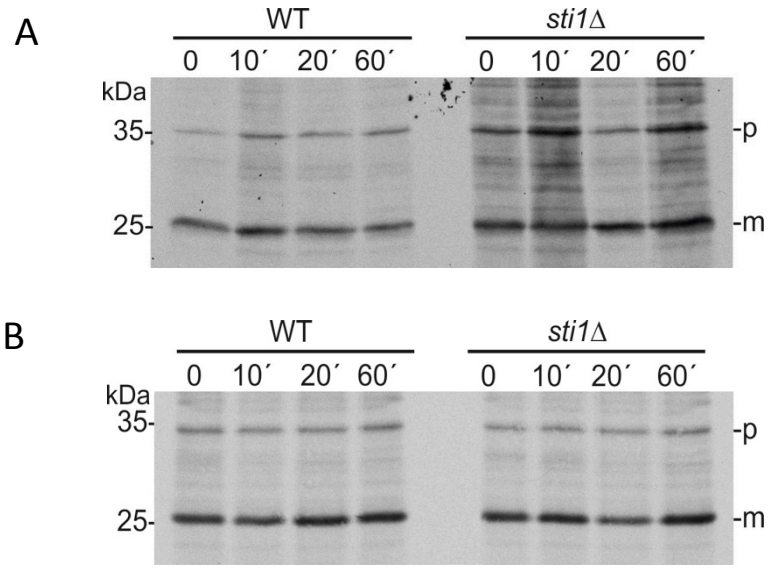


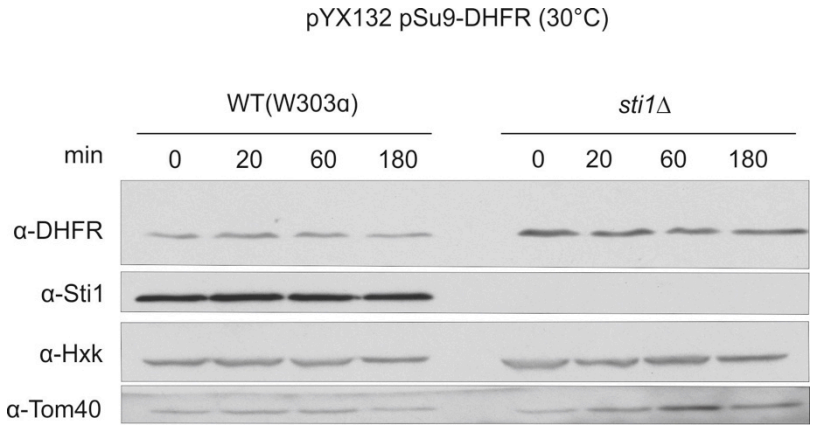
Fig. 3.7: Sti1 does not influence the *in vivo* import of pSu9-DHFR-HA; A and B) WT and *sti1Δ* cells were pulsed with radiolabeled Met at 30°C (A) or 37°C (B**)** and then cold Met was added and samples were removed after the indicated time periods. P; Precursor protein; m; Mature protein.

The deletion of *STI1* did not significantly decrease the import efficiency of pSu9-DHFR-HA as reflected by the amounts of the mature form of the protein. Taken together, a specific and physiological role of Sti1 in the import of mitochondrial preproteins could not be verified by these *in vivo* experiments.

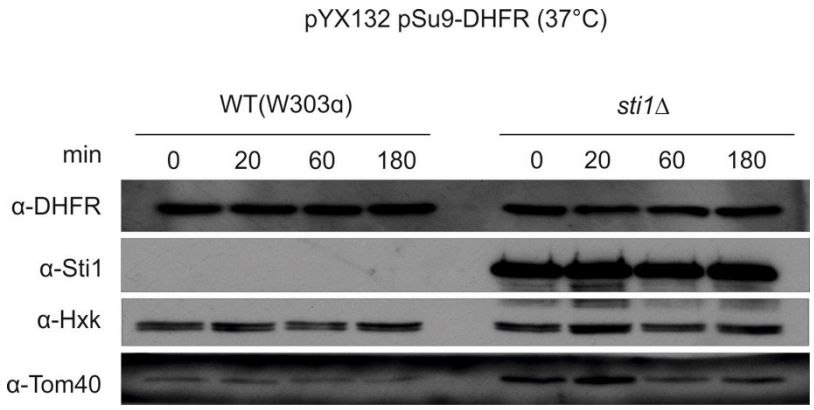
3.4 The role of Sti1 in the stability of mitochondrial precursor proteins

To test whether Sti1 has an effect on the stability of presequence-containing mitochondrial precursor proteins, the life span of pSu9-DHFR or pCyb2-DHFR were monitored in WT and *sti1*Δ strains. To that goal the translation inhibitor cycloheximide was added to the cultures at a certain time point and samples where then removed after various time periods. The cells were harvested, lysed and the whole cell lysates were subjected to SDS-PAGE and immunodecoration with antibodies against DHFR (Fig. 3.8). The results show that the stability of pSu9-DHFR was not affected in the *sti1*Δ strain (Fig. 3.8 A and B) while for pCyb2-DHFR no signal was detected in the deletion strain (Fig. 3.8 C). In the absence of Sti1, pCyb2-DHFR was either degraded immediately after translation or it was not expressed at all. Since previous experiments showed that the type of the vector could affect the protein expression, we suspected that the absence of pCyb2-DHFR in the whole cell lysate of *sti1*Δ cells might be related to the expression system rather than to enhanced protein degradation. In order to distinguish between these two possibilities, we tested if expression of pCyb2-DHFR from other vectors will result in the same result. Since pSu9-DHFR expression was from the pYX132 plasmid (CEN origin) and pCyb2-DHFR expressed from the pYES2 plasmid (2μ origin), we checked other plasmid (pES426) with 2μ origin. To this end, WT and *sti1*Δ strains from two different backgrounds (W303α and YPH499) were transformed with pES426-pCyb2-DHFR and the protein stability at 34°C was analyzed as before (Fig. 3.8 D and E). The results indicate that the stability of pCyb2-DHFR is not affected by the absence of Sti1 in the YPH499 background, except for the time point one hour at which the precursor form of pCyb2-DHFR could not be detected in the *sti1*Δ cells. This result was, however, not reproducible. But in the W303α background, it seems that the precursor form of pCyb2-DHFR was degraded or expressed at lower levels in the *sti1*Δ strain. Unfortunately, these experiments are not conclusive enough to determine whether the stability of mitochondrial precursor proteins is dependent on Sti1.

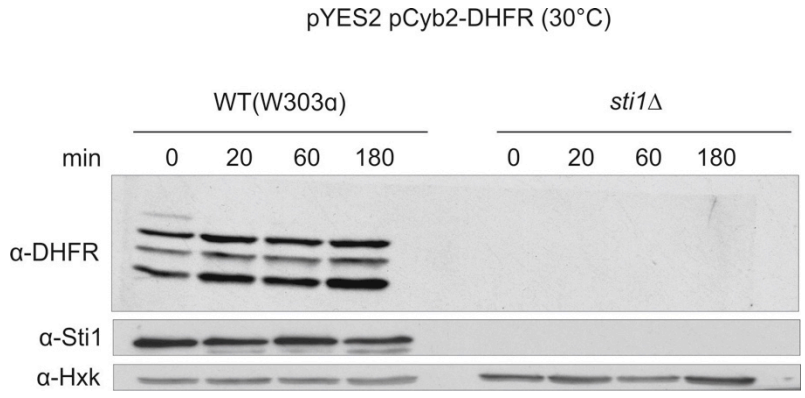
A


pYX132 pSu9-DHFR (30°C)
WT(W303α)
sti1Δ
min
0
20
60
180
0
20
60
180
α-DHFR
α-Sti1
α-Hxk
α-Tom40

B


pYX132 pSu9-DHFR (37°C)
WT(W303α)
sti1Δ
min
0
20
60
180
0
20
60
180
α-DHFR
α-Sti1
α-Hxk
α-Tom40

C


pYES2 pCyb2-DHFR (30°C)
WT(W303α)
sti1Δ
min
0
20
60
180
0
20
60
180
α-DHFR
α-Sti1
α-Hxk

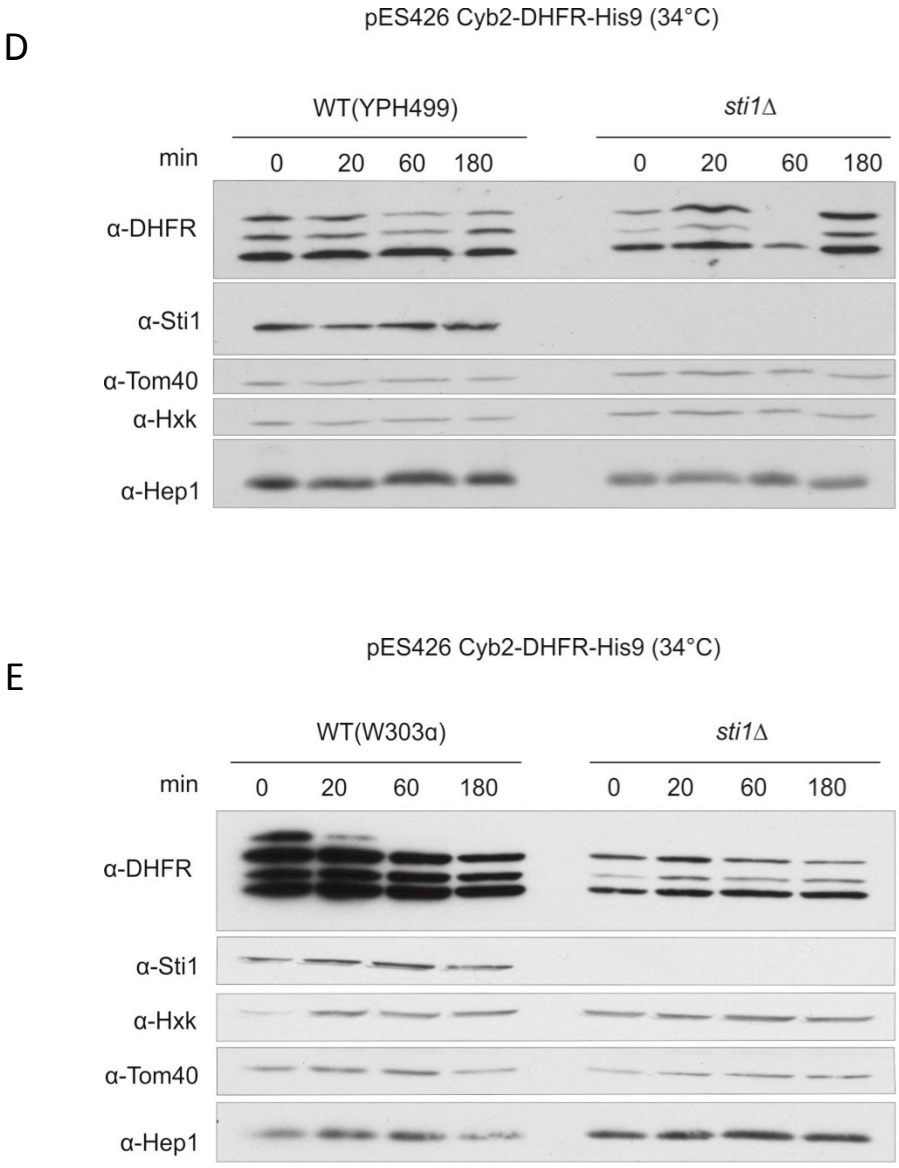


Fig. 3.8: Role of Sti1 in the stability of mitochondrial precursor proteins; Yeast cells expressing pCyb2-DHFR or pSu9-DHFR were grown in liquid medium cycloheximide was added to the cultures (*t*=0), and cells were further incubated for the indicated time periods. Cells were harvested and lysed and the whole cell lysates were analyzed by SDS-PAGE. A and B) pYX132-pSu9-DHFR expressed in W303α cells A) at 30°C. B) at 37°C. C) pYES2-Cyb2-DHFR expressed in W303α cells at 30°C. D) pES426-Cyb2-DHFR expressed in YPH499 cells at 34°C. E) pES426-Cyb2-DHFR expressed in W303α cells at 34°C.

3.5 Deletion of *STI1* affects the steady-state levels of a subset of mitochondrial proteins

To further investigate the effect of the deletion of *STI1* on mitochondrial proteins, whole cell lysates of both WT and *sti1Δ* strains which were grown on glucose-containing medium (YPD or SD) were prepared and the steady-state levels of several

mitochondrial proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 3.9 and Fig. 3.10). The steady-state levels of most of the examined mitochondrial proteins were not significantly altered upon the deletion of *STI1* some proteins showed increased or decreased levels in *sti1*Δ cells. Importantly, only Fis1 and Hep1 showed a major decrease in their steady-state levels in *sti1Δ* cells on both YPD and SD medium. However, this effect is only seen at 37°C. Additionally, Hsp70 and Hsp60 were slightly decreased, but the reduction was not as pronounced as for Fis1. Taken together, we can conclude that only a small subset of mitochondrial proteins is affected by the absence of Sti1 under heat stress conditions. Since the result for other mitochondrial matrix protein was very variable, it is not completely clear whether Sti1 is necessary for the biogenesis of mitochondrial proteins containing an N-terminal targeting signal.

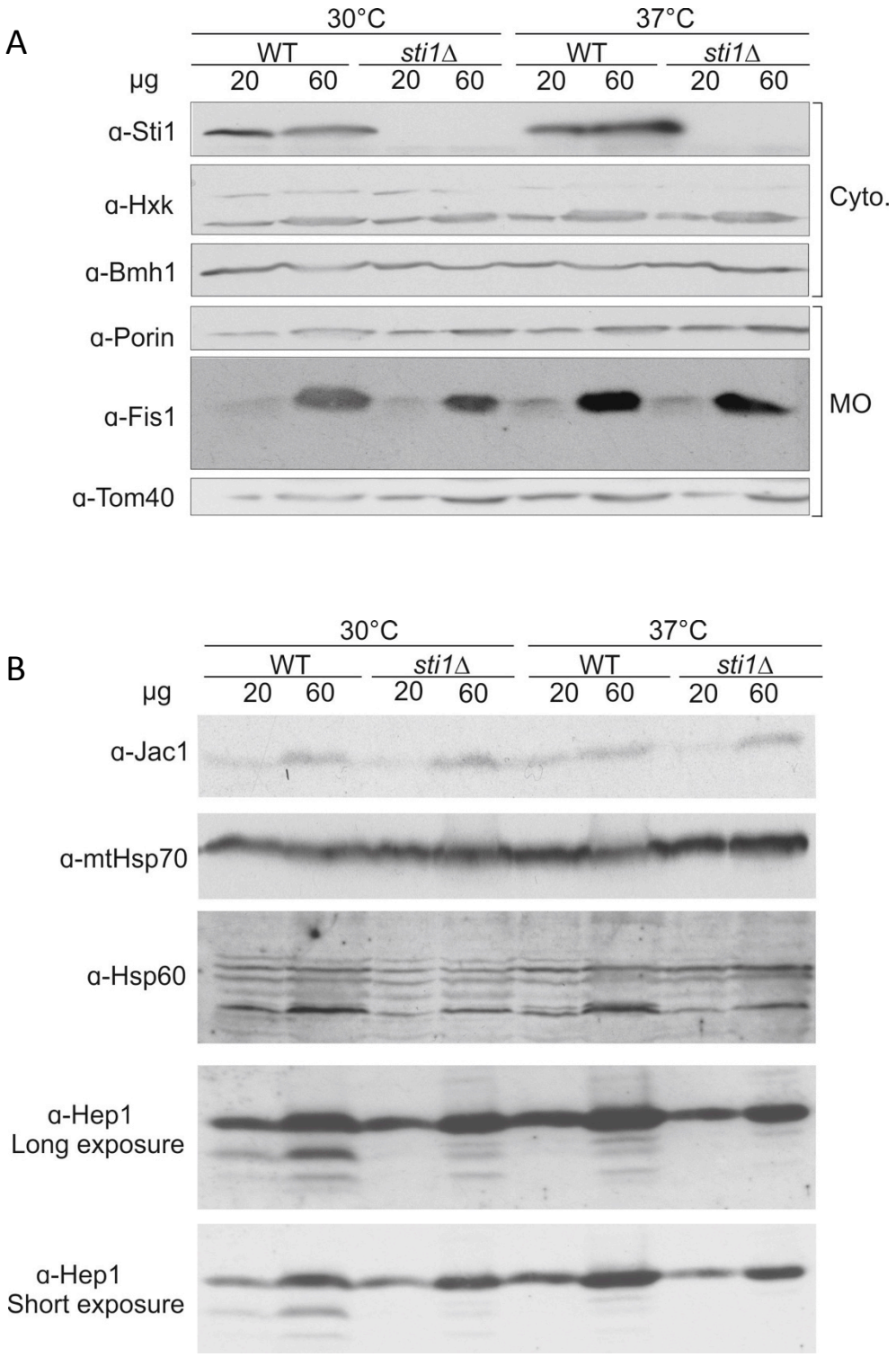


Fig. 3.9: Steady-state levels of various mitochondrial proteins in WT and *sti1Δ* cells grown on YPD medium; Wild type (W303α) cells as well as *sti1Δ* cells were cultivated in YPD medium at 30 or 37°C, subsequently harvested and lysed. Whole cell extracts were subjected to SDS-PAGE analysis and immunoblotting with antibodies against various mitochondrial matrix proteins as well as against Sti1 to confirm its deletion. The cytosolic proteins hexokinase and Bmh1 were used as loading control. A) Mitochondrial outer membrane proteins. B) Mitochondrial matrix proteins.

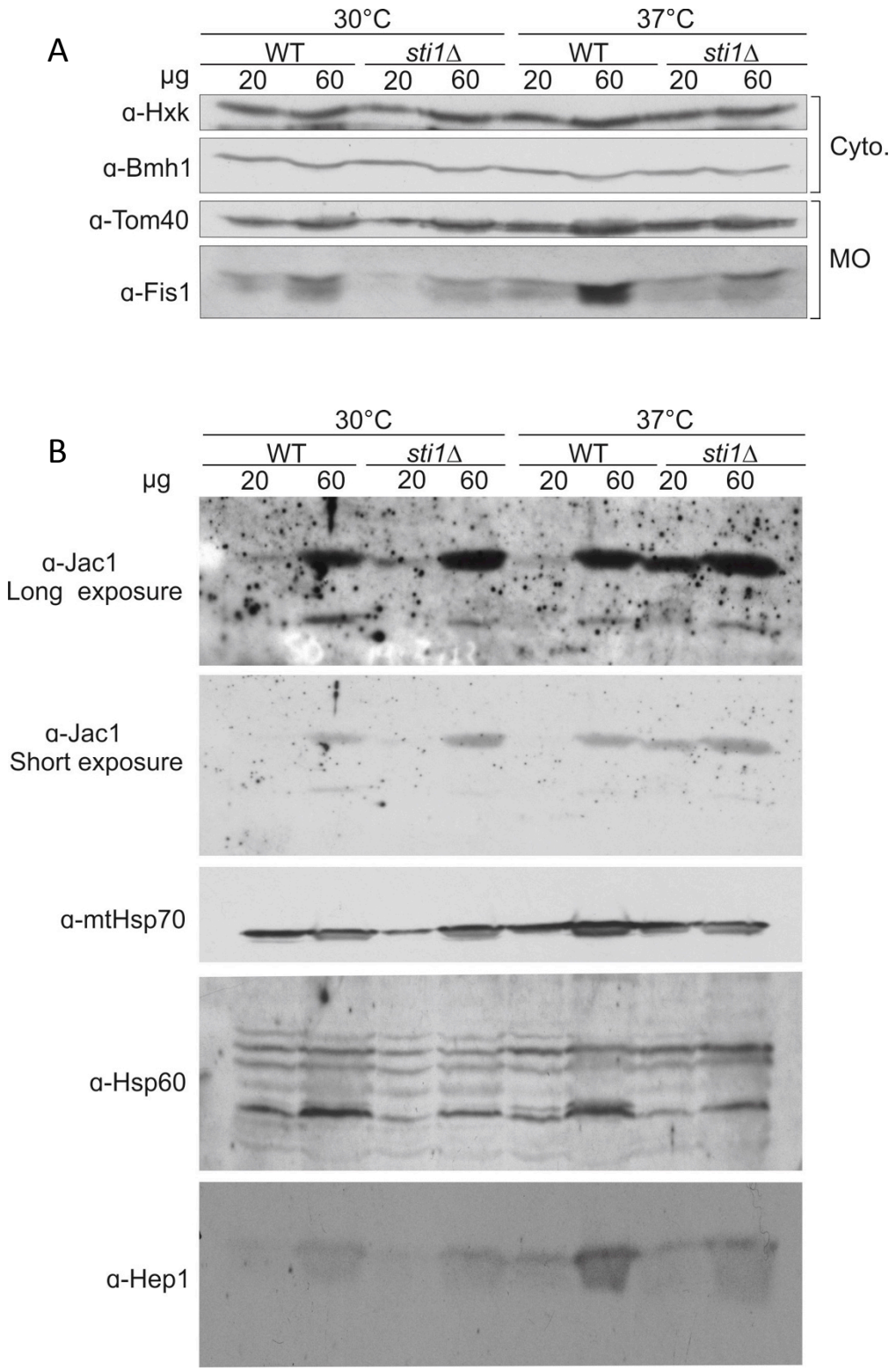


Fig. 3.10: Steady-state levels of mitochondrial matrix proteins in WT and *sti1Δ* cells grown on SD medium; Wild type (W303α) cells as well as *sti1Δ* cells were cultivated and analyzed as described in the legend to Fig. 3.9. A) Mitochondrial outer membrane proteins (MO). B) Mitochondrial matrix proteins.

3.6 Sti1 affects mitochondrial morphology

In addition to the importance of Sti1 for proteins biogenesis we were interested to investigate the influence of the deletion of *STI1* on mitochondrial morphology. To that goal WT and *sti1Δ* cells were transformed with a plasmid encoding matrix-targeted GFP (mt-GFP) to visualize their mitochondria. The mitochondrial morphology of these cells was analyzed by fluorescence microscopy (Fig. 3.11).

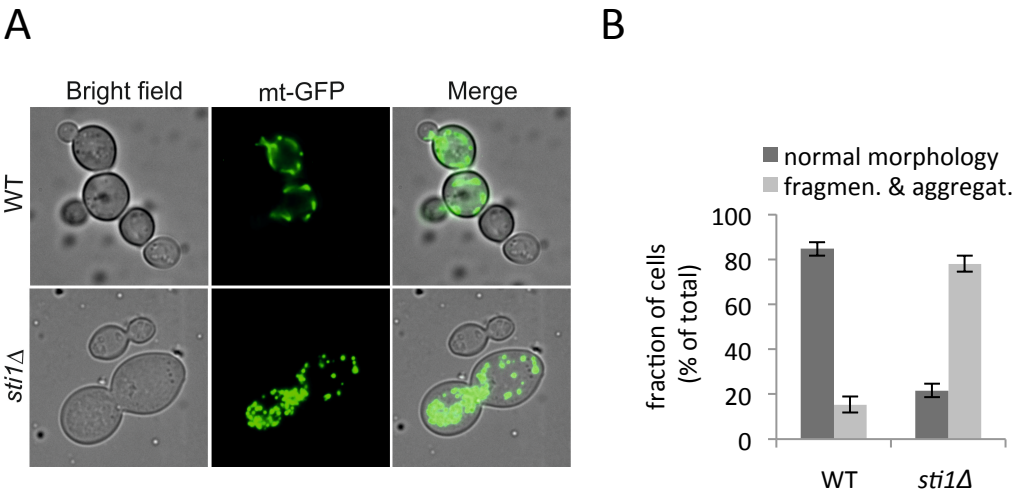


Fig. 3.11: Deletion of *STI1* affects mitochondrial morphology; A) Mitochondrial morphology was analyzed by fluorescence microscopy in WT and *sti1Δ* yeast cells that were grown at 37°C on SD-Leu medium. B) Statistical analysis of three independent experiments. Fragmen. & aggregate. Fragmented and aggregated mitochondria.

This analysis indicated that the mitochondria in the deletion strain were altered and fragmented as compared to the WT strain. Mitochondria of WT yeast form branched or tubular networks distributed around the cell but in the *STI1* deletion strain they were mostly fragmented and aggregated. Statistical analysis of the mitochondrial morphology showed that most *sti1Δ* cells harbor mitochondria with an abnormal morphology is much more than normal morphology (Fig. 3.1 B). Moreover, the *sti1Δ* cells are larger in size in comparison with WT cells. Taken together, these results show that Sti1 plays an important role in mitochondrial and cell morphology.

3.7 *STI1* deletion affects cell growth at elevated temperature

To further study the function of Sti1, the effect of this co-chaperone on the cell growth of yeast cells was investigated. WT (W303α) and *sti1*Δ cells were grown on liquid YPD or SD media at 30°C or 37°C and their growth was measured. As shown in Fig. 3.12 A,C, the *sti1Δ* strain showed the same growth behavior as the WT at 30°C. However, at

elevated temperature the mutant strain grew slower than the WT strain (37°C) on both YPD and SD media (Fig. 3.12 B and D). These findings show that the deletion of *STI1* affects cell growth at elevated temperature.

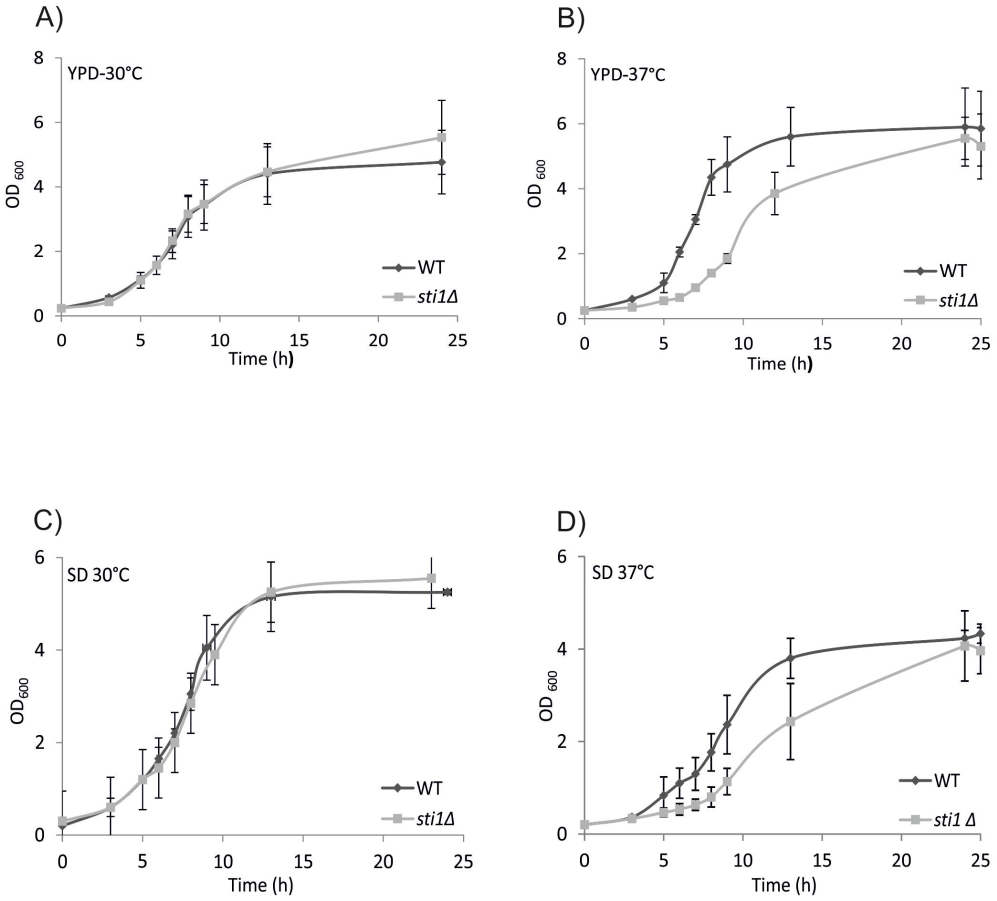


Fig. 3.12: Deletion of *STI1* affects cell growth at elevated temperature; WT and *sti1Δ* cells were grown on YPD (A, B) or SD (C, D) at 30°C and 37°C and the growth was analyzed by measuring the OD_{600} at various time points. A) YPD medium at 30°C, B) SD at 30°C, C) YPD at 37°C, D) SD at 37°C.

3.8. Genetic interaction between *STI1* and *MIM1*

Fluorescence microscopy and cell growth studies indicated that the absence of Sti1 could affect mitochondrial morphology and cell growth. To better understand the physiological role of Sti1 the growth of *sti1Δ* cells was analyzed also on solid medium. Furthermore, the genetic interaction of *STI1* with the import factor *MIM1* was investigated. To that goal the double deletion strain *mim1Δsti1Δ* was created and the growth phenotype of the single deletion strains as well as the double deletion strain was monitored by drop-dilution assay (Fig. 3.13).

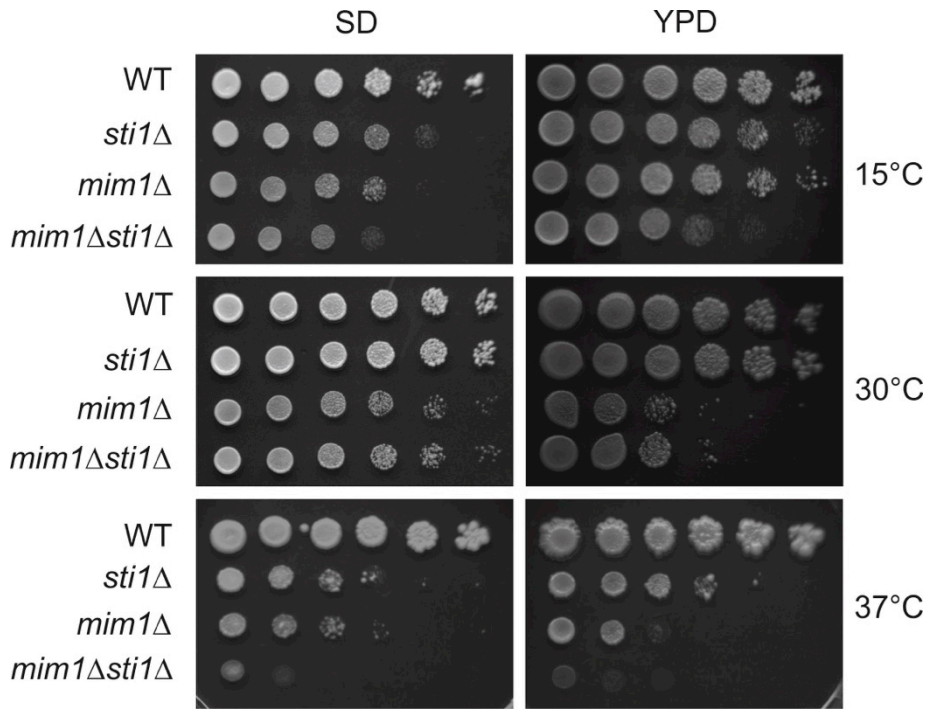


Fig. 3.13: Drop dilution assay of wild type, *sti1Δ*, *mim1Δ* and *mim1Δsti1Δ* cells; Drop dilution assay was performed using knockout strains and the corresponding wild type cells (W303α) on YPD and SD medium at 15°C, 30°C and 37°C.

The results show that *sti1Δ* and the corresponding WT (W303α) grow equally well on YPD and SD medium at 30°C. However, at lower temperature (15°C) *sti1Δ* cells displayed a slightly reduced growth rate compared to the corresponding wild type strain. This growth defect phenotype was even more pronounced at 37°C on both SD and YPD media. It can be concluded that Sti1 is necessary for cells in stress conditions especially at elevated temperatures and on synthetic media. Moreover, the double knockout strain (*mim1Δsti1Δ*) shows severe growth defect at 37°C in both YPD and SD medium that are by far more elaborated than the single deletion strains. It can be concluded that *MIM1* is a genetic interaction partner of *STI1*.

3.9 Deletion of *MIM* components affects association of Sti1, Ssa1 and Hsp82 with mitochondria

The drop dilution assay showed that there might be a genetic interaction between *STI1* and *MIM1*. To better understand the role of the MIM complex in relation to Sti1 and to other cytosolic factors strains with a deletion of Mim1 or Mim2 and wild-type cells as a control were grown on YPG medium. Their mitochondria were isolated and subjected to the SDS-PAGE and immunoblotting (Fig. 3.14).

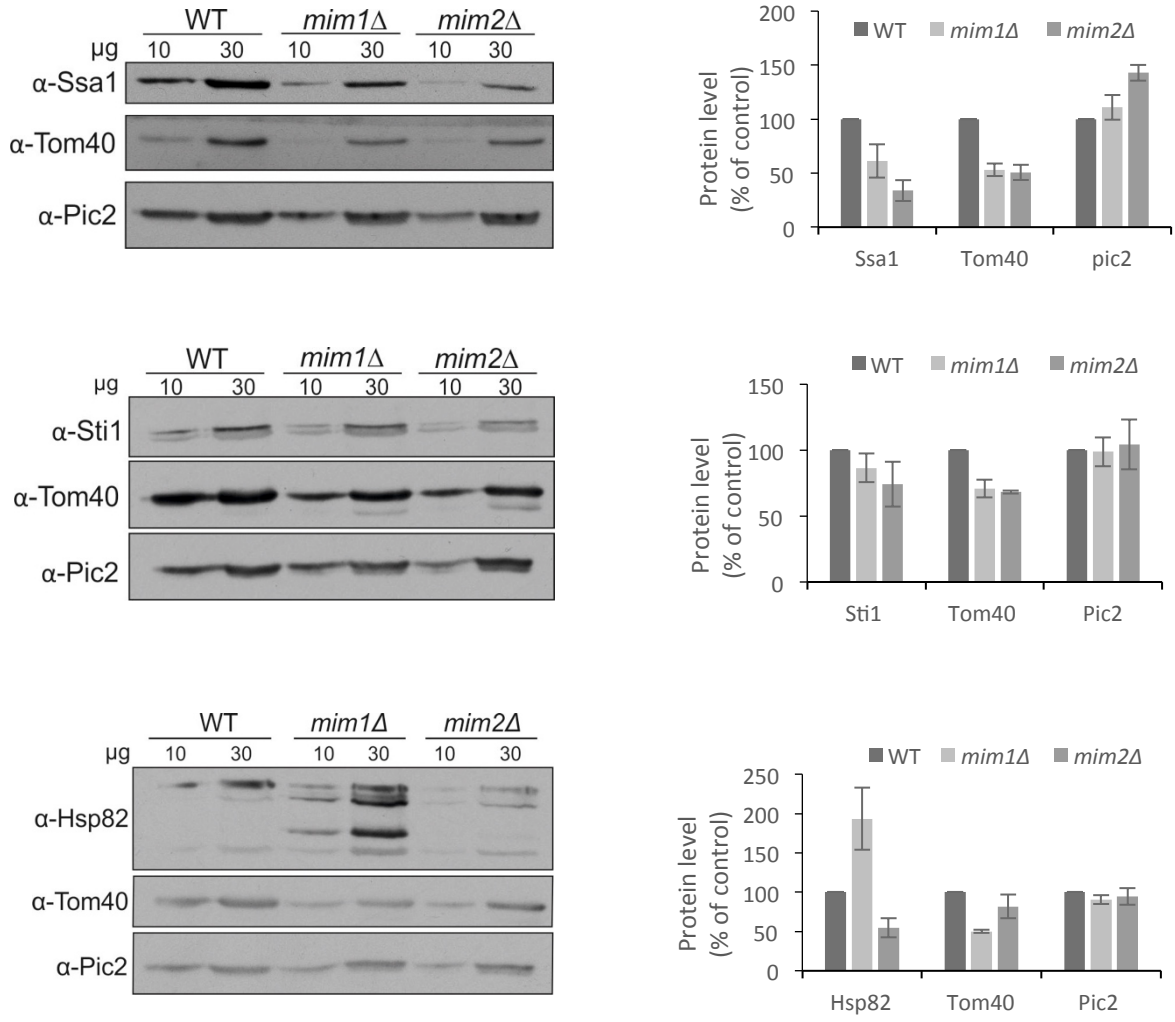


Fig. 3.14: Deletion of *MIM* complex components affects association of cytosolic factors with mitochondria; *mim1Δ, mim2Δ* and the corresponding WT (W303α) cells were grown on YPG medium. Their mitochondria were isolated and analyzed by SDS-PAGE and immunodecoratin with the indicated antibodies. Pic2 was used as a loading control whereas Tom40 is known to be affected by deletion of Mim subunits.

The results suggest that in the absence of Mim1 or Mim2, the amount of Sti1 and Ssa1 on the mitochondrial surface is reduced in comparison to WT cells. Interestingly, in the absence of Mim1 the level of associated Hsp82 is dramatically increased whereas in the absence of Mim2 it is decreased. Taken together, the components of the MIM complex affect the association of these cytosolic factors with the mitochondrial surface. It seems that the deletion of Mim2 has a stronger effect than the deletion of Mim1.

3.10 The role of the import receptors Tom20 and Tom70 in the association of cytosolic factors with the mitochondrial surface

3.10.1 Cytosolic factors interact differentially with Tom20 and Tom70

It is known that cytosolic Hsp70 and Hsp90 chaperones are involved in the import of carrier proteins into mitochondria and are docking on the import receptor Tom70 (Young et al, 2003). In order to screen the yeast cytosol for specific cytosolic binding partners of Tom20 and Tom70, pull-down experiments with N-terminally GST-tagged recombinant versions of the cytosolic domains (cd) of Tom70 ($Tom70_{cd}$-GST) and Tom20 ($Tom20_{cd}$-GST) were performed (Fig. 3.15). Samples representing the bound material were subjected to SDS-PAGE and immunodecoration with antibodies against different cytosolic factors (Fig. 3.15 A).

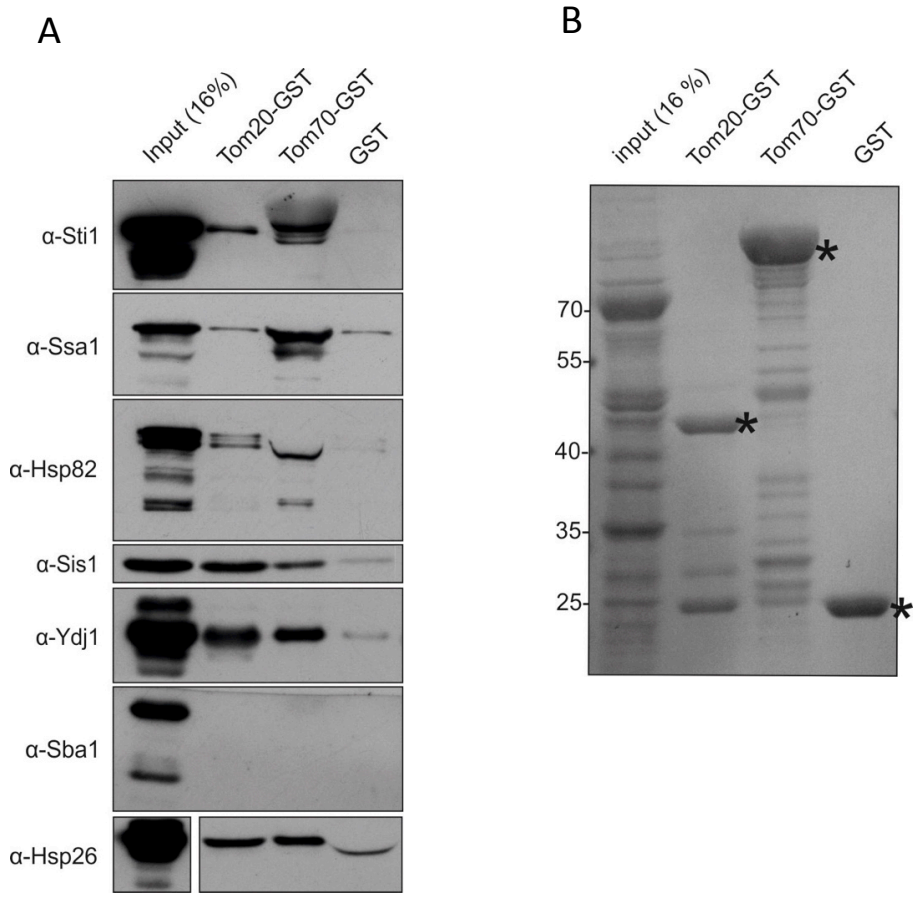


Fig. 3.15: Interaction of cytosolic factors with $Tom70_{cd}$-GST and $Tom20_{cd}$-GST; A) The indicated recombinant GST-tagged proteins were incubated with GSH-sepharose beads and subsequently with cytosol isolated from yeast WT cells (W303α). The beads were washed and bound material was analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. B) The same membrane as in part A was stained with Ponceau S to detect the GST-tagged proteins (indicated with asterisk).

The results show that Sti1, Ssa1 and Hsp82 bind specifically to the Tom70 receptor. This was shown before in the case of Ssa1 (Young et al. 2003). On other hand, Sis1 and Ydj1 bind better to Tom20 receptor. Sba1 did not bind to any of the receptors and Hsp26 binds rather equally weak to both receptors. Hence, we can conclude that similarly to Ssa1, Sti1 and Hsp82 can be recognized by Tom70 while Sis1 and Ydj1 are recognized by Tom20.

3.10.2 Genetic interaction of *STI1* and *TOM20*

The pull-down experiments showed that under *in vitro* conditions Sti1 binds better to Tom70 while so far it is known that presequence-containing mitochondrial proteins are recognized mainly by Tom20. If Sti1 is interacting with such preproteins, one can expect that Sti1 interacts also with Tom20. To test this possibility, the growth phenotype of cells harboring a double deletion of *STI1* and *TOM20* or their single deletion were assessed by drop-dilution assay on YPD and YPG medium at 15°C, 30°C or 37°C (Fig. 3.16). The results indicate that the double deletion causes a severe growth defect in comparison with the single deletions of either *STI1* or *TOM20*. As functional mitochondria are necessary for yeast cells to utilize non-fermentable carbon sources such as glycerol, a reduced growth rate on YPG medium is an indicator for a defect in mitochondrial function and/or biogenesis. The impaired growth rate exhibited by *sti1Δtom20Δ* cells on glycerol-containing medium implies a reduced mitochondrial function in the double knockout strain.

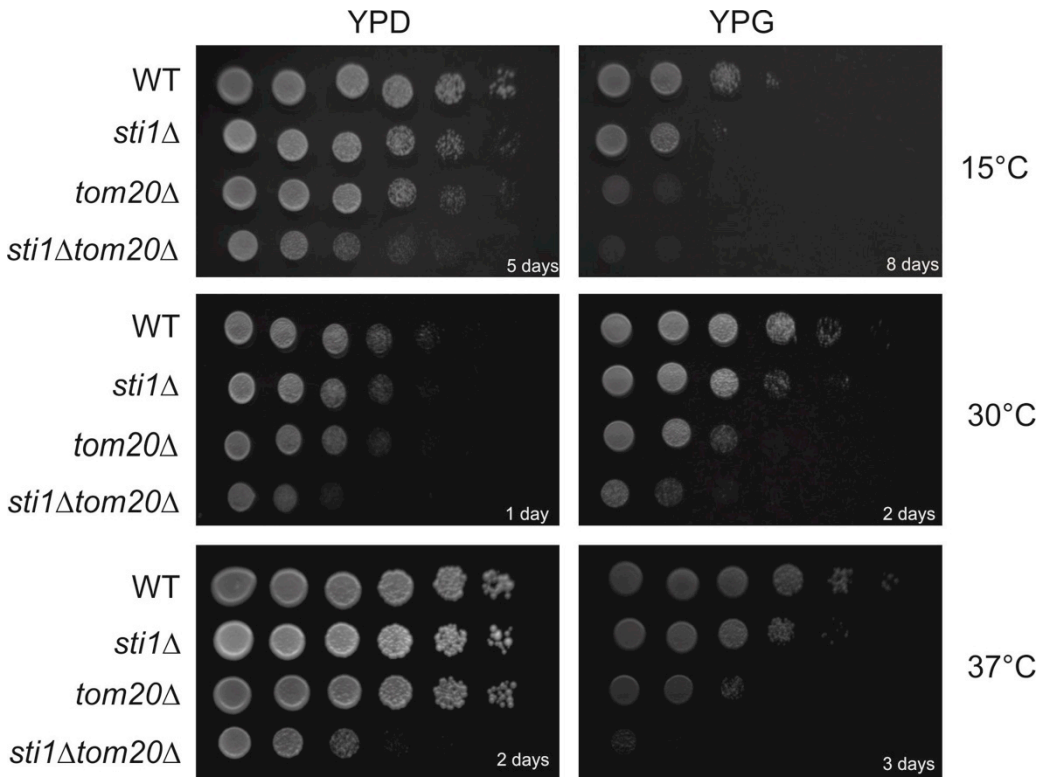


Fig. 3.16: Double deletion of *TOM20* and *STI1* causes severe growth defect; A drop dilution assay was performed using WT, *sti1Δ, tom20Δ* and *sti1Δtom20Δ* (W303α) strains on YPD and YPG medium at 15°C, 30°C and 37°C.

3.10.3 Steady state levels of mitochondrial proteins and cytosolic factors in *sti1Δtom20Δ* cells

To further investigate the function of Sti1 in mitochondrial protein import, steady state levels of mitochondrial proteins and cytosolic factors associated with mitochondria were checked in *sti1Δ*, *tom20Δ*, *sti1Δtom20Δ* and the corresponding WT (W303α) strain. Cells were grown on YPG medium and after harvesting and lysis, mitochondrial and cytosolic fractions of all strains, were subjected to SDS-PAGE and immunodecoration with antibodies against cytosolic factors (Fig. 3.17) and mitochondrial proteins (Fig. 3.18). The results demonstrate that the steady state levels of Sba1 and Hsp82 are the same in all strains suggesting that Sti1 and/or Tom20 are not involved in the mitochondrial association of these chaperones. However, the steady state levels of some cytosolic factors like Ydj1, Sis1 and Hsp26 were elevated in *sti1Δ* or *sti1Δtom20Δ*. Importantly, the steady state levels of Hsp26 and Sis1 were increased in the mitochondrial fraction of the double deletion strain (Fig. 3.17 C and D) while the Ydj1 level increased in the cytosolic fraction but not in the mitochondrial one. However, it was also elevated in *sti1Δ* cells (Fig. 3.17 B).

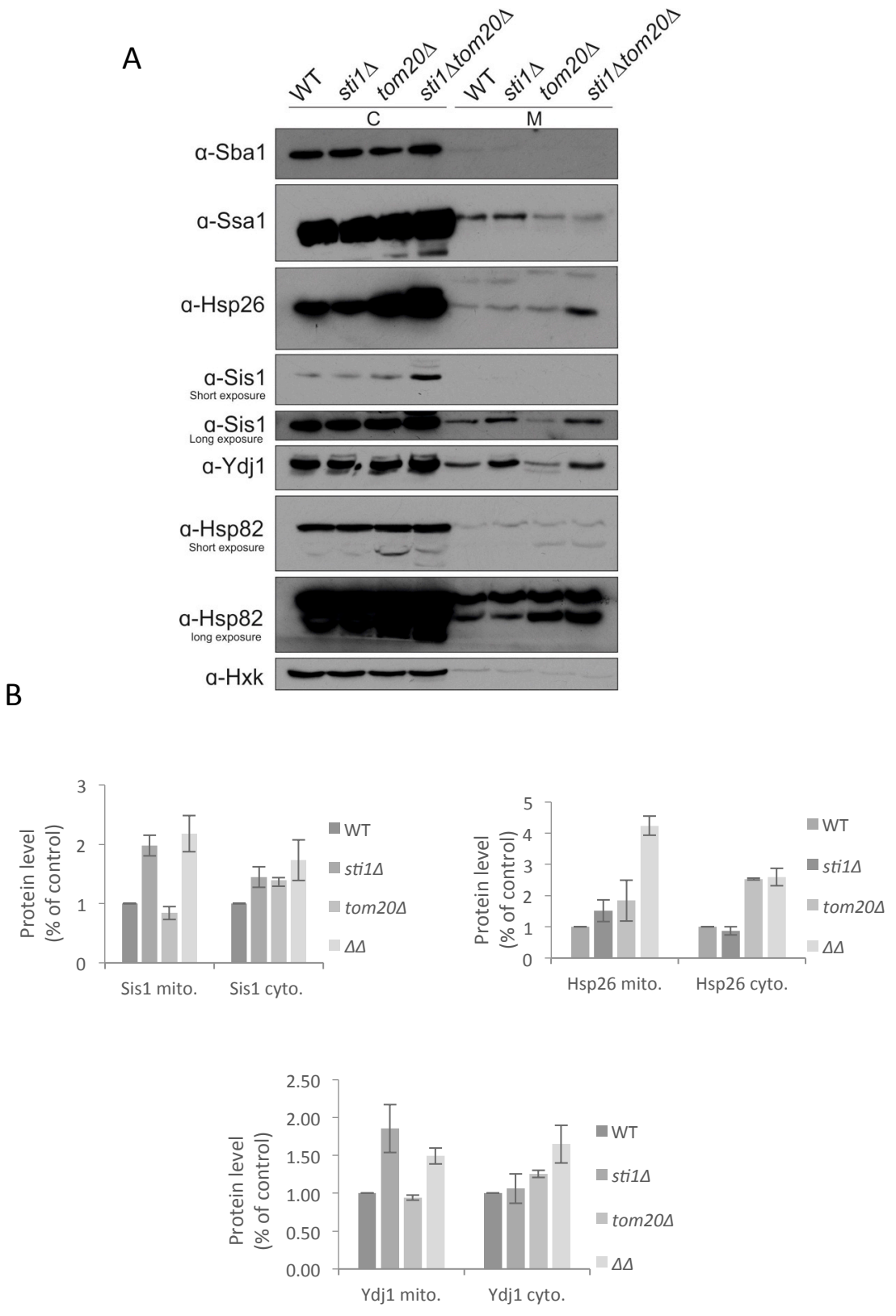


Fig. 3.17: Steady state levels of cytosolic factors in cytosolic and mitochondrial fractions; A) Cells from the indicated strains were grown on YPG medium, harvested and lysed to separate cytosolic (C) and mitochondrial (M) fractions. Each fraction was subjected to SDS-PAGE followed by immunodecoration with the indicated antibodies. Hexokinase (Hxk) was used as a loading control. B) Quantification of the steady state levels of Ydj1, Sis1 and Hsp26.

These results indicate that Hsp26, Sis1 and Ydj1 might play an important role in the absence of both Tom20 and Sti1 to compensate for the missing activity of the co-chaperone and receptor. Alternatively, the steady state level of these proteins could be increased because of stress conditions that occur in the absence of Tom20 and Sti1.

The steady state levels of most of the examined mitochondrial proteins did not change in response to the deletion of *STI1* and *TOM20*. Only the Pic2 and Porin levels were significantly decreased in mitochondria from the *sti1Δtom20Δ* strain (Fig.3.18). Taken together, we could not observe major alteration in the steady-state levels of mitochondrial proteins in the absence of Sti1 or Tom20. Even when both proteins were deleted the steady state levels of only some mitochondrial proteins were affected.

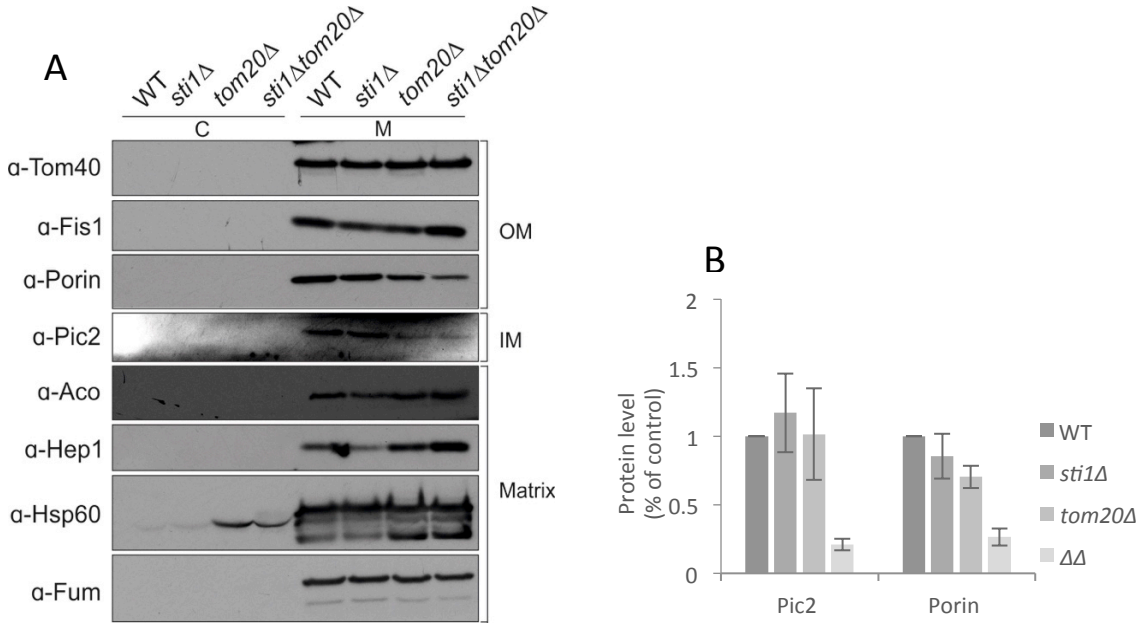


Fig. 3.18: Steady state levels of the mitochondrial proteins Pic2 and porin are reduced in mitochondria from *sti1*Δ*tom20*Δ cells; A) Cells from the indicated strains were grown on YPG medium, harvested and lysed to separate cytosolic (C) and mitochondrial (M) fractions. Each fraction was subjected to SDS-PAGE followed by immunodecoration with the indicated antibodies. B) Quantification of the steady state levels of Porin and Pic2. The levels of Fis1 were taken as loading control. (ΔΔ; *sti1*Δ*tom20*Δ).

3.11 Hsp90 affects *in vitro* import of Mpp1 into mitochondria

To investigate the role of Hsp90 in the import of mitochondrial precursor proteins, radiolabeled Mpp1 was imported into isolated mitochondria in the presence or absence of Radicicol. Radicicol inhibits the activity of Hsp90 in the rabbit reticulocyte lysate and therefore enables us to analyze its role in mitochondrial protein import. Import of Mpp1 was analyzed by SDS-PAGE and autoradiography (Fig. 3.19).

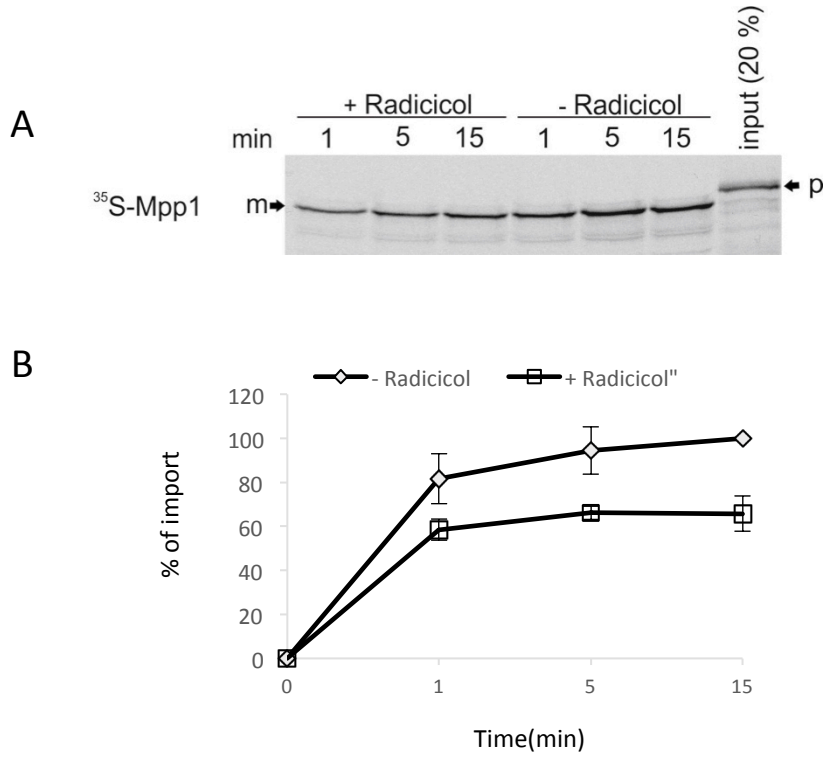


Fig. 3.19: *In vitro* import of Mpp1 is reduced when Hsp90 is inhibited; A) *In vitro* import of Mpp1 in the presence or absence of Radicicol. p, precursor protein; m, mature form. B) Statistical analysis of part A.

The results show that the import of Mpp1 was inhibited by the presence of Radicicol. Thus, it appears that Hsp90 affects the import of Mpp1 at least under these *in vitro* conditions. However, since this result was not reproducible with other mitochondrial precursor proteins we cannot conclude that Hsp90 plays a general role in the import of mitochondrial precursor proteins.

4. Discussion

4.1 Identification of molecular chaperones that interact with mitochondrial preproteins

Cytosolic factors such as molecular chaperones are required to prevent mitochondrial precursor proteins from folding and aggregation and to keep them in an import-competent state. Moreover, chaperones are also involved in the interaction with the mitochondrial import machinery. For instance, the recognition of chaperone-precursor molecule complexes can be mediated by the import receptor Tom70 that is known to have chaperone activity. So far, only little is known about the identity and the potential function of cytosolic chaperones assisting in import of presequence-containing mitochondrial proteins. Additionally, the mechanism by which newly synthesized precursor proteins are targeted to the surface of the organelle still remains largely unresolved (Beddoe and Lithgow, 2002; Young et al., 2003; Zara et al., 2009). In this study, cytosolic factors interacting with the presequence of the model protein pCyb2-DHFR were identified in yeast cells by site-directed *in vivo* photo-crosslinking. It has been demonstrated that the cytosolic factors Sti1, Ssa1, Hsp82 and Ydj1 interact *in vivo* with pCyb2-DHFR.

Ssa1, one of the cytosolic isoforms of Hsp70 was previously suggested to be involved in mitochondrial protein import. In fact, Ssa1 has been reported to bind to the amphiphilic presequences of many mitochondrial precursor proteins (Endo et al., 1996) and has been

shown to interact with the mitochondrial surface receptor Tom70 (Young et al., 2003). Ydj1, a Hsp40 co-chaperone, plays undefined role in protein import into mitochondria and mitochondrial protein biogenesis was defect in cells harboring mutated *YDJ1*. Interestingly, it was shown that overexpression of Sis1 (another Hsp40 co-chaperone) can rescue this mutation (Caplan et al., 1992). Both Ydj1 and Sis1 belong to the Hsp40 family of co-chaperones and interact with Hsp70 family proteins (Caplan and Douglas, 1991; Luke et al., 1991). Thus, their role in mitochondrial biogenesis might be related to the involvement of Hsp70 in mitochondrial protein import.

In this study it was demonstrated that Ydj1 interacts with both mitochondrial preproteins and the import receptor Tom20. However, the precise role of this co-chaperone in targeting and import of mitochondrial proteins could not be evaluated. Hence, at this stage, it can be concluded that Ydj1 probably functions as a regulatory partner protein of Ssa1. Additionally, Ydj1 was suggested to assist targeting of Hsp70 proteins to both mitochondria and ER via its farnesyl membrane-anchor (Caplan et al., 1992; Becker et al., 1996). Hence, Ydj1 might be involved indirectly in mitochondrial protein import as a co-chaperone or might assist the specific recognition of precursors by Tom20 receptor.

Additionally, Sti1 and Hsp82 were identified as interaction partners of pCyb2-DHFR. These findings indicate that the co-chaperone Sti1 seems to be involved in import of preproteins into mitochondria. Since so far nothing has been reported about the role of this co-chaperone in mitochondrial protein import, I mainly focused in this study on this co-chaperone. The role of these cytosolic factors will be further discussed in section 4.2.

In addition to cytosolic factors, we expected to find interactions of the presequence with components of the mitochondrial import machinery at the outer membrane such as Tom20, Tom40, Tom22 and Tom70. Especially interactions with the import receptors Tom20 and Tom22, which are known to bind presequence-containing proteins was anticipated. However, no cross-linked species specific for the main import receptors Tom70 and Tom20 were detected by immunodecoration. Only for Tom22 an interaction was shown, but this was not reproducible. Several reasons might be responsible for the absence of such detected interactions. First, the association of the hydrophobic face of pCyb2-DHFR with Tom20 is mediated by only short stretch of amino acids. Since the photo-reactive probe was located on the verge of this interacting region it might not be in the vicinity of Tom20. Second, the interaction of the presequence with receptors

might be very transient and temporary whereas the binding to the abundant cytosolic chaperones is probably more stable. Thus, a larger fraction of the precursor proteins are bound to cytosolic factors and therefore crosslinked to them. The small amount of precursor proteins that might be bound to outer membrane import factors and X-linked to them is apparently under these conditions too low to be detectable by immunodecoration.

4.2 Involvement of Sti1 in mitochondrial import of preproteins

As mentioned above, cytosolic chaperones are often involved in either binding to unfolded substrates keeping them in an import-competent state or in association with aggregate substrates to induce their refolding or degradation. My results identified Sti1 as an interaction partner of preproteins. Thus, I had a special interest to evaluate its potential role in precursor targeting and in the mitochondrial import process. Sti1 is an Hsp90 co-chaperone, homolog of mammalian Hop that regulates spatial organization of amyloid-like proteins in the cytosol (Frydman and Höfeld, 1997). This protein not only functions as a co-chaperone but also acts as an adapter between Hsp70 and Hsp90 and mediates their assembly to form a functional complex. It works as a linker, which specifically recognizes the C-terminal end of Hsp70 and Hsp90 by its two TPR clamp domains. In this way Sti1 assists the substrate transfer from Hsp70 to the Hsp90 chaperone machinery (Scheufler et al., 2000; Wegele et al., 2003). Additionally Sti1 interacts with the Ssa group of the cytosolic Hsp70 chaperones and activates Ssa1 ATPase activity. In contrast, upon its interactions with Hsp90 chaperones it inhibits their ATPase activity (Nicolet et al. 1989, Wolfe et al. 2013, Richter et al. 2003, Wegele et al. 2003).

While Hsp90 null mutations are lethal, deletion of *STI1* does not affect cell growth at 25°C or 30°C, but causes a slight defect of growth at higher and lower temperatures (Chang et al., 1997). In the present study we could confirm this report and extend it by observing a slight growth defects on non-fermentable carbon source (YPG) even at 30°C. Furthermore, we observed also alterations in mitochondrial morphology upon deletion of *STI1*. Despite these observations neither the steady-state levels of most mitochondrial proteins nor their *in vivo* import, as studied by pulse-chase experiments, was affected upon such a deletion. Our results indicate that among matrix proteins only the steady-state levels of Hep1 decreased in *sti1Δ*. Among the other mitochondrial proteins the levels of Fis1, a tail-anchored protein, was affected as well. Taken together,

my findings imply that Sti1 is not an essential import factor. It might be that in the absence of Sti1 another protein is taking over its function. Indeed, a previous study has suggested that Tom34 is a specific cytosolic factor for mitochondrial protein import in mammalian cells. The protein was found in a complex of at least five different proteins (Hsp90, Hsp70, Hop, Cdc37 and Tom34) and this complex associates with mitochondrial preproteins (Faou et al. 2011). As Hop is the mammalian homologue of Sti1, it seems likely that Sti1 works in mitochondrial protein import within a complex. Further investigation is required to find the exact role of Sti1 in mitochondrial protein import.

In this study it was observed that the levels of certain proteins were affected in *sti1Δ* cells when these proteins were encoded by a high-copy plasmid (2μ). Plasmids, which carrying 2μ origin of replication, encode proteins that allow cells to maintain 20-50 copies of the plasmid. Since this type of plasmids is maintained at such high copy numbers, they provide a convenient way to monitor the effects of overproduction of a particular gene product. Such a high production of a single protein puts the cell under high stress conditions. It seems that in the absence of Sti1 cells have difficulties to handle this over-production. In contrast, the amount of expressed precursor protein and its import efficiency were similar in WT and *sti1Δ* cells when the protein was encoded by a vector with CEN origin which provide low copy number. Therefore, the results obtained in this study do not allow precise conclusions concerning the exact function of Sti1 in mitochondrial protein import. They do however indicate that Sti1 is required to allow cells to deal with high amount of expressed proteins.

Although Sti1 was reported in some cases to functionally interact with Ssa proteins independently of Hsp90 chaperones (Jones et al., 2004), the majority of the Sti1 molecules in yeast cells are found in complex with Hsp90 (Chang et al., 1997; Chang and Lindquist, 1994). Thus, the identification of Sti1 as an interaction partner of the presequence of pCyb2-DHFR strongly suggests an involvement of yeast Hsp90 (Hsp82) as well. Hsp90 chaperones function in mammalian cells downstream of Hsp70 and have been reported to control the activity, turnover, and trafficking of a variety of client proteins (Pearl and Proromou, 2000; Zhao et al., 2005). The Hsp90 chaperone cycle involves highly dynamic and transient multi-chaperone complexes. Thus, the client proteins interact directly with several chaperones and co-chaperones (Buchner, 1999). Hsp82 was found in our photo-crosslinking experiments to be an interaction partner of pCyb2-DHFR. Moreover *in vitro* import experiments showed that Hsp82 might be

important for import of certain mitochondrial matrix protein like MPP. Conversely, other presequence-containing proteins like pSu9-DHFR or F1β were not affected. Thus, the contribution of Hsp90 might be substrate specific.

Since both Hsp70 and Hsp90 can bind to a variety of proteins and do not specifically recognize mitochondrial precursor proteins, other factors like co-chaperones might play a crucial role in the specificity of precursor recognition and targeting to mitochondria. Many of these co-chaperones bind to Hsp70 and Hsp90 by the help of three or more TPR domain (Scheufler et al., 2000). Therefore it is possible that Sti1 functions in cooperation with Hsp70 and Hsp90 to mediate mitochondrial protein import. However, it remains still unclear if and how mitochondrial precursor proteins can be specifically recognized. Additional experiments have to be performed in combination of Sti1 with Hsp70 and Hsp90 or other co-chaperone like Ydj1 and Sis1.

4.3. Physiological role of Sti1 in *S. cerevisiae*

Beside the function of Sti1 in mitochondrial protein import, Sti1 was also observed to affect mitochondrial morphology and cell growth under elevated temperature Furthermore it was shown that Hsp90 interacts with Sti1 (Hop) in lysates of yeast and vertebrate cells (Chang et al.1997). The overexpression of Sti1 has allele-specific effects on cells carrying various *hsp90ts* point mutations. These genetic interactions provide strong evidence that Hsp90 and Sti1 interact *in vivo* and their functions are closely allied. Indeed, deletion of *STI1*, *in vivo* reduces the activity of the Hsp90 target protein, glucocorticoid receptor (GR). Mutations in GR that eliminate interaction with Hsp90 also eliminate the effects of HOP deletion. Examination of GR protein complexes in the *STI1* deletion mutant reveals a selective increase in the concentration of GR-Ydj1 complexes, supporting previous hypotheses that Ydj1 functions at an early step in the maturation of GR and that Sti1 acts at an intermediate step. Hence, it appears that Sti1 is a general factor in the maturation of Hsp90 target proteins (Chang et al.1997). In this study we found that *STI1* has genetic interaction with *MIM1*. Mim1 is a mitochondrial outer membrane protein that is required for the assembly of the TOM complex of mitochondria (Waizenegger et al. 2005, Ishikawa D, et al. 2004). It also plays an important role in import of multispan mitochondrial outer membrane proteins like Ugo1 and Om14 (Papic et al, 2011, Becker et al., 2011). Recently it has shown that Mim1 is necessary for integrating Tom20 into the Tom complex as well (Hulett JM, et al 2008). Since mutation in both *STI1* and *MIM1* genes produced growth defect

phenotype at elevated temperature it can conclude that there is genetic interaction between *STI1* and *MIM1* under stress conditions. This genetic interaction can reveal functional relationship between genes and pathways but direct physical interaction between these two proteins was not examined in this study.

Moreover our observations indicate that *STI1* also genetically interact with *TOM20.* Double deletion of these two genes produced a synthetic growth phenotype as well. However, the steady state levels of only some mitochondrial protein such as Porin and Pic2 were affected in this double deletion strain. Porin is a high abundance protein in mitochondrial outer membrane that was reported to be recognized by Tom20 (Krimmer et al. 2001). Pic2 is a mitochondrial copper and phosphate carrier that is located in the inner membrane. Usually, the targeting and translocation of such carrier proteins are mediated by the Tom70 import receptors. Thus, a common characteristic for both proteins is not obvious.

Tom20 is the main import receptor for mitochondrial preproteins. It has no TPR clamp domain that can mediate the interaction between Hsp70 and Hsp90 and it binds directly to the mitochondrial targeting signal (Bhangoo et al., 2007; Abe et al., 2000). In addition, Tom20 is also involved in mitochondrial localization of mRNAs encoding mitochondrial proteins (Eliyahu et al., 2010). In yeast cells, an interaction of Tom20 with other components of the chaperone-precursor complex could not be demonstrated. In contrast, in mammalian cells the co-chaperone AIP was found to bind directly to Tom20 via its TPR clamp domain and transfers preprotein-Hsc70 complexes to the import receptor (Yano et al., 2003). Moreover, cytosolic domains of Tom20 and Tom22 have a chaperone-like activity (Yano et al., 2004). This might explain why the involvement of cytosolic chaperones in Tom20-mediated import seems to be less pronounced (Young et al., 2003).

Since Sti1 has TPR domain it might also interact with Tom20 for targeting mitochondrial protein to the organelle. We showed in this study that Sti1 interacts *in vitro* stronger with Tom70 than with Tom20. However, other cytosolic factors like Ydj1 and Sis1 were found to interact *in vitro* with Tom20. Both proteins are Hsp40 co-chaperones. Sis1 is a Type II Hsp40 co-chaperone that interacts with the Hsp70 protein Ssa1. It shuttles between cytosol and nucleus to mediate delivery of misfolded proteins into the nucleus for degradation and is also involved in proteasomal degradation of misfolded cytosolic protein and protein abundance increases in response to DNA

replication stress. Sis1 functions similar to bacterial DnaJ proteins and mammalian DnaJB (Luke, et al. 1991, Summers DW, et al. 2013). The second protein, Ydj1 is a DnaJ-like type I Hsp40 co-chaperone that is involved in regulation of Hsp90 and Hsp70 functions. It acts as an adaptor that helps Rsp5p to recognize cytosolic misfolded proteins for ubiquitination after heat shock and is critical for determining cell size. Indeed, Ydj1 was reported to facilitate proteins translocation across different intracellular membranes (Caplan et al. 1992; Atencio and Yaffe 1992). A role of Sis1 in mitochondrial protein import was not reported so far.

4.4 Conclusions

Taken together, the results of this study are not conclusive regarding a specific involvement of Sti1 or Hsp82 in import of mitochondrial preproteins. However, the current findings demonstrate that Sti1 can affect mitochondrial morphology. Moreover the absence of Sti1 causes growth defect in yeast cells. The slow growth of *Sti1Δ* cells on non-fermentable carbon source support the notion that absence of Sti1 can cause mitochondrial deficiency. The direct reason for this defect is not clear as the steady state level of most tested mitochondrial proteins did not changed in *Sti1Δ* cells. However, since the levels of some proteins like Hep1 and Fis1 were decreased in the absence of Sti1, it might be that the lower levels of only some key proteins are sufficient to cause such a growth phenotype. Furthermore in this study it was found that *STI1* genetically interacts with *MIM1* and *TOM20* and pull-down experiment showed that Sti1 has physical interaction with Tom70 as well. In summary, Sti1 plays, a yet to be defined, role in mitochondrial biogenesis.

4.5 Outlook

It might be that the *in vivo* conditions used in most of the assays are not optimal to detect a specific contribution of a single protein due to the redundancy among the various chaperones. Furthermore, the reticulocyte lysate that was used for *in vitro* translation and import is of mammalian origin and hence was not the best system to test the effect of yeast cytosolic proteins on mitochondrial import. Hence, an appropriate yeast system for *in vitro* synthesis and import of radiolabeled precursor proteins should be employed. Such a system could then allow the addition of recombinant Sti1, Hsp82, Ydj1 and Sis1or the usage of lysate from cells deleted for a certain factor.

Moreover to pursue the general aim of this study namely, the identification of cytosolic factors, which are involved in mitochondrial protein import, additional approaches should be considered. For example, it could be investigated whether other co-chaperones like Sis1 or Ydj1 interact directly with mitochondrial precursor proteins. Furthermore, it should be examined whether such factors function within a complex with other chaperones. In addition, it will be better to use additional methods like mass spectrometry and yeast two-hybrid system, to identify with high precision cytosolic factors, which interact with mitochondrial preproteins.

5. Summary

During the evolutionary evolvement of mitochondria, the mitochondrial genome got reduced and has almost completely been transferred to the host genome. Therefore most mitochondrial proteins are synthesized in the cytosol and delivered to the organelle by the help of molecular chaperones in an unfolded state. Cytosolic chaperones like Hsp90 and Hsp70 in mammalian cells act in a large cytosolic complex that helps in the delivery of some mitochondrial proteins to the organelle by docking on the import receptor Tom70. These chaperones are abundant ones, which have many other cellular functions, suggesting that the specificity for the targeting of mitochondrial proteins probably requires addition of specific factors within the targeting complex. Most mitochondrial precursor proteins comprise a cleavable N-terminal targeting sequence, also known as presequence, that targets them to the mitochondrial import receptor Tom20. Many of these proteins associate during or after synthesis with cytosolic chaperones to keep them in an import competent state. So far, only little is known about the identity and the potential function of specific cytosolic chaperones assisting in import of mitochondrial precursor proteins containing N-terminal targeting sequence.

The aim of this study was to identify cytosolic factors that interact with presequence-containing mitochondrial precursor proteins, to characterize their involvement in stabilization and targeting of precursor proteins and to study their physiological role in mitochondrial biogenesis.

To this end, *in vivo* site-directed photo-crosslinking method in *Saccharomyces cerevisiae* was performed. I identified the (co)-chaperones Sti1, Ssa1, Ydj1, and Hsp82 as cytosolic factors, which interact specifically with mitochondrial precursor proteins. Sti1 as a co-chaperone of Hsp90/Hsp70 complex was further investigated. I could demonstrate that *sti1Δ* cells displayed a decreased growth rate in comparison to wild type. Additionally mitochondrial morphology was affected in cells lacking Sti1. I also found genetic interaction of Sti1 with the import components Tom20 and Mim1. Furthermore pull-down experiments with recombinant GST-tagged versions of Tom70 and Tom20 revealed association of Sti1 with Tom70 whereas Sis1 and Ydj1 interacted with Tom20. Based on the results from this study, I can conclude that Sti1, Sis1, Ydj1, Ssa1 and Hsp82 are involved in the import of presequence-containing proteins to mitochondria.

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7. List of Abbreviations

aaRS	Aminoacyl-tRNA synthetase
ADP	adenosine diphosphate
AIP	Arylhydrocarbon receptor interacting protein
Amp	Ampicillin
APS	Ammonium persulfate
ATP	adenosine triphosphate
bis-aa	bis-acryl amide
Bpa	p-benzoyl-L-phenylalanine
BSA	bovine serum albumine
DHFR	Dihydrofolate reductase
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiotreitol
E. coli	*Escherichia coli*
ECL	enhanced chemoluminescence reagent
EDTA	ethylendiamine tetraacetate
Fig.	figure
GFP	green fluorescent protein
GST	Glutathion-S-transferase
GTP	guanosine triphosphate
HOP	Hsp70 and Hsp90 organizing prtoein
Hxk	hexokinase
His	histidine
HRP	horse raddish peroxidase
Hsc	heat shock chaperone

IMP	Inner membrane peptidase
IMS	Intermembrane space
IPTG	Isopropyl β-D-1 thiogalactopyranoside
LB	Luria-Betani medium
Leu	leucine
LiAC	Lithium acetate
M	mature
MIA	Mitochondrial intermembrane space import and assembly
MIM	Mitochondrial inner membrane
MOM	Mitochondrial outer membrane
MPP	mitochondrial processing peptidase
mtHSP	mitochondrial heat shock protein
OD	optical density
OD600	optical density at 600 nm
Om45	outer membrane protein 45
p	Precursor
PCR	Polymerase chain reaction
PEG	polyethyleneglycol
phe	Phenylalanine
PK	Proteinase K
PMSF	Phenylmethylsulfonylfluoride
PPIase	Peptidyl-prolyl cis-trans isomerase
pSU9-DHFR	Presuequense of ATP synthetase subunit 9 dihydrofolate reductase
RNase	Ribonuclease
S. cerevisiae	*Saccharomyces cerevisiae*
SAM	Sorting and assembly machinery
SAP	Shrimp alkaline phosphatase

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ssa	Stress-seventy subfamily A
Sti1	Stress inducible 1
TAG	Amber stop codon
TBS	Tris buffered saline
TEMED	N,N,N´,N´-tetramethylene diamine
TIM	Translocase of the inner mitochondrial membrane
TOB	Topogenesis of outer membrane ß-barrel proteins
TOM	Translocase of the outer membrane
TPI	Triose phosphate isomerase
TPR	Tetratricopeptide repeat
Tris	Tris-(hydroxymethyl)-aminomethane
trp	Tryptophan
ura	Uracil
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
WT	Wild type
α	Antibody
Δ	Mutant
h	Hour
min	Minute