Ergosterol Content Specifies Targeting of Tail-Anchored Proteins to the Mitochondrial Outer Membrane

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Abbreviations

aa	acryl amide
AAC	ADP/ATP carrier
Aco2	aconitase 2
ADP	adenosine diphosphate
amp	ampicillin
APS	ammonium peroxodisulfate
Asna	Arsenical pump-driving ATPase
ATP	adenosine triphosphate
bis-aa	bis-acryl amide
Bmh1	brain modulosignalin homologue
Bpa	p-benzoyl-L-phenylalanine
BSA	bovine serum albumine
C	cytosol
Caf	CCR4 Associated Factor
CHX	cycloheximide
Cox4	cytochrome C peroxidase
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
Dnm	Dynamin-related GTPase
dNTP	deoxyribonucleoside triphosphate
DTT	dithiotreitol
E. coli	*Escherichia coli*
ECL	enhanced chemoluminescence reagent
EDTA	ethylendiamine tetraacetate
ER	endoplasmic reticulum
Erv	Flavin-linked sulfhydryl oxidase localized to the endoplasmic reticulum lumen
FADH	flavine adenine dinucleotide
Fig.	figure
Fis1	mitochondrial fission protein 1
Fis1cyt	cytosolic domain of mitochondrial fission protein 1
Fis1-TMC	mitochondrial fission protein 1 containing a single cysteine residue within the Transmembrane domain
Fum1	fumarase
Fzo	FuZzy Onions homolog
Gem1	GTPase EF-hand protein of mitochondria
Get/GET	Guided Entry of Tail-anchored proteins

GFP	green fluorescent protein
GST	Glutathion-S-transferase
GTP	guanosine triphosphate
HA	heme agglutinin
HEPES	N-2-hydroxyl piperazine-N´-2-ethane sulphonic acid
Hex	hexokinase
hFis1	human Fis1
his	histidine
Hmg2	3-Hydroxy-3-MethylGlutaryl-coenzyme a reductase
HRP	horse raddish peroxidase
Hsc	heat shock chaperone
Hsp	heat shock protein
IASD	4-acetamido-4´-[(iodoacetyl)amino]stilbene-2,2´-disulfonic acid
IMP	inner membrane protease
IMS	intermembrane space of mitochondria
leu	leucine
m	mature
Mba	Multi-copy Bypass of AFG3
Mcr1	Mitochondrial NADH-Cytochrome b5 Reductase
Mdj	Mitochondrial DnaJ
Mdv	mitochondrial division
Mge	Mitochondrial GrpE
Mgm	Mitochondrial Genome Maintenance
MIA	Mitochondrial intermembrane space Import and Assembly
MIM	mitochondrial inner membrane
Mim	mitochondrial import
MOM	mitochondrial outer membrnae
MOPS	N-morpholinopropane sulphonic acid
MPP	mitochondrial processing peptidase
mRNA	messenger ribonucleic acid
MSF	mitochondrial stimulation factor
mt	mitochondrial
MT	mitochondria
mtHSP	mitochondrial heat shock protein
NADH	nicotine amide adenine dinucleotide
OD	optical density
OD600	optical density at 600 nm
OEP	outer envelope protein
Om45	outer membrane protein 45

Oxa1	cytochrome OXidase Activity protein 1
p	precursor
p. A.	per analysis
PCR	polymerase chain reaction
Pex19	peroxin 19
phe	phenylalanine
PK	Proteinase K
PM	plasmamembrane
PMSF	phenylmethylsulfonylfluoride
Por1	mitochondrial porin 1
pSU9-DHFR	presequence of ATP synthetase subunit 9 dihydrofolate reductase
Qcr6	ubiQuinol-cytochrome C oxidoReductase
RFP	red fluorescent protein
rNTP	ribonucleoside triphosphate
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SAM	sorting and asembly machinery
SDS-PAGE	sodium dodecyl sulfate polyacryl amide gel electrophoresis
Sec61	essential subunit of Sec61 complex
Sgt	Small Glutamine-rich Tetratricopeptide repeat-containing protein
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
Spf1	P-Type ATPase of endoplasmic reticulum
Ssa	Stress-Seventy subfamily A
Ssb	Stress-Seventy subfamily B
TA	tail-anchored
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N´,N´-tetramethylene diamine
TIM	translocase of the inner mitochondrial membrane
Tim10	essential protein of the mitochondrial intermembrane space
Tim23	subunit of TIM23 complex 23
Tim44	sunbunit TIM23 complex 44
TMD	transmembrane domain
TOB	topogenesis of outer membrane β-barrel proteins
Tob55	subunit of TOB complex 55
Toc/TOC	translocase of the outer chloroplast envelope
TOM	translocase of the outer mitochondrial membrane
Tom20	subunit of TOM complex 20
Tom40	subunit of TOM complex 40

Tom70	subunit of TOM complex 70
TPI	triose phosphate isomerase
TRC	transmembrane domain recognition complex
Tris	tris-(hydroxymethyl)-aminomethane
trp	tryptophan
TX100	Triton X-100
Ugo	UGO (Japanese for fusion)
ura	uracil
UV	ultra violet
v/v	volume per volume
VAMP	vesicle-associated membrane protein
w/v	weight per volume
WC	whole cell lysate
WT	wildtype
Ydj1	Yeast dnaJ 1
Zim	Zinc finger Motif protein
α	antibody
Δ	deletion

1 INTRODUCTION

1.1 Origin of mitochondria

According to the endosymbiont theory mitochondria are derived from an α-proteobacterium taken up by an ancestor cell about 1.5 billion years ago (Anderson et al. 2003; Dyall et al. 2004; Lang et al. 1999; van der Giezen and Tovar 2005; van der Giezen et al. 2005). During evolution and transformation of the bacterium to an organelle of the host cell most of its genes were transferred to the nucleus of the host and needless genes were deleted. Today yeast cells express about 1000 mitochondrial proteins and mammalian cells contain ca. 1500 mitochondrial proteins (Lang et al. 1999). Only eight of the yeast mitochondrial proteins are still encoded by the organelle´s genome. These are hydrophobic inner membrane proteins that belong to the complexes of the respiratory chain (Lithgow 2000; von Heijne 1986).

1.2 Mitochondrial structure

Mitochondria possess a double membrane system and are therefore subdivided in four compartments: The mitochondrial outer membrane (MOM), the intermembrane space (IMS), the mitochondrial inner membrane (MIM) and the matrix. The MOM is permeable for small molecules (< 5 kDa) because this membrane harbors VDAC/porin pore-forming proteins that allow the passage of small metabolites. This membrane contains 1-1.5 µg proteins / µg phospholipids and constitutes the outer boundary of the mitochondrial structure. The MIM is relatively rich of proteins (5-6 µg / µg phospholipids), partially because the respiratory chain is localized in this membrane. Accordingly, the MIM is nearly impermeable to small solutes since it has to maintain the electrochemical gradient of H^+. The MIM is intensely folded and can further be differentiated into the inner boundary membrane which is parallel to the MOM and the cristae structures that are large invaginations of the membrane.

1.3 Structural dynamics of mitochondria

Mitochondria are morphologically very diverse organelles that constantly change their shape by fission and fusion events (reviewed in Hoppins et al. 2007). Mitochondrial fission in yeast cells is mediated by four proteins: The dynamin-related GTPase Dnm1, the adaptor protein Mdv1 (and its paralog Caf4) and the membrane-anchor Fis1 (Mozdy et al. 2000; Otsuga et al. 1998; Sesaki and Jensen 1999; Tieu et al. 2002). Dnm1 can undergo a self-assembly and GTP-induced conformational switch turning it into extended multimeric spiral structures (Ingerman et al. 2005; Labrousse et al. 1999; Mozdy et al. 2000; Sesaki and Jensen 1999; Tieu et al. 2002). Fis1 is anchored to the MOM and harbors a cytosolic tetratricopeptide repeat fold for interaction with Mdv1 and Caf4 (Dohm et al. 2004; James et al. 2003; Mozdy et al 2000; Suzuki et al. 2003, Yoon et al. 2003). Mdv1 interacts additionally also with Dnm1 and thus serves as an adaptor between Fis1 and Dnm1 (Ceverny and Jensen 2003; Ceverny et al. 2001; Mozdy et al. 2000; Sesaki and Jensen 1999; Tieu et al. 2002). Dnm1 builds multimeric spirals around the mitochondria and its dynamic interaction with Mdv1 leads to constriction of the Dnm1 spiral and fission of mitochondria via mechanochemical forces (Ceverny and Jensen 2003; Suzuki et al. 2003; Tieu et al. 2002).

In yeast mitochondrial fusion is performed by three proteins: The dynamin-related GTPases Fzo1 and Mgm1 and the outer membrane protein Ugo1. Fzo1 is localized in the MOM and promotes membrane fusion by GTPase-regulated self-interaction with other Fzo1 molecules in an opposite MOM resulting in a tethering of two opposing MOMs (Griffin and Chan 2006; Ishihara et al. 2004, Meeusen et al. 2004; Santel and Fuller 2001). Mgm1 can be found as two isoforms, a long one anchored to the MIM and a short soluble one in the IMS. Both isoforms are required for fusion of the inner membrane (Herlan et al. 2003; Meeusen et al. 2006; Sesaki et al. 2003). The Mgm1-induced tethering of opposite MIMs is followed by a fusion reaction that results in the formation of a single lipid bilayer. The molecular mechanism of the fusion reaction itself is currently unknown. The third yeast fusion protein Ugo1 is embedded in the MOM and might be an adaptor element that coordinates the fusion complexes within the inner and outer membrane by interacting with both Fzo1 and Mgm1 (Sesaki and Jensen 2001 and 2004; Sesaki et al. 2003; Wong et al. 2003).

1.4 Functions of mitochondria

Mitochondria carry out various metabolic functions in the eukaryotic cell. The most outstanding activity is probably the oxidative phosphorylation-dependent ATP synthesis which provides energy for many cellular processes. Besides this, the citric acid cycle and fatty acid metabolism take place in the mitochondrial matrix. Fatty acids are processed to acetyl CoA which is further broken down in the citric acid cycle. NADH and $FADH_2$ from the citric acid cycle provide the electrons that fuel the respiratory chain. Furthermore, mitochondria are involved in the synthesis of iron-sulfur clusters, nucleotides, amino acids, heme and co-enzymes (Lill and Muhlenhoff 2008, Saraste 1999, Scheffler 2001). Mitochondria also participate in the biogenesis of phospholipids. Cardiolipin is made within the mitochondria from phosphatidic acid and phosphatidylethanolamine from phosphatidylserine. Moreover, apoptosis, cancer and ageing are influenced by mitochondrial processes and mitochondrial mutations. Mitochondrial dysfunctions are the cause of many human diseases (Chan 2006; Galonek and Hardwick 2006; Youle 2007; Youle and Karbowski 2005).

1.5 Protein translocation into mitochondria

Most mitochondrial proteins are encoded in the nucleus, synthesized on cytosolic ribosomes and imported into the different mitochondrial compartments in a post-translational manner. The proteins have to be kept in an import competent state; a requirement that is especially important for hydrophobic membrane proteins that tend to aggregate in an aqueous environment. Therefore cytosolic chaperones like Hsp70, Hsp90, mitochondrial stimulation factor (MSF) and Hsp40-related J-proteins bind mitochondrial precursor proteins and assist them in their import (Bhangoo et al. 2007; Hachiya et al. 1994; Murakami et al. 1988; Young et al. 2003). For a reliable sorting to their destined mitochondrial compartment precursor proteins contain specific targeting signals. A large group of mitochondrial proteins harbor a cleavable N-terminal presequence. A typical presequence consists of 10-80 amino acids that can form an amphipathic helix with a hydrophobic phase and an opposite positively charged side. All matrix proteins and some inner membrane and intermembrane space proteins contain such a cleavable presequence (Neupert and Herrmann 2007). The removal of

the presequence is performed after complete import by the mitochondrial matrix processing peptidase (MPP). A second class of mitochondrial proteins contains internal non-cleavable targeting signals. This group comprises all outer membrane proteins and some inner membrane and intermembrane space proteins.

Although most mitochondrial proteins are imported in a post-translational manner (Borgese et al. 2007; Borgese et al. 2003; Hallermeyer et al. 1977; Harmey et al. 1977; Neupert 1997; Reid and Schatz 1982), there is evidence that at least some proteins are imported co-translationally (Fujiki and Verner 1991 and 1993, Yogev et al. 2007). In this case targeting of the mRNA can be a crucial sorting mechanism (Marc et al. 2002; Sylvestre et al. 2003). It was shown that the mRNA coding for the mitochondrial ATP2 accumulates close to the surface of mitochondria and provides the basis for protein synthesis in the vicinity of the TOM complex (Margeot et al. 2002). The mRNA can simultaneously be translated by polysomes, so that a bunch of newly synthesized presequences emerge from the ribosomes. These presequences can then be recognized by the import receptor Tom20 and imported co-translationally into mitochondria before they are completely synthesized on the cytosolic side (Eliyahu et al. 2009). Such a scenario would overcome the problem of aggregation of hydrophobic domains in the aqueous cytosol.

Mitochondrial proteins are guided to their final destinations by six different import pathways (Fig.1.1 and reviewed in Becker et al. 2011; Chacinska et al. 2009; Endo and Yamano 2009; Neupert and Herrmann 2007). Three import machineries reside in the outer membrane: the translocase of the outer mitochondrial membrane (TOM complex), the topogenesis of mitochondrial outer membrane β-barrel proteins (TOB complex) also known as sorting and assembly machinery (SAM complex), and the mitochondrial import (MIM) complex. The inner membrane contains also three translocation complexes: the presequence translocase of the inner mitochondrial membrane (TIM23), the carrier translocase of the inner mitochondrial membrane (TIM22) and a dedicated machinery for those proteins encoded by the mitochondrial genome (OXA1 complex). In addition, mitochondria contain several other import-assisting systems. One of those are the IMS chaperone complexes Tim9-10 and Tim8-13 that accompany hydrophobic precursor proteins in the IMS on their way from the TOM complex to either the TIM22 and or the TOB machinery. Additional

components are the mitochondrial intermembrane space import and assembly (MIA) complex, mitochondrial processing peptidases (MPPα/β, IMP and others) and chaperones in the matrix responsible for protein folding and stability (mtHsp70, Mdj1, Hsp60/10, Hsp78 and Zim17).

Almost all mitochondrial proteins enter the mitochondria through the TOM complex. Exceptions are signal- and tail-anchored proteins of the outer membrane, outer membrane helical multispan proteins and Mas37/Sam37. β-barrel proteins are initially translocated across the MOM through the TOM complex and are then passed over to the TOB complex with the help of the small Tim proteins in the IMS (Fig.1 and Kozjak et al. 2003; Paschen et al. 2003). The TOB complex finally inserts the β-barrel proteins into the outer membrane. Presequence containing proteins also first pass through the TOM complex and are then handed over in a coordinated manner to the TIM23 complex which translocates the proteins over the inner membrane into the matrix (Fig. 1.1 and Mokranjac and Neupert 2005). Multispan inner membrane proteins with internal targeting signals are transferred from the exit of the TOM channel to the TIM22 complex by the small Tim proteins in the IMS (Neupert and Herrmann 2007). The TIM22 machinery finally inserts these proteins into the inner membrane. The import of small IMS proteins involves a translocation across the MOM via the TOM import pore and then these proteins are trapped in the IMS by formation of disulfide bonds and complete folding. This last step is facilitated by Mia40 and its partner protein Erv1 (Hell 2008; Hermann et al. 2009). MIM proteins encoded by the mitochondrial DNA are inserted from the matrix side into the MIM by the OXA1 complex.

1.5.1 The TOM complex

The TOM-complex constitutes the major entry-gate for proteins in the MOM. The TOM holo-complex has an estimated size of 490-600 kDA (Künkele et al. 1998; Model et al. 2002). Tom40 forms the protein conducting channel and is present in several copies in the complex (Ahting et al. 2001; Hill et al. 1998; Stan et al. 2000; Rapaport et al. 1998). Precursor proteins coming from the cytosol are first recognized by the receptor subunits of the TOM complex that face the cytosol. The receptors Tom20 and Tom22 bind to presequence containing proteins and β-barrel proteins and assist in threading

them to the Tom40 import pore (Krimmer et al. 2001; Mayer et al. 1995; Schleiff et al. 1999; Yamano et al. 2008). Moreover Tim22 also plays a role in substrate release on the IMS side of the complex once the precursor proteins reached the exit of the pore. The Tom70 receptor interacts with polytopic MIM proteins, such as metabolite carriers and with multispan helical MOM proteins on the cytosolic side of the TOM complex and transfers them into the Tom40 pore or relays them to the MIM complex, respectively (Brix et al. 1999; Schlossmann et al. 1994; Papic et al. 2011). Tom5, Tom6 and Tom7 are small subunits of the TOM complex that are not directly involved in protein translocation but rather play various roles in the biogenesis and assembly of the TOM complex. Tom5 is necessary for assembly and stability of the complex (Ahting et al. 1999; Wiedemann et al. 2003), while Tom6 and Tom7 regulate assembly and dissociation of the complex. Tom6 stabilizes the complex whereas Tom7 has a destabilizing effect (Alconada et al. 1995; Dembowski et al. 2001; Hönlinger et al. 1995; Sherman et al. 2005).

1.5.2 The TOB/SAM complex

The TOB/SAM complex plays a key role in the biogenesis of β-barrel proteins (Gentle et al. 2004; Kozjak et al. 2003; Paschen et al. 2003). The complex has a size of 200-250 kDa and consists of the two essential subunits Tob55/Sam50 and Tob38/Sam35 as well as the non-essential subunit Mas37/Sam37 (Gratzer et al. 1995; Ishikawa et al. 2004; Milenkovic et al. 2004; Waizenegger et al. 2004; Wideman et al. 2010). The TOB complex is evolutionary conserved; its bacterial counterpart is the BAM complex, which can be found in the outer membrane of all Gram-negative bacteria (reviewed in Hewitt et al. 2011). Tob55, which is derived from the bacterial BamA constitutes the central component of the complex (Gentle et al. 2004; Kozjak et al. 2003; Paschen et al. 2003). Tob38 forms with Tob55 the TOB core complex and stabilizes the complex (Waizenegger et al. 2004). Mas37 is proposed to facilitate the release of the substrates into the MOM. Moreover Mas37 plays also a role in the stability of the complex (Chan and Lithgow 2008, Dukanovic et al. 2009).

1.5.3 The MIM complex

It has recently been shown that a third import complex in the MOM exists. The MIM complex comprises at least two different proteins: Mim1 and the recently identified Mim2 (Dimmer et al. 2012). The complex is not fully characterized until now, but it was shown to be involved in the insertion of multispan helical proteins (like Ugo1) into the outer membrane. The precursor proteins are first bound by the Tom70 receptor and then handed over to the MIM complex that facilitates the membrane insertion of the protein (Becker et al. 2011, Dimmer et al. 2012; Papic et al. 2011). Moreover, the MIM complex plays also a crucial role in assembly of the TOM complex.

1.5.4 The MIA40 machinery

A subset of IMS proteins is rich in cysteines residues that are arranged in conserved twin Cx_3C or twin Cx_9C motifs. These proteins form intramolecular disulfide bridges that result in hairpin-like structures (Arnesano et al. 2005; Webb et al. 2006). The formation of these bonds and the resulting stable folding is crucial for the vectorial translocation of the proteins from the cytosol into the IMS. The last step of translocation is accomplished by the MIA40 machinery (Deponte and Hell 2009). Mia40 itself exists in two redox states, a partially reduced and an oxidized state (Mesecke et al. 2005; Terziyska et al. 2009). The precursor-proteins are imported from the cytosol through the TOM complex in a reduced state. Oxidized Mia40 binds these proteins upon their exit from the TOM pore and intermolecular disulfide bonds are established. In a last step the substrate is released in an oxidized and fully folded state to the IMS, while Mia40 is reduced (Muller et al. 2008).The folding of the substrate protein prevents a retrograde transport back to the cytosol. Erv1 (flavin-dependent oxidase), the second component of the machinery, regenerates oxidized Mia40 and reestablishes its own oxidized state by electron transfer to molecular oxygen via cytochrome c (Grumbt et al. 2007; Terzyiska et al. 2009).

1.5.5 The TIM23 complex

The import of all matrix proteins, most MIM proteins and some IMS proteins is carried out with the help of the TOM and the TIM23 complex (reviewed in Chacinska et al. 2009; Mokranjac and Neupert 2005). The TIM23 complex is built by two functional modules, the membrane-integrated translocation channel and the import motor on the matrix side of the MIM. Tim50 is the major receptor for presequence containing proteins coming through the TOM channel (Geissler et al. 2002; Mokranjac et al. 2003; Yamamoto et al. 2002). It relays the incoming proteins to the protein-conducting channel which is built by Tim23 and TIM17 that are multispan transmembrane proteins (Meier et al. 2005; Milisav et al. 2001). Tim23 also contains a receptor domain facing the IMS. For coordination of the TOM and the Tim23 complexes, Tim21 interacts with Tom22 to pose the TIM23 and the TOM complexes in vicinity to each other to facilitate a concerted action of both complexes (Chacinska et al. 2005; Mokranjac et al. 2005). Translocation through the TIM channel is energy-dependent and requires the membrane potential ($\Delta\Psi$) as a driving force. To couple the protein conducting channel to the import motor in the matrix Tim44 on the internal surface of the MIM provides the platform for the import motor (mtHsp70, Mge1, Tim14/16) to interact with the channel components (Moro et al. 1999). This chaperone harbors an ATP-binding domain and a substrate-binding domain (Bukau et al. 2006; Young et al. 2004). In an ATP-bound state the substrate can be easily bound or released, while the bond between the chaperone and its substrate is tight in an ADP-bound state. The substrate peptide is bound by Tim44 after emerging from the TIM channel and passed over to mtHsp70 which tightly binds the substrate upon ATP hydrolysis and releases it again after turning back to an ATP-bound state. By this mechanism and with multiple molecules of mtHsp70 the substrate is pulled step by step into the matrix. Mge1 is the nucleotide exchange factor for mtHsp70 that regenerates the ATP-bound state of mtHsp70. Additional two J-domain containing proteins regulate the activity of mtHsp70. Tim14 stimulates the ATPase activity of mtHsp70, while Tim16 regulates the activity of Tim14 (Kozany et al. 2004; Li et al. 2004; Mokranjac et al. 2006).

1.5.6 The TIM22 complex

The TIM22 complex inserts multispan inner membrane proteins, like carriers, Tim23, Tim22 and Tim17 into the MIM. After passage through the TOM complex the substrate proteins are bound by small Tim proteins in the IMS. Tim54 binds the substrate-loaded Tim9/10/12 complex and takes over the substrate to conduct it to Tim22 that constitutes the translocation channel and mediates insertion of substrate proteins into the inner membrane (Mokranjac and Neupert 2008; Peixoto et al. 2007; Rehling et al. 2003). Tim18 is a component of the complex with unknown function. Translocation through the TIM22 complex is energy-dependent and driven by the membrane potential ($\Delta\Psi$).

1.5.7 The OXA1 complex

The OXA1 complex facilitates integration of inner membrane proteins that are synthesized in the mitochondrial matrix and should be inserted from the matrix side into the inner membrane (Baumann et al. 2002; Hell et al. 1997, 1998 and 2001; Herrmann et al. 1997; Rojo et al. 1995). Oxa1 together with Cox18 can insert proteins into the membrane in a co- and posttranslational manner, while Mba1 together with Oxa1 facilitates association of translating ribosomes with the inner mitochondrial membrane to provide the basis for co-translational insertion (Ott et al. 2006; Stuart 2002; Yi and Dalbey 2005).

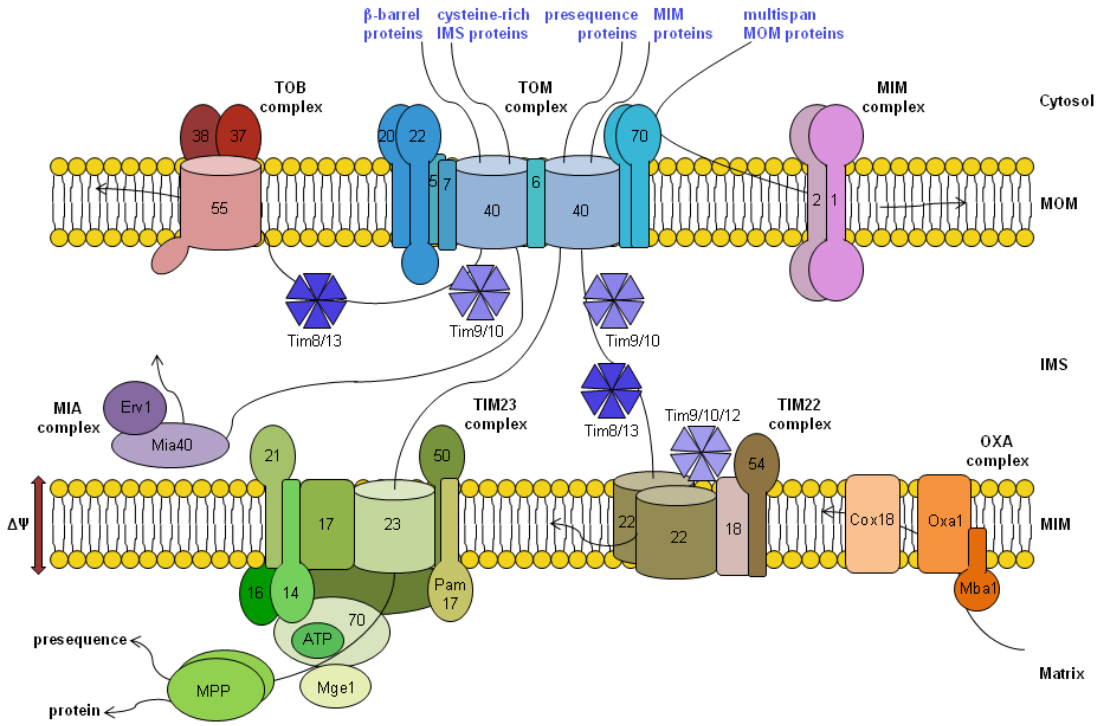


Figure 1.1 Translocation machineries in mitochondria

Most proteins enter the mitochondria via the TOM complex. Presequence-containing proteins are handed over to the TIM23 complex that imports the proteins into the matrix or inserts them into the MIM. Cysteine-rich IMS proteins are trapped in the IMS by the MIA40 complex after passage through the TOM channel. Polytopic MIM proteins pass through the TOM complex, are handed over to the TIM22 complex by small Tim chaperones and finally inserted into the MIM. Beta-barrel proteins are inserted into the MOM by a coordinated action of the TOM complex, small Tim chaperones and the TOB complex. Outer membrane helical multispan proteins are recognized by Tom70 and inserted into the MOM with the help of the MIM complex. Oxa1 inserts proteins that are synthesized in the mitochondrial matrix into the MIM.

1.6 Protein classes of the outer mitochondrial membrane

The outer mitochondrial membrane comprises around 40 different integral proteins in *S. cerevisiae* (Burri et al. 2006; Zahedi et al. 2006). These proteins can be divided into four classes by their topology (Fig. 1.2). One class is represented by the β-barrel proteins that reside only in the outer membrane of Gram-negative bacteria, mitochondria and chloroplasts. Beta-barrel proteins form cylindrically shaped pore-like

structures within the membrane by formation of an even number of antiparallel β-strands (Wimley 2003). The residues that face the inside of the pore are hydrophilic and the outside of the β-barrel consists of hydrophobic residues (Schulz 2000). This amphiphilic character allows membrane integration and conduction of hydrophilic substrates across the membrane. Another class of MOM proteins is represented by the helical multispan proteins. These proteins span the membrane with multiple α-helices. Members of this class are proteins are for example the fusion mediators Fzo1 and Ugo1 (Coonrod et al. 2007) and the peripheral benzodiazepine receptor in the MOM of mammalian cells (Joseph-Liauzun et al. 1998; Otera et al. 2007). The integral membrane proteins spanning the bilayer with only a single α-helix can be divided into three groups. The first are the signal-anchored proteins. They contain a moderately hydrophobic stretch of about 20 amino acids often flanked by positively charged residues very close to their N-terminus. This transmembrane region anchors the protein to the MOM and also includes the targeting signal (Shore et al. 1995; Waizenegger et al. 2003). The large hydrophilic domain is facing the cytosol. Examples for signal-anchored proteins are Tom20, Tom70, OM45 and the MOM isoform of cytochrome b5 reductase Mcr1 (Hahne et al. 1994; Yaffe et al. 1989). The second group is the class of the tail-anchored proteins. Tail-anchored proteins comprise a class of proteins characterized by a single transmembrane domain very close to a polar C-terminus and a larger N-terminal domain facing the cytosol (Borgese et al. 2003). Hence, TA-proteins are structured a similar same way as signal-anchored proteins. The only difference is that the transmembrane region is close to the C-terminus (Borgese et al. 2007; Wattenberg and Lithgow 2001). Examples for mitochondrial tail-anchored proteins are mitochondrial fission protein Fis1, Gem1 (GTPase EF-hand protein of mitochondria) and the small TOM subunits Tom5, 6 and 7 (Beilharz et al. 2003; Frederick et al. 2004; Mozdy et al. 2000). Mammalian cells contain additional mitochondrial TA-proteins like cyt b5, synaptojanin-binding protein OMP25, VAMP-1B and the members of the Bcl-2 family, that are involved in apoptosis (Beilharz et al. 2003; Cory and Adams 2002; DÁrrigo et al. 1993; Isenmann et al. 1998; Mozdy et al. 2000; Nemoto et al. 1999). Proteins that harbor the single TMD domain in their central region form the third group of single-span proteins. Members of this group are Mim1, Tom22 and ATG32.

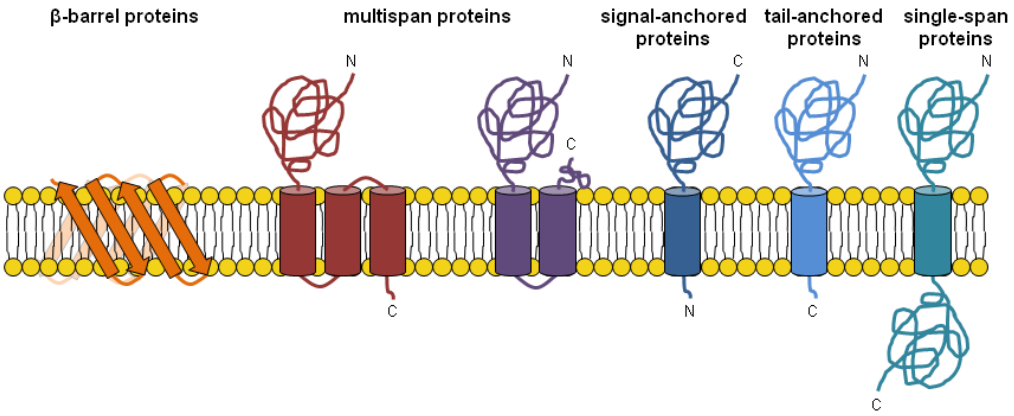


Fig 1.2 Topogenesis of outer mitochondrial membrane proteins

The mitochondrial outer membrane harbors different classes of membrane proteins. Beta-barrel proteins, like Tom40 or Porin, are anchored to the membrane by multiple β-strands. Helical multi-span proteins, like Ugo1 or Fzo1 contain two or more transmembrane domains. Single-span proteins harbor only one transmembrane domain that anchors them to the MOM. This class can be sub-divided into the classes of signal- and tail-anchored proteins, like OM45 or Fis1, respectively, and a class that has larger soluble domains protruding to both sides of the MOM into the cytosol and the IMS, like Mim1.

1.7 Functions and biogenesis of tail-anchored proteins

TA-proteins can be found in all kingdoms of life and make up 3-5% of all membrane proteins. In yeast 50 TA-proteins are predicted and in humans even 300 (Beilharz et al. 2003; Borgese and Righi 2010; Kalbfleisch et al. 2007). Multiple functions are carried out by TA-proteins, for example SNARE proteins are involved in vesicular trafficking and Bcl-2 family proteins are regulators of apoptosis. Moreover, some subunits of ER, plastidial and mitochondrial translocation machineries are TA-proteins (Borgese et al. 2007; Chen and Scheller 2001; Cory and Adams 2002). All membranes abutting to the cytosol, like the plasma membrane, ER, compartments of the secretory pathway, peroxisomes, lysosomes, mitochondrial outer membrane and in plants the outer envelope of the chloroplasts contain TA-proteins. These proteins do not contain an N-terminal targeting sequence but rather the targeting information is encoded in the TMD and its flanking regions (reviewed in Borgese et al. 2003). Therefore the targeting

signal does not emerge from the ribosome until translation is almost finished, implying membrane insertion in a post-translational mode (Johnson and van Waes 1999; Kutay et al. 1995). The variety of cellular locations of TA-proteins implies that the cell faces the problem how to assure specific sorting of the TA-proteins to their final destinations. ER-, mitochondria- and plastid-resident TA-proteins are directly sorted and inserted into their target membranes. This is also true for some peroxisomal TA-proteins. Other peroxisomal TA-proteins and also all TA-proteins destined for the secretory pathway are first inserted into the ER membrane and then sorted to their target membranes (Borgese et al. 2007).

1.7.1 Targeting of TA-proteins to the endoplasmic reticulum

Different pathways for targeting of TA-proteins to the ER membrane have been proposed in the last years. It was shown that TA-proteins follow different routes and modes of insertion depending on the TMD´s properties, such as length, hydrophobicity and charge of the flanking regions (Ng et al. 1996; Rabu et al. 2008). The ER membrane accepts TA-proteins with transmembrane domains of varying hydrophobicity followed by polar regions of different length and charge. TA-proteins with relatively short transmembrane domains with low hydrophobicity remain in the ER membrane, while TA-proteins with longer and more hydrophobic transmembrane domains, such as SNAREs, will be passed on into the secretory pathway (Bulbarelli 2002; Pedrazzini et al. 1996 and 2000; Rayner and Pelham 1997; Reggiori et al. 2000).

TA-proteins with a transmembrane domain of moderate hydrophobicity such as cytochrome b5 and PTB-1B can insert into the ER membrane in an unassisted manner (Borgese et al. 2007). It was shown that both proteins do not need membrane associated proteins to insert into lipid vesicles with an ER-like lipid composition (Brambillasca et al. 2006; Colombo et al. 2009). Higher cholesterol levels hampered the insertion, maybe due to the higher order of the lipid bilayer (Brambillasca et al. 2005). There is also some evidence that Hsp40 and Hsc70 play a role in the biogenesis of ER TA-proteins (Abell et al. 2007; Rabu et al. 2008). *In vitro* assays with Hsc70 inhibitors showed a dependence on Hsp40/Hsc70 for a subset of ER TA- proteins, including Cyt b5 (Fewell et al. 2004; Rabu et al. 2008). *In vivo*

confirmation of these results is still lacking, but it is most probable that cytosolic factors are needed to keep the TA-proteins in a soluble and import-competent state during their passage through the aqueous cytosol (Hartl and Hartmeyer 2002, Yabal et al. 2003). Potential involvement of cytosolic factors is supported by the requirement for ATP that was observed for the insertion of most TA-proteins (Abell et al. 2007; Favaloro et al. 2008; Kim et al. 1999).

Other TA-proteins with more hydrophobic transmembrane region are incapable of unassisted insertion. There is some evidence that a subset of ER TA-proteins might utilize the signal recognition particle (SRP) for targeting although they are inserted post-translationally. Synaptobrevin 2 (Syb2) was shown to interact with SRP *in vitro* and it also needs an intact SRP receptor for proper targeting, while for insertion another membrane-localized protein might be necessary. Of note, the GTP-dependent insertion of Syb2 is independent of the Sec61 translocon (reviewed in Rabu et al. 2009).

The GET-pathway (guided entry of TA-proteins) constitutes the third possibility for TA-proteins to enter the ER. The first discovered component of this pathway is the cytosolic ATPase Get3 in yeast (Jonikas et al. 2009; Schuldiner et al. 2005) or its homologue Asna1/TRC40 in mammals (Favaloro et al. 2008; Stefanovic and Hedge 2007). It was shown that Get3 is necessary for the biogenesis of TA-proteins (Schuldiner et al. 2005 and 2008). Soon after, other components of the pathway were identified: Get1 and Get2 form the membrane-bound receptor in the ER membrane and Get4 and Get5 form the cytosolic recognition complex for newly synthesized TA-proteins. Furthermore Sgt2, Hsp104 and Hsp70s can associate with the cytosolic Get proteins (Angeletti et al. 2002; Fleischer et al. 2006; Fleischer et al. 2006). It was proposed that Sgt2 mediates association of the recognition complex which is formed by Get4 and Get5 with ribosomes synthesizing TA-proteins (Wang et al. 2010). Get5 mediates the interaction of Sgt2 with Get4. Upon release of the transmembrane region of the TA-protein from the ribosome a symmetric homodimer of Get3 is recruited by Get4 and Get5 and binds directly via hydrophobic interactions to the TMD of the TA-protein. Get3 then delivers it to the Get1/2 receptor heterodimer that mediates the insertion of the TA-protein into the ER membrane (Jonikas et al. 2009; Schuldiner et al. 2008). This pathway is used in yeast cells by Sec22, Pex15 and the SNARE proteins

Sed5 and Tlg2 (Schuldiner et al. 2008). Components of the GET machinery in yeast cells are not essential, thus TA-proteins that normally use the GET-pathway might also be able to use other pathways (Schuldiner et al. 2008). In higher eukaryotes Asna1/TRC40 is an essential protein (Mukhopadhyay et al. 2006). Also other components of the mammalian pathway differ from the yeast system. Bat3 has no homologue in yeast, but in mammals it was shown to be involved in the TRC40 pathway. Bat3 interacts with ribosomes loaded with transmembrane domains and binds to the TMD of the substrate protein Sec61β (Leznicki et al. 2010). The role of Bat3 in targeting TA-proteins to the ER is not yet clear. It might be involved in formation of a stable Trc40-substrate complex or in the recycling of Trc40. The TRC40 pathway is used by Sec61β, VAMP2 and syntaxins (Favaloro et al. 2008; Stefanovic and Hedge 2007). Get3 and Asna1/TRC40 homologues exist also in bacteria and archaea. The bacterial ArsA is an arsenite transporter while the archaeal ArsA might be involved in TA-protein biogenesis and therefore might be the ancestor of Get3 and Asna1/TRC40 (Borgese and Righi 2010).

The choice of the pathway of ER TA-protein insertion is mainly dictated by the properties of the TA-protein´s TMD and its flanking regions. Ribosomes and associated cytosolic factors might also play a crucial role in directing the substrate protein to a certain pathway (Berndt et al. 2009; Bornemann et al. 2008; Ng et al 1996; Schuldiner et al. 2008). Of note, the choice of the pathway is probably not completely strict as TA-proteins can use an alternative insertion pathway in the absence of their original route.

1.7.2 Targeting of TA-proteins to peroxisomes

The group of peroxisomal TA-proteins contains short transmembrane regions of moderate hydrophobicity flanked by positively charged residues. Some TA-proteins are first inserted into the ER and then transported to peroxisomal membranes (Hoepfner et al. 2005; Kim et al. 2006). Other peroxisomal TA-proteins are directly guided to their target membrane (Halbach et al. 2006; Heiland et al. 2005). It has been shown that the major cytosolic chaperone for peroxisomal membrane proteins, Pex19 binds the peroxisomal TA-proteins hFis1 and Pex26 (Hallbach et al. 2006; Delille et al. 2008).

1.7.3 Targeting of TA-proteins to plastids

For plants the challenge of sorting TA-proteins is even more difficult as plastids provide an additional type of membranes that has to be considered (reviewed in Abell and Mullen 2010). Chloroplasts outer envelope contains a variety of TA-proteins like subunits of the TOC translocase Toc33 and Toc34, as well as OEP9 and OEP61. Until now the targeting and insertion of TA-proteins into chloroplasts is poorly understood. At least for OEP9 it was shown that a membrane-bound protein factor is required for insertion (Dhanoa et al. 2010). The ankryin repeat-containing protein (AKR2A) was suggested as a possible targeting factor for chloroplast TA-proteins, but this still needs to be proven (Bae et al. 2008; Dhanoa et al. 2010).

1.7.4 Targeting of TA-proteins to mitochondria

Mitochondrial TA-proteins usually contain relatively short transmembrane regions (<20 amino acids) of moderate hydrophobicity flanked by positively charged residues (Borgese et al. 2001; Horie et al. 2002; Isenmann et al. 1998; Kuroda et al. 1998). However, some ER TA-proteins share these features and mitochondrial located Bax for example has a longer transmembrane domain. Different experimental approaches showed that the major protein import complexes of the MOM, TOM and TOB are not involved in the biogenesis of mitochondrial TA-proteins (Setoguchi et al. 2006). Moreover the insertion into the outer membrane is energy-independent. So far, no protein component as a mediator of mitochondrial TA proteins was identified. Hence, it was proposed that, like cyt b5 in the ER, mitochondrial TA-proteins might be able to insert into the membrane in an unassisted process.

The unique lipid composition of the MOM might play a role in ensuring the specifity of the process. Of note, the MOM has the lowest sterol content among all membranes that are facing the cytosol (Zinser 1991). Indeed, higher ergosterol content than typical for the MOM is inhibiting insertion (Kemper et al. 2008). Until now it is not clear how mitochondrial TA-proteins are targeted to the MOM and avoid a mis-targeting to other membranes. The involvement of cytosolic factors is anticipated as exposure of the

hydrophobic TMDs in the cytosol might lead to aggregation of the TA-proteins in the aqueous environment.

1.8 Fis1, a model TA-protein of the outer mitochondrial membrane

Fis1 is a tail-anchored protein of 17.7 kDa that is localized in the MOM and involved in mitochondrial fission (Mozdy et al. 2000). In yeast Fis1 recruits the fission complex to the membrane (Karren et al. 2005), whereas the role of its human homologue (hFis1) in fission of mitochondria is not clearly defined (Otera and Mihara 2011). In addition, hFis1 was suggested to be involved in apoptosis by transmitting an apoptosis signal from mitochondria to the ER by interaction with Bap31 on the ER (Iwashara et al. 2011). Fis1 is also present in peroxisomal membranes. The dual localization to mitochondria and peroxisomes is facilitated by properties of the transmembrane region and the C-terminus (Koch et al. 2005). In humans hFis1 is targeted to the peroxisomes upon interaction with Pex19, a cytosolic membrane import factor (Delille and Schrader 2008).

1.9 Lipid composition of cellular membranes in *S. cerevisiae* and its importance for the biogenesis of membrane proteins

Most cellular membranes are built up mainly by phospholipids. The predominant structural lipids of most cellular membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phophatidic acid (PA). Moreover membranes contain sterols and sphingolipids, which harbor ceramide as hydrophobic backbone (van Meer et al. 2008). The lipid composition of different membranes within one cell can strongly differ. The plasma membrane (PM) is enriched with sphingolipids and sterols (Di Paolo and De Camilli 2006). This makes the PM more rigid and resistant to mechanical stress than other cellular membranes. Moreover phosphatidylserine accounts for 30% of all phospholipids of the PM.

The ergosterol content of the various cellular membranes also significantly differs. Whereas the PM, secretory vesicles and the nuclear membrane possess a relatively high ergosterol / phospholipid ratio, this ratio is very low in the mitochondrial outer membrane (Zinser 1991). The inner mitochondrial membrane has also a unique lipid composition that includes high percentage of cardiolipin and high PE / PC ratio, both features the bacterial origin of the MIM (Daum 1985; Strauss et al. 2003).

The lipid composition of a membrane influences its physical parameters like fluidity or curvature. The proteins embedded and associated with the membrane are influenced by the surrounding lipids. Their efficiency of translocation, topology, stability and enzymatic activity are all influenced by the lipid components of the membrane. Also assembly of protein complexes, sorting and transport of proteins along the secretory pathway can be regulated by lipids (Schneiter and Toulmay 2007). The presence of non-bilayer lipids like PE, which have an overall conical shape can cause membrane curvature and may create insertion sites for proteins due to a reduced packing density at the membrane-water interface (van den Brink-van der Laan et al. 2004). It has also been shown that mitochondrial presequences can bind to cardiolipin containing vesicles by interaction of the positively charged residues with the polar headgroup of cardiolipin (Ou et al. 1988). As the MOM contains a certain percentage of cardiolipin, this may provide a sorting mechanism for mitochondrial precursor proteins. Moreover cardiolipin influences stability of respiratory chain complexes and carriers in the MIM (Jiang et al. 2000; Wenz et al. 2009; Zhang et al. 2005). It has also been shown that cardiolipin affects the organization of TOM and TOB complexes in the MOM (Gebert et al. 2009).

A large number of proteins were suggested to associate with specific lipids for a proper transport and insertion into their target membrane. For example, yeast arginine permease Can1 requires PE for its transport to the cytoplasma membrane (Malinska et al. 2003), while yeast tryptophan permease Tat2 depends on ergosterol for surface transport and association with lipid rafts (Umebayashi and Nakano 2003). It has also been shown that ergosterol levels can influence the ability of tail-anchored proteins to insert *in vitro* into lipid vesicles. Low ergosterol levels are a prerequisite for the insertion of mitochondrial and some TA-proteins into liposomes (Kemper et al. 2008). Sterols

also play an important role in protein sorting along the exocytic pathway (Bagnat and Simons 2002; Proszynski et al. 2005).

1.10 Cytosolic chaperones and their role in protein translocation

Chaperones form a diverse group of proteins that can bind to unfolded proteins and support a correct folding or refolding of proteins. Chaperones can also keep proteins in a partly-folded translocation-competent state or can help to avoid aggregation of hydrophobic transmembrane proteins during their passage through the cytosol. Moreover chaperones assist in protein degradation and disassembly of protein complexes (Bukau et al. 2006; Hartl and Hayer-Hartl 2002). One major subset of chaperones is comprised by the Hsp70 family that harbors an ATPase activity. Yeast cells contain nine cytosolic Hsp70 proteins that are divided in two functionally distinct classes, the Ssa and Ssb chaperones (Craig and Huang 2005).

Hsp70s can interact with a number of Hsp40 co-chaperones (Cheetham and Caplan 1998). These so-called J proteins stimulate the ATPase activity of the Hsp70 partner (Craig and Huang 2005). The interplay between a certain pair of Hsp70 and its co-chaperone can influence the activity and substrate specifity of the Hsp70 partner (Fan et al. 1998; Lu and Cyr 1998). For example, a complex of Hsp70 family Ssa1 and the J protein Ydj1 is involved in protein refolding and translocation of proteins into ER and mitochondria (Tutar and Tutar 2007). It has been also shown that yeast Hsp70 (and mammalian Hsp70 and Hsp90) is involved in precursor delivery to mitochondria and interacts with Tom70 receptor subunit of the TOM complex upon relay of the substrate protein to the TOM machinery. Although in yeast docking of chaperones to Tom70 is not essential for initial binding of the precursor, it is necessary for the formation of a productive high-molecular weight TOM-preprotein complex (Young et al. 2003). Of note, a subset of mammalian ER tail-anchored proteins uses an Hsp40/Hsc70 complex for their biogenesis. The transmembrane regions of these proteins are of a relatively low hydrophobicity and they do not use the Asna1/TRC40 pathway for insertion into ER (Rabu et al. 2008). Despite the aforementioned progess, the role of cytosolic factors in the biogenesis of mitochondrial TA-proteins is currently unclear.

1.11 Aim of this study

Tail-anchored proteins are present in almost all membranes and fulfill a great variety of functions. Currently it is still unclear how mitochondrial TA-proteins are targeted from the cytosol to the organelle and how they are integrated into the outer membrane. The general aim of this study is to identify the factors necessary for targeting and insertion of mitochondrial TA-proteins. Two specific questions will be addressed while using Fis1 as a model mitochondrial TA-protein. These questions are:

(i) Are cytosolic factors involved in the biogenesis of Fis1? If yes, which are these factors?

(ii) How is Fis1 inserted into the mitochondrial membrane?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Media

Media for *S. cerevisiae*

Carbon source and amino acid stock solutions were autoclaved separately and added to media before use.

YPD-medium

2% (w/v) bacto peptone, 1% (w/v) yeast extract, 2% (v/v) glucose, pH adjusted to 5.5 with NaOH

YPG-medium

2% (w/v) bacto peptone, 1% (w/v) yeast extract, 3% (v/v) glycerol, pH adjusted to 5.5 with NaOH

Lac-medium

0.3% (w/v) yeast extract, 0.05% (w/v) glucose, 0.05% (w/v) NaCl, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgCl_2x6H_2O$, 0.05% (w/v) $CaCl_2x2H_2O$, 2.5% (v/v) lactic acid 80%, 0.8% (w/v) NaOH, pH adjusted to 5.5 with NaOH

SD-medium

0.17% (w/v) yeast nitrogen base without ammonium sulfate, 0.5% (w/v) ammonium sulfate, 0.0055% (w/v) adenine sulfate, 0.0055% (w/v) uracil, 2% (v/v) glucose, 1% (v/v) amino acid stock solution, pH adjusted to 5.5 with NaOH. In some selective media specific amino acids or nucleotides were omitted from this mixture

SD-trp-leu-phe-medium + Bpa

0.17% (w/v) yeast nitrogen base without ammonium sulfate, 0.5% (w/v) ammonium sulfate, 0.0055% (w/v) adenine sulfate, 0.0055% (w/v) uracil, 2% (v/v) glucose,

1% (v/v) amino acid stock solution without tryptophan, leucine and phenylalanine, 0.6 mM Bpa, pH adjusted to 5.5 with NaOH; Bpa was solved in 1 M NaOH and added before use.

YNBGO-medium

0.1% (w/v) yeast extract, 0.17% (w/v) yeast nitrogen base without ammonium sulfate, 0.5% (w/v) ammonium sulfate, 0.1% (w/v) glucose, 0.1% (v/v) oleic acid, 0.002% (w/v) uracil, 0.002% (w/v) adenine sulfate, 1% (v/v) tergitol, 1% amino acid stock solution, pH adjusted to 6.0 with KOH. In some cases uracil was omitted from the medium.

Amino acid stock solution

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

Glucose stock solution

40% (w/v) glucose

Glycerol stock solution

100% glycerol

For solid media 2% agar was added to media before autoclaving.

Media for *E. coli*

LB-medium

2% (w/v) LB-medium (Lennox)

LB_{amp}-medium

2% (w/v) LB-Medium (Lennox), 100 µg/ml ampicillin. Ampicillin was added after autoclaving the media and agar solutions when those were not warmer than 50°C.

For solid media 1.5% agar was added to media before autoclaving.

2.1.2 Buffers and solutions

Buffers for agarose-gel electrophoresis

TAE-buffer

40 mM Tris-Base, 1.14 ml/l acetic acid, 1 mM EDTA, pH 8.0

Loading dye

6% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol

Buffers for PCR

10xTaq-buffer with $(NH_4)_2SO_4$

200 mM $(NH_4)_2SO_4$, 0.1% Tween20, 750 mM Tris, pH 8.8

10xPfu-buffer with $MgSO_4$

100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% Triton X-100, 1 mg/ml BSA, 20 mM $MgSO_4$, 200 mM Tris, pH 8.8

Buffers for SDS-PAGE, Western blotting and immunodecoration

2x Lämmli buffer

4% (w/v) SDS, 20% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 5% (v/v) 2-mercaptoethanol, 160 mM Tris, pH to 6.8 adjusted with HCl

SDS-Running buffer

50 mM Tris, 1.61 M glycine, 1 g/l SDS

Blotting buffer

20 mM Tris, 150 mM glycine, 0.02% (w/v) SDS, 20% (v/v) ethanol

Ponceau staining solution

8.5 ml 72% TCA, 0.4 g Ponceau for 200 ml total volume

TBS buffer

10 mM Tris, 154 mM NaCl, pH adjusted to 7.5 with HCl

TBS buffer + TX100

TBS buffer + 0.05% Triton X-100

Blocking buffer

5 % (w/v) powdered skim milk in TBS buffer

ECL

0.2 mM p-coumaric acid, 1.25 mM Luminol, 100 mM Tris, pH adjusted with HCl to 8.5; 30% H_2O_2 were added before use in ratio 1:1000 to the ECL.

Buffers for silver staining of gels

Fixation solution

40% ethanol p.A., 10% acetic acid

0.02% sodium thiosulfate

0.5% glycin

0.2% silver nitrate

Developing solution

3% Na_2CO_3, 0.05% H_2CO, 0.0004% sodium thiosulfate

Buffers for preparation of chemically competent *E. coli* cells

Tfb1 buffer

30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, pH adjusted with acetic acid to 5.8

Tfb2 buffer

10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) glycerol, pH adjusted with NaOH to 6.5

Buffers for small-scale plasmid-DNA preparation from *E. coli*

E1 buffer

50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/ml RNAse

E2 buffer

200 mM NaOH, 1% (w/v) SDS

E3 buffer

3 M potassium acetate, pH adjusted with acetic acid to 5.5

Buffers for preparation of mitochondria and sub-cellular fractionation

Resuspension buffer

100 mM Tris, 10 mM DTT

Spheroblasting buffer

1.2 M sorbitol, 20 mM KPI pH 7.2, 4 mg/g harvested cells of zymolyase

Homogenization buffer

0.6 M sorbitol, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2% (w/v) fatty acid free BSA, 1 mM PMSF

SEM buffer

250 mM sucrose, 10 mM MOPS, 1 mM EDTA, pH adjusted with KOH to 7.2

Percoll gradient solution

25 % (v/v) Percoll (Sigma-Aldrich), 250 mM sucrose, 10 mM MOPS, 1mM EDTA, 1 mM PMSF

Buffers for *in vitro* transcription, translation and import of proteins

Transcription buffer

40 mM Tris-HCl pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine

F5-import buffer

250 mM sucrose, 10 mM MOPS, 80 mM KCl, 5 mM $MgCl_2x6H_2O$, 3% (w/v) fatty acid free BSA, pH adjusted with KOH to 7.2

SEM-K80 buffer

SEM buffer + 80 mM KCl

Labeling buffer

0.6 M sorbitol, 20 mM HEPES, 50 mM Tris, 4 M Urea, 1mM DTT, pH adjusted with KOH to 7.4

IASD buffer

5 mM (4-acetamido-4'-((iodoacetyl) amino)stilbene-2,2'-disulfonic acid (IASD) in Labeling-buffer

IASD buffer + TX-100

5 mM IASD in Labeling-buffer + 1% Triton X-100

Buffers for purification of GST-tagged proteins

GST-basis buffer

100 mM NaCl, 20 mM HEPES, 1.25 mM $MgCl_2$, 1% (v/v) Triton X-100, pH adjusted with NaOH to 7.25

Elution buffer

15 mM reduced glutathione in GST-basis buffer + 0.05% Triton X-100

Lysis buffer

1 mM DTT, 2 mM PMSF, 3 mM EDTA, 0.2 mg/ml lysozyme, 0.2 µl/ml aprotinin in GST-basis buffer

Binding buffer for immunoprecipitation of HA-tagged proteins

Binding buffer

50 mM NaH_2PO_4, 100 mM NaCl, pH 7.5

2.1.3 Enzymes

Restriction enzymes and appropriate buffers were provided by New England Biolabs. T4-DNA-Ligase, Shrimp Alkaline Phosphatase, Taq-Polymerase, Pfu-Polymerase and buffers were purchased from Fermentas. Zymolyase (Seikagaku), Lysozyme (Serva), Proteinase K (Roche), Apyrase (Sigma) and SP6-Polymerase (Biozym) were obtained from the indicated manufacturers. Reactions were set up according to recommended protocols of the producers.

2.1.4 Antibodies

Specific antibodies were used to detect immobilized proteins on nitrocellulose membranes. All primary antibodies were raised in rabbits, except α-Flag that was raised in mice. Antisera were diluted in TBS buffer. Used antibodies and the dilutions are listed below. The secondary antibodies goat anti-rabbit IgG (H+L)-HRP-conjugate and goat anti-mouse IgG (H+L)-HRP-conjugate were purchased from BIORAD.

name	**dilution**	**cellular localisation of antigen**
Aco2	1:50000	mitochondrial matrix
Bmh1	1:000	cytosol
Cox4	1:000	MIM
Erv2	1:1000	ER lumen
Fis1	1:500	MOM
Fis1 aff. pur.	1:200	MOM
Fum1	1:500	cytosol / mitochondrial matrix
Get3	1:4000	cytosol
GST-Pex19	1:500	cytosol
Hex	1:5000	cytosol
Mcr1	1:1000	MOM / IMS
mtHSP60	1:1000	mitochondrial matrix
Om45	1:1000	MOM / IMS
Oxa1	1:1000	MIM
Porin	1:4000	MOM
Qcr6	1:1000	MIM
Sec61	1:2000	ER membrane
Tim10	1:1000	IMS
Tim23	1:250	MIM
Tim44	1:1000	MIM
Tob55	1:1000	MOM
Tom20	1:1000	MOM
Tom40	1:2000	MOM
Tom70	1:3000	MOM
Ydj1	1:10000	cytosol
Flag	1:200	

secondary antibodies

Goat anti-Rabbit IgG (H+L)-HRP Conjugate, BIORAD, # 172-1019

Goat anti-mouse IgG (H+L)-HRP Conjugate, BIORAD, # 172-1011

2.1.5 Yeast strains

Name	**Background**	**Genotype**	**Reference**
W303α	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52;	Deutsche Stammsammlung für Mikroorganismen
W303α:pRS426xTPI	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52;	this study
W303α:pRS426xTPI-Pex19	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52;	this study
W303α:pRS316-CherryFis1+ pYX142mtGFP	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52;	this study
W303α:pRS316-CherryFis1+ BFGIII-Get3-OE	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52;	this study
W303α: pRS426xTPI-Fis1	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52;	this study
W303α:pYX142-3xHA-Fis1-139stop +pPR1-PGK1-3SUP4-tRNAcua	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52;	this study
spf1Δ	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52; SPF1::HIS3	this study
spf1Δ:pYX142-mtGFP+pRS316-CherryFis1	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52; SPF1::HIS3	this study
spf1Δ:pRS316-CherryFis1+BFGIII-Get3-OE	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52; SPF1::HIS3	this study

Name	Background	Genotype	Reference
pex19Δ	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52; PEX19::HIS3	this study
fis1Δ:pRS316-FisPr-Fis1-T	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
fis1Δ:pRS316-FisPr-Fis1-T +pYX142-mtGFP	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
fis1Δ:pRS426xTPI-Fis1	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
fis1Δ:pRS316-FisPr-Fis1Tom70-T	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
fis1Δ:pRS316-FisPr-Fis1Tom70-T +pYX142mtGFP	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
fis1Δ:pRS316-FisPr-Fis1Tom70M166R-T	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
fis1Δ:pRS316-FisPr-Fis1Tom70M166R-T +pYX142mtGFP	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
fis1Δ:pRS426xTPI-Fis1Tom70	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
fis1Δ:pYX142-Fis1-139stop + pPR1-PGK1-3SUP4-tRNAcua+pRS416-mtsRFP	ADM552	MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200; FIS1::HIS3	this study
BY4741	BY4741	MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0	Euroscarf (Frankfurt)
spf1Δ	BY4741	MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; SPF1::KanMX4	Euroscarf (Frankfurt)
Ssa2-4Δ SSA1	JN516	MATa leu2-3,112; his3-11; ura3-52; trp1Δ1 lys2; SSA1; ssa2::LEU2 ssa3::TRP1 ssa4::LYS2	kind gift from Ophrey Pines

Name	Background	Genotype	Reference
Ssa2-4Δ ssa1 ts	JB67	MATa leu2-3,112; his3-11; ura3-52; trp1Δ1 lys2; ssa1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2	kind gift from Ophrey Pines
M3	M3	MATα lys2 his4 trp1 ade2 leu2 ura3	Blachly-Dyson et al. 1997
M3 *por1Δ*	M3	MATα lys2 his4 trp1 ade2 leu2 ura3; por1::LEU2	Blachly-Dyson et al. 1997
M3 *por1Δ por2Δ*	M3	MATα lys2 his4 trp1 ade2 leu2 ura3; por1::LEU2 por2	Blachly-Dyson et al. 1997
por1 Δ	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52; Por1::HIS3	made by H. Kato
por1Δ:pRS426xTPI-Fis1	W303α	MATα; ade2-1; can1-100; his3-11; leu2 3_112; trp1Δ2; ura3-52; Por1::HIS3	this study

2.1.6 *E. coli* strains

Name	Background	Plasmids	Reference
BL21	BL21		Stratagene
BL21:pGEX4T	BL21	pGEX4T	this study
BL21:pGEX4T-Fis1cyt	BL21	pGEX4T-Fis1cyt	this study
BL21:pGEX4T-Fis1cyt	BL21	pGEX4T-Fis1	this study
BL21:pGEX4T-Pex19	BL21	pGEX4T-Pex19	this study

2.1.7 List of oligonucleotides

Name	Sequence
5-Fis-Pr-spel	5´ AAA *ACT AGT* TCA AAT AAC ATG TGT CCA TTA CC 3´
3-Fis-Pr-smaI	5´AAA *CCC GGG* GTT GTA TGG CTG TG 3´
5-Fis1-EcoRI	5´ AAA *GAA TTC* ATG ACC AAA GTA GAT TTT TGC AAC TC 3´
3-Fis1-HindIII	5´ AAA *AAG CTT* TTA CCT TCT CTT GTT TCT TAA GAA GAA AC 3´

5-Fis1-T-HindIII	5´CCC *AAG CTT* ATA AAA AAT CAG CAC ATA CGT ACA TAC 3´
5-FisTom70-EcoRI	5´AAA *GAA TTC* ATG ACC AAA GTA GAT TTT TGG CCA AC 3´
3-FisTom70-HindIII	5´GGG *AAG CTT* TTA CAT CTT GCT GAA AAT TG 3´
3-Fis1Tom70M166RHindIII	5´GGG *AAG CTT* TTA CCT CTT GCT GAA AAT TG 3´
c-Fis1-139	5´ C GCT GGA GGC GTA TAG GCC GGC GCT GTG G 3´
w-Fis1-139	5´ C CAC AGC GCC GGC CTA TAC GCC TCC AGC G 3´
5´-E-H-3xHAFis1	CCC *GAA TTC AAG CTT* ATG TAC CCA TAC GAT GTT C
3´-E-3xHAFis1	CCC *GAA TTC* AGC GTA ATC TGG AAC G
5-Pex19-EcoRI	5´ AAA CAC *GAA TTC* ATG AAT GAA AAC GAG TAC 3´
3-Pex19-BamHI	5´ A AAT *GGA TCC* ACC TTA TTG TTG TTT GC 3´
5-KO-Pex19	5´ AAG TAT TGA CGG AAA GAA GAA ATG CCA AAC ATA CAA CAC GAA GTA *CGT ACG CTG CAG GTC GAC* 3´
3-KO-Pex19	5´ TAC TTT TTT TTT TTT TTT TTT ACT GTT ATC ATA AAT ATA TAT ACC *ATC GAT GAA TTC GAG CTC G* 3´
Pex19-KO-K	5´ GAC TTG GCT TTG GCT TGC GG 3´
5-SPF1-KO	5´ GAC ATA GTT GAC ATA TCA GAC CTA CAG AAA CAT AGG AAT CGG TAA *CGT ACG CTG CAG GTC GAC*
3-SPF1-KO	ATA TAA GTA TAT AAA TAC AAA AAG GGG TAC TAC ATA AAA GAT TTA *ATC GAT GAA TTC GAG CTC G* 3´
5SPF1-KO-K	5´ CCC TGC ATC TTG CGC TGC C 3´
3SPF1-KO-K	5´ CCC GAA GCT ATT ATA ATT TTC GTA TAC 3´
His-K	5´ CTT GGT TTC ATT TGT AAT ACG CT 3´

2.1.8 List of plasmids

Name	Promotor	Marker (*E. coli*)	Marker (*S.cerevisiae*)	Reference
pRS426xTPI-Fis1	TPI	Amp	URA	this study
pRS316-Fis1Pr-Fis1-T	Fis1	Amp	URA	this study
pRS316-CherryFis1	TEF2	Amp	URA	this study
pRS426xTPI-Fis1Tom70	TPI	Amp	URA	this study
pRS316-FisPr-Fis1Tom70-T	Fis1	Amp	URA	this study
pRS316-FisPr-Fis1Tom70M166R-T	Fis1	Amp	URA	this study
pYX142-3xHA-Fis1-139stop	TPI	Amp	LEU	this study
pRS426xTPI-Pex19	TPI	Amp	URA	this study
BFGIII-Get3-OE	ADH2	Amp	LEU	kind gift from M. Schuldiner
pYX142-mtGFP	TPI	Amp	LEU	plasmid collection, Rapaport group
pRS416-mtsRFP	TPI	Amp	URA	plasmid collection, Rapaport group
pPR1-PGK1-3SUP4-tRNAcua	PGK1, ADH1	Amp	TRP	Chen et al. 2007
pRS426xTPI	TPI	Amp	URA	plasmid collection, Rapaport group
pGEX4T	LAC	Amp		Amersham Biosciences
pGEX4T-Fis1	LAC	Amp		this study
pGEX4T-Fis1cyt	LAC	Amp		this study
pGEX4T-Pex19	LAC	Amp		this study
pFA6a-HIS3MX6	TEF	Amp	HIS	Wach et al. 1997
pGEM4	AmpR, T7, SP6	Amp		Promega
pGEM4-Fis1TMC	AmpR, T7, SP7	Amp		this study
pGEM4-pSU9DHFR	AmpR, T7, SP6	Amp		Pfanner et al. 1987
pGEM4-AAC	AmpR, T7, SP6	Amp		Mayer et al. 1993

2.2 Methods

2.2.1 Molecular biology methods

2.2.1.1 Polymerase Chain Reaction (PCR)

PCR was applied for amplification of specific DNA sequences (Saiki et al. 1988). Both genomic and plasmid DNA were used as templates. The standard PCR reaction mix of 100 µl contained: 100 ng plasmid DNA or up to 1 µg genomic DNA as template, 20 pmol of both primers, 10 µl appropriate 10x buffer, 1.5 U of Taq- or Pfu-Polymerase, 2 µl of 10 mM dNTPs and addition of H_2O to 100 µl.

PCR reactions were performed in a Thermo Cycler (Biometra) according to the following program. Denaturation (5-10 min) at 95°C, 30 cycles of DNA denaturation for 1 min at 95°C followed by annealing for 1 min at 50-60°C and DNA extension for 1-4 min at 72°C, and a final extension step of 10 min at 72°C. Afterwards PCR samples were cooled down to 4°C.

2.2.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to their molecular mass. For this purpose gels with an agarose concentration between 0.5-2% in TAE buffer and 0.5 µg/ml ethidium bromide were utilized. Electrophoresis of samples mixed with Loading Dye was performed in gels submerged in TAE buffer. Fermentas Gene Ruler™ 1 kb DNA Ladder was applied as DNA fragment size marker. Separated DNA fragments were visualized by UV-light.

2.2.1.3 Isolation of DNA from agarose gels

DNA fragments were cut out from the gel under UV-light and isolated via a silica-column using peqGOLD Gel Extraction Kit (peqLAB). Procedures were carried out according to the recommended protocol of the producer. DNA was eluted from the column in 30 µl H_2O.

2.2.1.4 Restriction digestion of DNA

DNA was digested by restriction endonucleases for analytical and preparative purposes. The set up of restriction reaction, incubation and inactivation times were performed according to the enzyme manufacturer´s instructions. For analysis of obtained fragments agarose gel electrophoresis was applied or fragments were directly purified by using peqGOLD Gel Extraction Kit (peqLAB).

2.2.1.5 Dephosphorylation of DNA fragments

Shrimp Alkaline Phosphatase was employed to dephosphorylate 5´-ends of linearized plasmid DNA to avoid re-ligation of the vector in a subsequent ligation reaction. Reactions were carried out according to manufacturer's recommendations. Dephosphorylized plasmids were purified using peqGOLD Gel Extraction Kit (peqLAB).

2.2.1.6 Ligation

Linearized plasmid DNA (50-100 ng) and DNA fragments which should be inserted into the vector in a 1:3 ratio were incubated with T4-DNA-Ligase in 1xT4-Ligase buffer for 16 h at 14°C. Ligase was inactivated by incubation for 15 min at 65°C.

2.2.1.7 Preparation of chemically competent *E. coli* cells

XL1-blue *E. coli* cells were plated on solid LB-medium and grown for 16 h at 37°C. A single colony was picked and transferred to a 50 ml LB culture and incubated for 16 h at 37°C while shaking at 120 rpm. Cultures were diluted in 200 ml LB-medium to an OD_{600} of 0.1 and incubated at 37°C while shaking at 120 rpm until an OD_{600} of 0.5 was reached. Cultures were chilled on ice for 15 min. The cell suspension was transferred to a sterile centrifugation tube and cells were harvested by centrifugation (3000x g, 10 min, 4°C). The cell pellet was resuspended in 80 ml Tfb1 buffer and stored on ice for 15 min. Cells were sedimented (3000x g, 10 min, 4°C) and 80 ml of Tfb2 buffer were used to resuspend the cell pellet. After 15 min of incubation on ice 100 µl aliquots

of competent *E. coli* cells were shock-frozen in liquid nitrogen and stored at -80°C until use.

2.2.1.8 Transformation of *E. coli* cells

For amplification of plasmid DNA chemically competent *E. coli* cells were transformed. A solution of ligation reaction (1-10 µl) was mixed with 100 µl of cells and incubated on ice for 30 min. Afterwards the cells were subjected to a heat-shock for 45 s at 42°C and incubated on ice for 2 min. Next, 900 µl LB-medium were added to the cells and the mixture was incubated for 45 min at 37°C with mild shaking. Cells were collected by centrifugation (10000x g, 30 sec, RT), plated on solid LB_{amp}-medium and incubated overnight at 37°C.

2.2.1.9 Small scale plasmid DNA preparation from *E. coli* cells

For small-scale plasmid-DNA preparation the alkaline lysis method (Birnboim and Doly 1979) was applied. Single transformed colonies of *E. coli* were picked and cultured in 3 ml LB_{amp} for 16 h at 37°C. Portions (1.5 ml) of the cultures were harvested by centrifugation (10000x g, 1 min, RT). And cells were resuspended in 300 µl E1 buffer. For lysis of the cells 300 µl of E2 buffer were added and cells were mixed with the buffer by inverting the reaction tube 5 times. After 5 min of incubation at RT 300 µl of E3 buffer were added for neutralization and the tubes were inverted 5 times. After centrifugation (15000x g; 15 min, 2°C) supernatants were transferred to reaction tubes containing 600 µl of 96% 2-propanol for precipitation of the plasmids. Samples were centrifuged again (15000x g, 15 min, 2°C), the supernatants were discarded and the DNA pellet was washed with 70% ice-cold ethanol and dried for 5-10 min at 50°C. The plasmid-DNA was resuspended in 30 µl H_2O and stored until further use at -20°C.

2.2.1.10 Large-scale DNA preparation from *E. coli* cells

For large-scale plasmid-DNA preparation the PureYield Plasmid Midiprep System (Promega) was used. Procedures were carried out according to the producer´s protocol. DNA was eluted from the column in 500 µl H_2O. For determining the DNA concentration, plasmid-DNA from Midi-Preps was diluted 1:20 in H_2O and absorption was measured at a wavelength of 260 nm (Eppendorf BioPhotometer). An absorption of one unit corresponds to a concentration of 50 µg/ml double-stranded DNA.

2.2.2 Methods in yeast genetics

2.2.2.1 Cultivation of *S. cerevisiae*

Cultivation of yeast cells was performed according to published procedures (Sambrook et al. 1989) in YPD, YPG or Lac-medium. For selection on auxotrophic markers cells were grown in the appropriate SD-medium. Liquid cultures with volumes of 20-2000 ml were incubated at 24°C, 30°C or 37°C while shaking at 120 rpm. Culture plates were incubated at 15°C, 30°C and 37°C.

2.2.2.2 Transformation of yeast cells

The procedures for lithium-acetate transformation method of *S. cerevisiae* (Gietz et al. 1995) were slightly modified. Yeast cells were grown on appropriate solid medium at 30°C. Cells were scraped from the plate with a sterile inoculation loop, washed in 1 ml sterile water and collected by centrifugation (10000x g, 30 sec, RT) and resuspended in 1 ml 100 mM lithium-acetate. After incubation for 5 min at 30°C and shaking at 500 rpm, cells were collected again by centrifugation (10000x g, 30 sec, RT). Then solutions were added to the cells in the following order: 240 µl 50% (w/v) polyethylene glycol 3350, 55 µl water, 36 µl 1 M lithium-acetate, 10 µl 5 mg/ml heat denaturized salmon sperm DNA and 5 µl plasmid DNA (100-600 ng/µl). The mixture was incubated at 42°C with shaking at 800 rpm for 30 min. Cells were sedimented by centrifugation as above and streaked on a plate of the appropriate selective medium. The plates were

incubated at 30°C for 2-4-days until single colonies were visible. A few transformed colonies were streaked on fresh plates and further incubated at 30°C.

For transformation of knockout-cassettes with subsequent homologous recombination event cells from logarhythmic growing liquid cultures were used.

2.2.2.3 Construction of *S. cerevisiae* deletion strains

Deletion of the yeast genes *SPF1* and *PEX19* in the W303α background was performed by a PCR-based approach using the histidine-marker cassette amplified from the pFA6a-HIS3MX6 plasmid. The open reading frame of the genes was replaced by the His-cassette by homologous recombination. Cells were selected on SD-his plates at 30°C. Genotypes were confirmed by PCR.

2.2.3 Methods in cell biology

2.2.3.1 Cycloheximide treatment of yeast cells

To investigate the stability of yeast proteins in a living cell, cells were treated with cycloheximide that inhibits translation processes and synthesis of new proteins by the cell. Yeast cells were grown in 300 ml of appropriate liquid medium at 30°C or 37°C to an OD_{600} of 0.8-1. The culture (100 ml) was harvested by centrifugation (3000x g, 5 min, RT) and the pellet was frozen at -20°C. Cycloheximide (at conc. of 100 µg / ml) was added to the 200 ml of the residual culture and cells were incubated at 30°C or 37°C. After 2 h of incubation 100 ml of the culture were harvested by centrifugation (3000x g, 5 min, RT) and the pellet was frozen at -20°C. The rest of the culture was incubated further for another two hours and then also harvested. Whole cell lysate or crude mitochondria were isolated from the cells and proteins were analyzed by SDS-PAGE and Western blot.

2.2.3.2 Isolation of crude mitochondria from yeast

Cells were cultured overnight in appropriate medium and diluted the next day in 200 ml medium to an OD_{600} of 0.2 and further grown until the culture has reached an OD_{600} of 0.5-0.8. Cells were then harvested by centrifugation (3000x g, 5 min, RT). The pellets were resuspended in 300 µl SEM buffer supplemented with 1 mM PMSF. For opening of the cells 300 mg glass beads (diameter: 0.75-1 mm, Roth) were added and the mixture was vortexed 5 times for 30 s with pauses of 30 s on ice. After centrifugation (1000x g, 5 min, 2°C) the supernatant which represents the whole cell lysate was transferred to a new reaction tube and the protein concentration was determined. Crude mitochondria were harvested from specific amounts of whole cell lysate by centrifugation (20000x g, 20 min, 2°C). The crude mitochondrial pellet was resuspended in 2xLämmli buffer, heated for 5 min at 95°C and stored at -20°C until further analysis by SDS-PAGE.

2.2.3.3 Isolation of pure mitochondria and microsomes from yeast

Isolation of *S. cerevisiae* mitochondria was performed according to a previously described method (Daum et al. 1982). This method was expanded for the isolation of microsomes as well. Yeast cells were grown at 30°C in 2-8 l of appropriate medium to an OD_{600} of 1.0-2.0 and harvested by centrifugation (3000x g, 5 min, 20°C). The collected cells were washed with 200 ml H_2O (3000x g, 5 min, 20°C), the pellet was weighted and resuspended in 2 ml/g cells in resuspension buffer. After 10 min shaking at 30°C cells were re-isolated by centrifugation (5 min, 3000x g, 20°C) and resuspended in 100 ml 2.4 M sorbitol. Cells were collected by centrifugation (3000x g, 5 min, 20°C), resuspended in 2 ml / g cells of spheroblasting buffer and incubated while shaking for 50-60 min. The spheroblasting buffer contains zymolyase, an enzyme which digests fungal cell walls. The efficiency of spheroblasting was checked photometrically: 20 µl of the spheroblast solution were mixed with 1 ml of either H_2O or 1.2 M sorbitol. Spheroblasted cells burst in pure water and the solution becomes clear. Eight- to tenfold difference in the OD_{600} was considered as an indicator for sufficient spheroblasting. Spheroblasted cells were re-isolated by centrifugation (2000x g, 5 min, 2°C) and resuspended in 13 ml/g cells homogenization buffer. Cells were opened by 12

strokes in a tight fitting glass homogenisator on ice. Cell lysate was clarified two times by centrifugation (1000x g, 5 min, 2°C). Mitochondria were isolated from the cleared lysate by a following centrifugation step (15000x g, 15 min, 2°C). The post-mitochondrial supernatant was kept for isolation of microsomes. The mitochondrial pellet was resuspended in 100 ml SEM buffer and re-collected again (15000x g, 15 min, 2°C). For further purification, mitochondria were resuspended in 5 ml SEM buffer and loaded on top of 17.5 ml of Percoll gradient solution (25 % Percoll) in a 60Ti centrifugation tube (Beckman). The gradient was established by ultracentrifugation (80000x g, 45 min, 2°C) in an Optima^{TM} L 90-K ultracentrifuge (Beckman Coulter). The purified yellowish mitochondria were collected from the lower third of the gradient and resuspended in 30 ml SEM buffer. Re-isolation of mitochondria was done by centrifugation (15000x g, 15 min, 2°C). The mitochondrial pellet was resuspended in 500 µl SEM buffer. 50 µl aliquots were shock-frosted in liquid nitrogen and stored at -80°C until further use. Microsomes were isolated from post-mitochondrial supernatant. After a clearing centrifugation step (15000x g, 15 min, 2°C) the microsomes were collected by ultracentrifugation (130000x g, 1 h, 2°C) in an Optima^{TM} L 90-K ultracentrifuge (Beckman Coulter). Microsomes were resuspended in 500 µl SEM buffer and subjected to a clearing spin (8000x g, 10 min, 2°C). Aliquots (50 µl) were snap-frozen in liquid nitrogen and stored at -80°C until further use. Protein concentrations of mitochondria and microsomes were determined by a Bradford assay.

2.2.3.4 *In vivo* site-directed photo-crosslinking

For introduction of the photo-reactive cross-linking moiety p-benzoyl-L-phenylalanine (Bpa, Bachem) into the examined protein, yeast cells were transformed with two plasmids. One plasmid contains the coding sequence for the protein of interest containing an amber stop codon (TAG) in the desired position (in this study: 3xHA-Fis1-139stop). The other plasmid carries coding information for an amber suppressor tRNA and its cognate aminoacyl-tRNA synthetase that charges the tRNA with Bpa (Chin et al. 2003). The plasmid was kindly provided by Peter G. Schultz. A previously published protocol (Chen et al. 2007; Carvalho et al. 2010), which was slightly modified, was applied for *in vivo* site-directed photo-crosslinking procedures. Yeast

cultures grown at 30°C in SD-trp-leu medium were back-diluted to an OD_{600} of 0.2 in 200-1000 ml SD-trp-leu-phe- medium containing 0.6 mM Bpa and incubated further up to an OD_{600} of 0.8. During this time cells incorporated the modified amino acid Bpa into the protein of interest. Cells were harvested by centrifugation (3000x g, 5 min, RT) and resuspended in 800 µl H_2O / 80 ODs of cells. The total volume was halved and one half of the cells was stored in the dark at 4°C. The other half was applied to a 12-well plate (Becton Dickinson) at 80 OD units per well. The plate was placed on ice and the cells were exposed to UV light with a wavelength of 365 nm for 1 h at 4°C (Black-Ray Ultraviolet Lamp B-100 AP, UVP). The UV-lamp was at a distance of 10 cm to the plate. Irradiated and non-irradiated cells were transferred to 2 ml reaction tubes and re-isolated by centrifugation (15000x g, 1 min, RT). Whole cell lysate was prepared according to the method of cell-breakage with glass beads (see above). The lysate was analyzed by SDS-PAGE and immunodetection.

2.2.3.5 Fluorescence microscopy of yeast cells

Pre-cultures of yeast cells expressing fluorescent marker proteins were grown overnight in 20 ml of appropriate selective medium. Cultures were back-diluted to OD_{600} of 0.1 and incubated until an OD_{600} of 0.6-0.8 was reached. Cells (from 1 ml culture) were harvested by centrifugation (8000x g, 30 sec, RT) and resuspended in 100 µl water. The cell suspension (5 µl) was mixed with 5 µl of 1% low melting point agarose (65°C, Roth), placed on a microscope slide and covered with a cover glass. Microscopy was done with a ZEISS Axioscope microscope and pictures were taken with an Axio Cam MRm system. Fluorescence of GFP proteins was excited with blue light (488 nm) and red fluorescent proteins were excited with green light (580 nm).

2.2.4 Biochemical methods

2.2.4.1 Determination of protein concentration

Protein concentrations were determined by application of the Bradford method (Bradford, 1976). Protein solutions (10 µl) were diluted in 1 ml Roti-Quant solution (1:5 diluted in SEM buffer) and incubated for 10 min at RT. Absorbance at a wavelength of 595 nm was measured using an Eppendorf BioPhotometer. A standard calibration line was obtained by using known amounts (2-10 µg) of BSA. Protein concentrations were calculated according to the calibration line.

2.2.4.2 Carbonate extraction

Carbonate extraction was performed according to the procedures published by Fujiki (Fujiki et al. 1982). Isolated mitochondria or microsomes were mixed with 50 µl cold 20 mM HEPES/KOH pH 7.5 and 50 µl freshly prepared cold 200 mM sodium carbonate. Samples were incubated on ice for 30 min. Membranes with embedded proteins were isolated by ultracentrifugation (120000x g, 1 h, 2°C). Soluble proteins were precipitated with trichloroacetic acid (TCA, see below) from the supernatant.

2.2.4.3 Proteinase K treatment of yeast mitochondria and microsomes

Isolated organelles (30-50 µg) were resuspended in 100 µl SEM buffer supplemented with Proteinase K (200 µg/ml) and incubated on ice for 30 min. To stop the reaction 2 µl 200 mM PMSF were added and the mixture was incubated for further 15 min on ice. The organelles were re-isolated by centrifugation (20000x g, 20 min, 2°C for mitochondria; 90000x g, 1 h, 2°C for microsomes). The pellet was resuspended in 100 µl SEM buffer + 1 mM PMSF, centrifuged again (see above), resuspended in 2xLämmli buffer and incubated for 5 min at 95°C before analysis by SDS-PAGE.

2.2.4.4 TCA-precipitation of proteins

Proteins were precipitated from aqueous solutions by adding 72% trichloracetic TCA to 20% of the total volume. The samples were incubated for 30 min on ice and centrifuged (30000x g, 20 min, 2°C). The pellet was washed with 500 µl ice-cold 90% acetone (30000xg, 5 min, 2°C), dried on air for 5-10 min, resuspended in 2xLämmli buffer and incubated at 95°C for 5 min.

2.2.4.5 SDS-PAGE

For separation and analysis of denatured proteins in the range of 10-130 kDa one-dimensional glycine-SDS-PAGE was applied (Lämmli, 1970). Protein samples of 20-150 µg were dissolved in 20-50 µl 2xLämmli buffer. Gels were casted between glass plates (16.5 x 12 cm) separated by 1 mm plastic spacers. The bottom gel had a height of app. 8 mm, the separating gel 9 cm and the stacking gel 1 cm. Separation range was adjusted to the needs of the experiments by choice of different percentages of acryl amide (aa) in the separating gel. Amounts of solutions for different gels used in this study are listed below. The bottom gel usually contained 15% aa gel.

% acryl amide	**30% aa, 0,8% bis-aa**	**1 M Tris pH 8.8**	**H_2O**	**APS**	**TEMED**
10%	5 ml	3.75 ml	6.09 ml	150 µl	12 µl
12,5%	6.25 ml	3.75 ml	4.84. ml	150 µl	12 µl
15%	7.5 ml	3.75 ml	3.59 ml	150 µl	12 µ
		1 M Tris pH 6.8			
Stacking gel	1.5 ml	1 ml	7.39 ml	100 µl	8 µl

For separation of small proteins (10-20 kDa) with only higher resolution High-Tris urea-gels were applied:

	60% aa, 0.8% bis-aa	**1.825 M Tris-HCl + 1 mM NaCl pH 8.8**	**urea**	**10% SDS**	**H_2O**	**APS**	**TEMED**
Running gel	4.9 ml	6 ml	5.46 g	152 µl	-	100 µl	20 µl
		0.6 M Tris-HCl pH 6.8					
Stacking gel	500 µl	1.25 ml	2.16 g	60 µl	2.62 ml	30 µl	10 µl

The gels were adjusted vertically in the electrophoresis apparatus and both cathode and anode compartments were filled with SDS running buffer. Samples and protein size marker (pre-stained PageRuler from Fermentas) were loaded on the gel. Empty pockets were filled with 2xLämmli buffer. Electrophoresis was performed with a current of 25 mA.

2.2.4.6 Western Blotting

For transfer of proteins from the SDS-PAGE gel to a nitrocellulose membrane (Whatman) the previously described method of semi-dry blotting (Khyse-Anderson, 1984; Towbin et al. 1979) was applied. Two filter papers (Whatman, 3 mm) were placed on the bottom of the blot sandwich apparatus followed by a nitrocellulose membrane, the gel, two further filter papers and the top of the apparatus. Filter papers, membrane and gel were shortly soaked in blotting buffer before assembly. Transfer of proteins was performed for 1 h at 220 mA (app. 1.2 mA/cm^2 membrane). Efficiency of blotting was checked incubating the membrane in Ponceau staining solution for 1-2 min and washing in water several times. Then, visible bands were inspected.

2.2.4.7 Immunodetection of proteins on membranes

Unspecific binding-sites on the membranes were blocked by incubation in blocking buffer for 1 h at RT. Membranes were shortly washed with TBS before the primary antibody was applied for 1h at RT. Afterwards primary antibodies were removed and membranes were washed for 5 min at RT in TBS, then TBS +TX-100 and again in TBS. Membranes were incubated in secondary antibody (goat-anti-rabbit-HRP-conjugate or goat-anti-mouse-HRP-conjugate 1:10000 in blocking buffer) for 1 h at RT or overnight at 4°C. After washing (see above) the membranes were wetted with ECL reagent and chemoluminescence was detected with Super RX X-ray films (Fuji).

2.2.4.8 Silver-staining of SDS-PAGE gels

To visualize protein bands within a SDS-PAGE gel after electrophoresis the gel was subjected to a silver-staining. After a short wash with water the gel was incubated in fixation solution for 2 h or for overnight. Then, the gel was washed three times for 20 min in 30% ethanol p.A. Next the gel was soaked for 1 min in 0.02% sodium thiosulfate, followed by three short washing steps in water. The gel was then incubated for 1 h in 0.2% silver nitrate and afterwards shortly washed three times in water. Developing was performed for 10 min in developing solution. The reaction was stopped by removal of the developer, a short wash in water and soaking the gel 5 min in 0.5% glycine. The gel was scanned and stored in 1% acetic acid at 4°C.

2.2.4.9 *In vitro* synthesis of radio-labeled proteins

For the synthesis of ^{35}S-radiolabeled proteins genes of interest were first cloned into the transcription plasmid pGEM4 (Promega). Transcription into mRNA was performed with the help of SP6-Polymerase (Melton et al. 1984; Sambrock et al. 1989). The transcription reaction contained: 10 µl SP6-buffer, 5 µl 10 mM DTT, 2 µl (80U) RNAse inhibitor (Promega), 10 µl 2.5 mM rNTP-mix (GE Healthcare), 2.6 µl 0.8 mM methyl-G(5´)ppp(5´)G cap (Amersham), 5 µg plasmid DNA and 1.5 µl (75 U) SP6 Polymerase in a total volume of 50 µl. The mixture was incubated for 1 h at 37°C. Synthesized

mRNA was precipitated by adding 5 µl 10 M LiCl and 150 µl ethanol p.A. and the mixture was incubated for 3 h at -20°C. The RNA was isolated by centrifugation (37000x g, 20 min, 2°C) and washed with 500 µl ice-cold 70% ethanol. The pellet was dried on air for 5 min and resuspended in 37 µl H_2O supplemented with 2 µl RNAse inhibitor. Aliquots were stored at -80°C until use.

Translation of the mRNA into ^{35}S-labeled protein was done in rabbit reticulocyte lysate (Promega). The *in vitro* translation mix contained: 12.5 µl mRNA, 1.75 µl amino-acid mix (without methionine), 3.5 µl Mg-acetate, 0.5 µl RNAse inhibitor, 6 µl ^{35}S-methionine (10 mCi /ml) and 50 µl rabbit reticulocyte lysate. The reaction was incubated for 1 h at 30°C and after addition of 6 µl of 58 mM methionine incubated for another 10 min at 30°C. Then, 12 µl of 1.5 M sucrose were added. For removal of ribosomes the mixture was centrifuged in an ultracentrifuge (90000x g, 50 min, 4°C, TL45). The cleared supernatant was used for import experiments.

2.2.4.10 Import of proteins into mitochondria and microsomes

Mitochondria or microsomes were resuspended in F5-import buffer to a concentration of 300 µg/ml and 500 µg/ml, respectively. The organelle solution (30 µg / 50 µg in 100 µl) was supplemented with 2 µl of 0.2 M ATP and 1 µl of 0.2 M NADH. Import reaction was started by adding 6 µl rabbit reticulocyte lysate containing the synthesized ^{35}S-labeled protein. Incubation time and temperature varied for each investigated protein (Fis1-TMC: 5 min on ice, pSU9-DHFR: 1, 5, or 15 min at 25°C, AAC2: 1 or 15 min at 25°C). Import reaction was stopped by adding 400 µl SEM-K80 and centrifugation (20000x g, 20 min, 2°C for mitochondria; 90000x g, 1 h, 2°C for microsomes). To monitor import efficiency the import reaction was followed by an integration assay. For mitochondrial inner membrane proteins and matrix proteins, such as AAC2 and pSU9-DHFR, a Proteinase K treatment was applied.

A modification assay with (4-acetamido-4'-((iodoacetyl) amino) stilbene-2,2'-disulfonic acid (IASD) was used to monitor the membrane integration of Fis1-TMC (Kemper et al. 2008). Fis1-TMC harbors a single cysteine residue within the transmembrane domain. This residue can be modified by IASD which would result in a migration-shift on SDS-

PAGE if the transmembrane domain is not embedded into a membrane. Correctly imported Fis1-TMC is not accessible for IASD. Hence, the degree of labeling with IASD reflects the import efficiency of Fis1-TMC. The mitochondrial or microsomal pellets of three similar reactions were resuspended in 30 µl of labeling buffer, IASD buffer (5mM IASD) or IASD buffer + 1% TX-100 and incubated for 30 min on ice, followed by a second incubation step for 20 min at 25°C. The organelles were re-isolated by centrifugation (20000x g, 20 min, 2°C for mitochondria; 90000x g, 1 h, 2°C for microsomes). Samples containing Triton X-100 were precipitated with TCA. All pellets were resuspended in 30 µl 2xLämmli buffer and incubated for 5 min at 95°C. Samples were further analyzed by SDS-PAGE and autoradiography.

2.2.4.11 Apyrase treatment of mitochondria and rabbit reticulocyte lysate

Apyrase catalyzes the hydrolysis of ATP to AMP and inorganic phosphate. To deplete ATP, rabbit reticulocyte lysate and isolated mitochondria were treated with apyrase for 15 min at 25°C. ATP was removed from 40 µl of reticulocyte lysate by addition of 0.3 U apyrase, while 150 µg mitochondria dissolved in 600 µl F5-import buffer were treated with 1.8 U of apyrase.

2.2.4.12 Inhibition of Hsp40/Hsc70 complex from rabbit reticulocyte lysate by addition of inhibitors

The activity of the chaperones Hsp40 and Hsc70 can efficiently be hampered by addition of small molecule inhibitors (Rabu et al 2008). The reagents Mal3-101 and DMT002220 were solubilized in DMSO (5 mM) and added to the reticulocyte lysate to a final concentration of 250 µM. The reaction was incubated for 20 min at 30°C. The lysate was incubated with the corresponding volume of DMSO as a control.

2.2.4.13 Autoradiography and quantification of bands

Radio-labeled proteins were immobilized on nitrocellulose membrane and detected by exposure of the membranes to an X-ray film (Kodak Bio Max MM). Exposure time depended on the signal intensity and varied between 2 to 21 days. The exposed film was developed and scanned on a MICROTEK ScanMaker i800. For quantification of autoradiography and immunodetection films the AIDA Image Analyzer v 4.19 software was used.

2.2.4.14 Pulldown of 3xHA-Fis1 and cross-linking products

A pulldown via the HA-tag of 3xHA-Fis1-139stop was performed after photo-crosslinking experiments to identify potential interaction partners of the protein. UV-irradiated and non-irradiated yeast cells (300 OD units each) from a cross-linking experiment (see above) were resuspended in 400 µl binding buffer supplemented with 1 mM PMSF and protease inhibitor tablets (Roche). Glass beads (diameter 0.75-1 mm, Roth) at 2/3 volumes were added and the mix was vortexed 8 times for 30 sec with 30 sec pause on ice. After a clearing spin (1000x g, 5 min, 2°C) the lysate was transferred to a new tube and filled up to 1 ml with binding buffer supplemented with 1 mM PMSF and protease inhibitor mixture. Protein concentration was determined and accordingly equal amounts of +UV and –UV lysates were taken for further treatment. Membranes were lysed by addition of 25 µl of 20% Triton X-100 and incubation for 1 h at 4°C on an overhead-shaker. After a clarifying spin (30000x g, 20 min, 2°C) the lysate was loaded on 50 µl anti-HA-agarose (Pierce) that were pre-washed three times with 1 ml binding buffer and incubated for 1 h at 4°C on an overhead-shaker. Next, the agarose beads (and the bound proteins) were re-isolated from the lysate by centrifugation (10000xg, 30 sec, RT) and washed three times with 1 ml binding buffer + 0.05% Triton X-100. Bound proteins were eluted from the agarose matrix by incubation in 75 µl 2xLämmli buffer for 10 min at 95°C. The agarose beads were pelleted by centrifugation (10000x g, 1 min, RT) and the supernatant was divided into two fractions (30% and 70%) and both were loaded on a SDS-PAGE gel. One portion (30 %) was analyzed by Western blot and immunodetection, while the other portion (70%) was visualized by silver-stain. Bands containing potential cross-link products

were cut out from the silver-stained gel and analyzed by mass spectrometry in the Proteome Centre of the University of Tübingen.

2.2.4.15 Over-expression and purification of GST-tagged proteins

GST-tagged proteins were expressed in *E. coli* cells and purified via the GST-tag. An overnight pre-culture of *E. coli* cells (50 ml LB_{amp}, 37°C) harboring the plasmid pGEX4T encoding the desired protein was diluted in 500 ml LB_{amp} to an OD_{600} of 0.1 and grown to an OD_{600} of 0.6-0.8 at 30°C (for GST-Pex19, GST-Fis1cyt, GST) or 24°C (for GST-Fis1). Expression of the proteins was induced by adding 1 mM IPTG to the cultures. The cells were further incubated for 2 h at 30°C (for GST-Pex19, GST-Fis1cyt, GST) or 16 h at 14°C (for GST-Fis1). Then, cells were harvested by centrifugation (3000x g, 10 min, 10°C), resuspended in 15 ml lysis buffer and incubated for 45 min at 4°C and 12 rpm on an overhead-shaker. Sonification of 10 x 15 sec was applied to open the cells (Branson Sonifier, 80% duty cycle, output control 4). The lysate was clarified by centrifugation (15000x g, 15 min, 2°C) and loaded on a column (13.5 x 1.1 cm) with 2 ml settled glutathione sepharose (GE Healthcare). The sepharose was previously washed with 25 ml water and 20 ml GST basis buffer. The bacterial lysate was soaked through the column by gravity flow and the column was washed with 20 ml GST basis buffer. GST-proteins were eluted with 10 ml elution buffer. Fractions of 1 ml were collected and the protein concentration was determined. The three fractions with the highest protein content were pooled and 10% glycerol was added. Aliquots of 100 µl were snap-frozen in liquid nitrogen and stored at -80°C until use.

2.2.4.16 Extraction and analysis of phospholipids and ergosterol

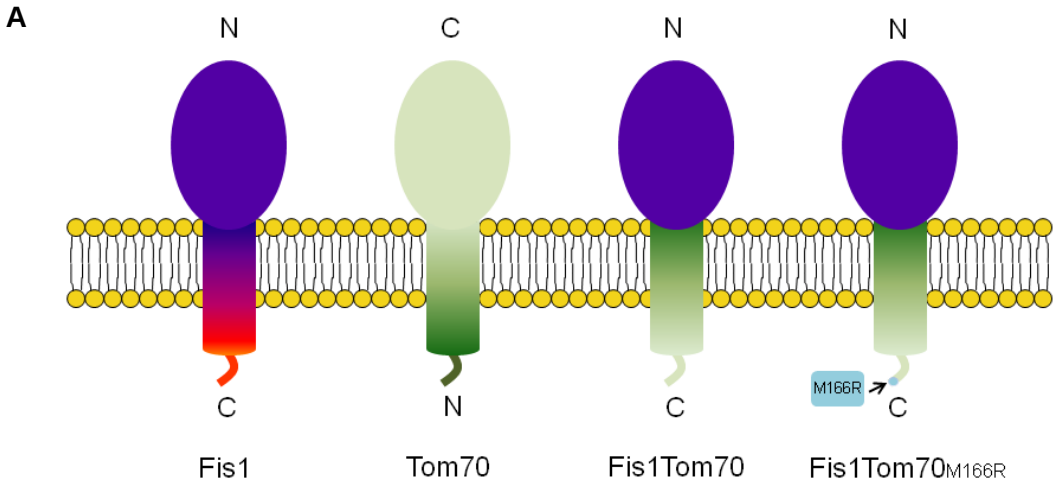
Lipids from purified mitochondria or microsomes were extracted and subjected to phosphate analysis as described (Brugger et al., 2000). Mass spectrometry analysis was performed in the laboratory of Dr. Britta Brügger, University of Heidelberg using the positive ion mode on a quadrupole time-of-flight mass spectrometer (QStar Elite, Applied Biosystems). Quantification of ergosterol was performed as described (Ejsing et al., 2009). Prior to extraction stigmasta-5,7,22-trienol was added as standard.

3 RESULTS

3.1 Cytosolic interactions partners of Fis1

3.1.1 The TMD of Tom70 can substitute the TMD of Fis1

Signal- and tail-anchored proteins share several topological features, the major difference is the opposite orientation of the termini. The targeting signal for both protein classes is located at their transmembrane domains and its flanking regions. In this study a hybrid protein of Fis1 and Tom70 was used to investigate if a transmembrane domain and its flanking regions of a signal-anchored protein can also serve as a targeting signal for a tail-anchored protein. For this purpose a Fis1-Tom70 chimera was constructed. It contained the cytosolic domain of Fis1 and the transmembrane domain and its flanking regions from Tom70 in reversed order. Thus, the original n-terminally located TMD of Tom70 became a C-terminal domain while the very N-terminal amino acids are still located in the mitochondrial IMS. In addition a variant of this chimera was made by mutation of the very last amino acid methionine into an arginine, which added an additional positive charge to the C-terminus of the protein (Fig. 3.1).



B Fis1Tom70 protein sequence

1 M T K V D F W P T L K D A Y E P L Y P Q

21 Q L E I L R Q Q V V S E G G P T A T I Q

41 S R F N Y A W G L I K S T D V N D E R L

61 G V K I L T D I Y K E A E S R R R E C L

81 Y Y L T I G C Y K L G E Y S M A K R Y V

101 D T L *F E H* E R N N K Q V G A L K S M V

121 E D K G P G K G R Q Q Q Q Q L Q N Y Y Y

141 Y A G I A T G T A A V T A L I A T K N R

161 T I F S K M/R

Fig. 3.1 The Fis1-Tom70 chimera proteins

(A) Model of the topologies of Fis1, Tom70 and the Fis1-Tom70 chimera proteins. (B) Protein sequence of the Fis1-Tom70 chimera proteins: straight black: sequence originating from the Fis1 cytosolic domain; gray italic: spacer; black underlined: sequence of Tom70 or $Tom70_{M166R}$ transmembrane domain in reversed order.

The chimera proteins Fis1-Tom70 and Fis1-$Tom70_{M166R}$ were expressed in *fis1Δ* yeast cells under control of the *FIS1* promotor and Fis1-Tom70 was also over-expressed under the control of the TPI promotor. Mitochondria were isolated from transformed yeast cells and subjected to a Proteinase K digestion and a carbonate extraction to evaluate the insertion state of the chimera proteins (Fig. 3.2). Both, Fis1-Tom70 and Fis1-$Tom70_{M166R}$ show a moderate sensitivity to Proteinase K which is consistent with the properties of WT Fis1. After carbonate extraction the chimerical proteins are found in the pellet fraction, thus both proteins behaved as integral membrane proteins. Fis1-Tom70 showed the same behavior when it was over-expressed. These results indicate that the chimerical Fis1-Tom70 proteins are targeted to and inserted into the mitochondrial outer membrane. Thus, the targeting signals of signal- and tail-anchored proteins share common features.

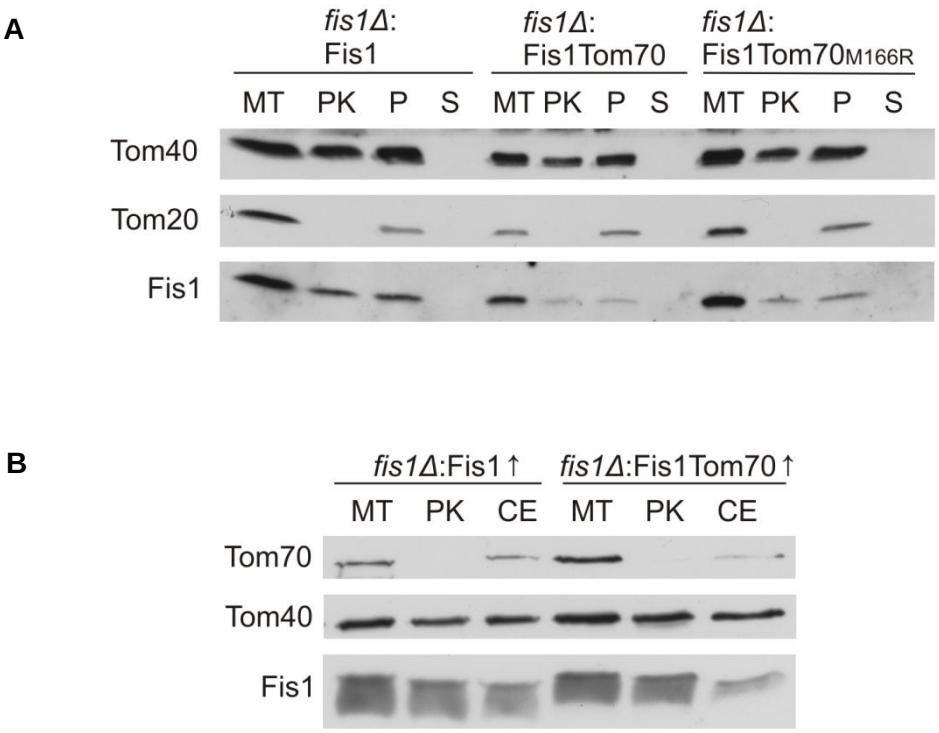


Fig. 3.2 The Fis1-Tom70 chimera proteins are integral proteins of the MOM

(A) Mitochondria were isolated from *fis1Δ* cells expressing Fis1, Fis1-Tom70 or Fis1-Tom70$_{M166R}$ under control of the *FIS1* promotor. The isolated organelles were subjected to Proteinase K treatment (PK) or carbonate extraction (S = soluble fraction, P = pellet / membrane fraction) or directly loaded (MT) on SDS-PAGE. Proteins were analyzed by immunodecoration with the indicated antibodies. (B) Isolated mitochondria from *fis1Δ* cells over-expressing either Fis1 or Fis1-Tom70 were subjected to Proteinase K treatment (PK) or carbonate extraction (CE = pellet / membrane fraction) or directly loaded (MT) on SDS-PAGE. Proteins were analyzed by immunodecoration with indicated the antibodies.

3.1.2 Fis1-Tom70$_{M166R}$ can functionally substitute native Fis1

Next, Fis1-Tom70 chimera proteins were checked for functionality by introducing them into cells lacking Fis1 and expressed mtGFP. The mitochondrial morphology was analyzed by fluorescence microscopy to test wether the chimera proteins are able to rescue the hyperfused mitochondrial *fis1Δ* phenotype (Fig.3.3). While the Fis1-Tom70 containing cells showed a typical *fis1Δ* phenotype, cells expressing the Fis1Tom70$_{M166R}$ cells showed tubular WT-like mitochondria. These findings indicate that not only a functional N-terminal cytosolic Fis1 domain but also the right number of positive

charges of the very C-terminus of the protein is essential for the protein´s function in mitochondrial fission.

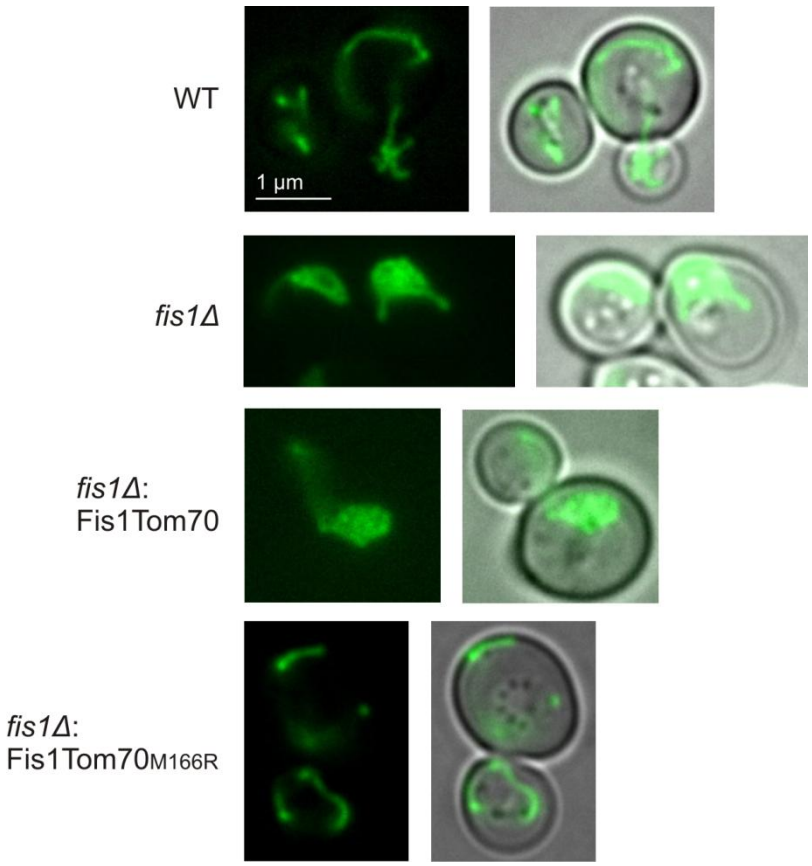


Fig. 3.3 Fis1-Tom70$_{M166R}$ can rescue the *fis1Δ* phenotype

WT, *fis1Δ* and *fis1Δ* cells expressing either Fis1-Tom70 or Fis1-Tom70$_{M166R}$ were transformed with a plasmid encoding mtGFP. Mitochondrial morphology was analyzed by fluorescence microscopy.

Taken together, these results imply that the TMD and its flanking regions of a signal-anchored protein can also function as targeting signal for a TA protein and lead to a correct membrane insertion of the proteins. Moreover Fis1-Tom70$_{M166R}$ with its positively charged C-terminus can also take over the functions of Fis1 in mitochondrial fission.

3.1.3 Overexpression of Pex19 stabilizes Fis1

Fis1 exhibits a dual localization to mitochondria and peroxisomes in yeast and mammalian cells. It was reported that human hFis1 interacts with Pex19, the major import chaperone for peroxisomal membrane proteins (Delille and Schrader 2008). Thus, it might be postulated that Pex19 stabilizes Fis1 molecules that are destined to either peroxisomes or mitochondria. In this study a possible interaction of Fis1 and Pex19 in yeast cells was investigated.

Wildtype cells (W303α) and cells over-expressing Pex19 were grown on either glucose- or oleate-containing medium. Growth on oleate as a carbon source induces proliferation of peroxisomes in yeast while there are barely any peroxisomes in cells grown on glucose. Whole cell lysates of both yeast strains were prepared and divided into a crude mitochondrial fraction (that probably also contained the peroxisomes) and a post-mitochondrial fraction, considered as cytosol. The samples were analyzed by SDS-PAGE and Western blotting (Fig. 3.4). Pex19 could only be detected under overexpression conditions. Thus normal Pex19 levels are too low to be detected with the used antibody. It was observed that growth on oleate medium resulted in higher Fis1 steady-state levels as compared to those under growth on glucose. This is probably due to a stronger requirement for Fis1 by additional peroxisomes. Furthermore, overexpression of Pex19 caused higher Fis1 steady-state levels. This effect is more pronounced in cells grown on glucose than on oleate (Fig. 3.4). These increased levels support the notion that Pex19 acts as a chaperone for Fis1 during its passage through the cytosol and stabilizes Fis1 in an import-competent state. The stabilizing effect of Pex19 may be less pronounced in cells growing on oleate because Fis1 might be imported faster due to an increased need for the protein in peroxisomes and the Fis1 levels in cells with normal expression levels of Pex19 are already higher than in cells grown on glucose.

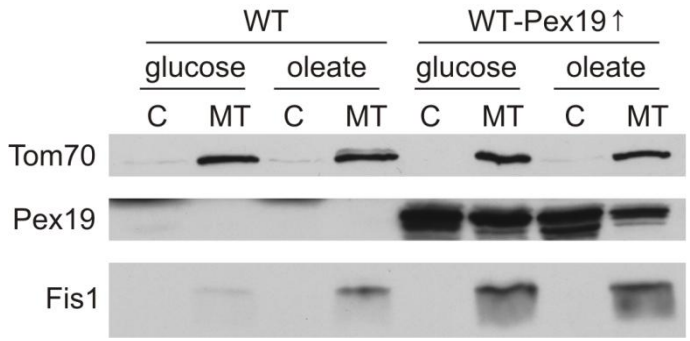


Fig. 3.4 Overexpression of Pex19 stabilizes Fis1 in *S. cerevisiae*

Wild-type (W303α) cells and WT cells over-expressing Pex19 were grown in glucose- or oleate-containing media. Crude mitochondria (MT) and cytosol (C) were isolated and analyzed by SDS-PAGE and immunodecoration with the indicated antibodies.

3.1.4 Deletion of Pex19 does not affect the steady-state levels of Fis1

As overexpression of Pex19 leads to increased steady-levels of Fis1 in yeast, it was investigated wether deletion of the protein would cause an opposite effect. For this purpose *PEX19* was deleted and crude mitochondria were prepared from both WT (W303α) and *pex19Δ* cells grown on YPD medium. No differences in the steady-state levels could be observed between the WT and the deletion strain (Fig. 3.5). Thus Pex19 appears not to be essential for the biogenesis of Fis1. It is very likely that in the absence of Pex19 other chaperones are able to keep Fis1 in an import-competent state during its passage through the cytosol.

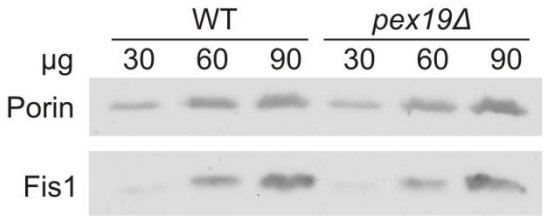


Fig. 3.5 Fis1 steady-state levels are not reduced in *pex19Δ*

Mitochondria from WT (W303α) and *pex19Δ* cells were isolated and the indicated amounts were subjected to SDS-PAGE analysis followed by Western blot using antibodies against the indicated proteins.

3.1.5 Pex19 does not improve the *in vitro* import of Fis1

To investigate the role of Pex19 in import of Fis1 into mitochondria GST-Pex19 (or GST alone as control) were purified from bacterial lysate and added to *in vitro* import reactions. ^{35}S-Fis1-TMC was imported into isolated WT mitochondria in presence of GST-Pex19 or GST. Import of Fis1-TMC was analyzed by an IASD-assay, SDS-PAGE and autoradiography (Fig. 3.6). Addition of GST-Pex19 did not influence the import efficiency in comparison to the addition of GST. Thus Pex19 does not seem to influence the import of Fis1-TMC into mitochondria, at least under the employed *in vitro* conditions. This absence of effect by addition of Pex19 suggests that the protein probably does not bind Fis1-TMC and thus neither stabilizes the Fis1 newly synthesized molecules nor competes off any other component. The rabbit reticulocyte lysate contains a certain set of mammalian chaperones that might interact with Fis1-TMC. These interactions could mask possible effects of Pex19 on Fis1-TMC under these conditions.

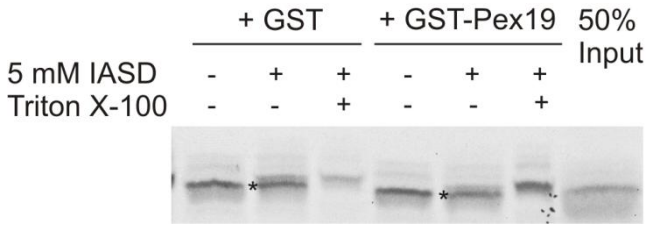


Fig. 3.6 GST-Pex19 does not affect the *in vitro import* of Fis1-TMC

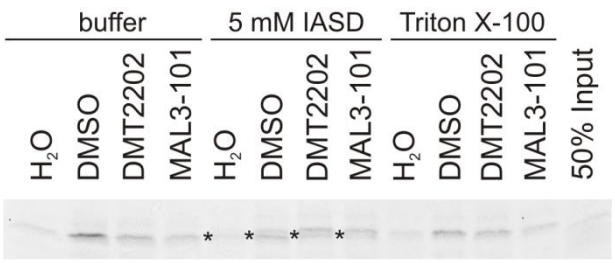
GST and GST-Pex19 were purified from bacterial lysates. Fis1-TMC was imported into isolated WT mitochondria in the presence of either GST (3.3 µg) or GST-Pex19 (10 µg), followed by an IASD-assay and SDS-PAGE. The import efficiency was monitored by autoradiography. Bands resulting from correctly inserted Fis1-TMC are marked by asterisks.

Taken together, these findings demonstrate that Pex19 has a stabilizing effect on Fis1. However, it is not essential for the biogenesis of Fis1 into yeast mitochondria.

3.1.6 Import of Fis1 is influenced by inhibitors of the Hsc70/Hsp40 family

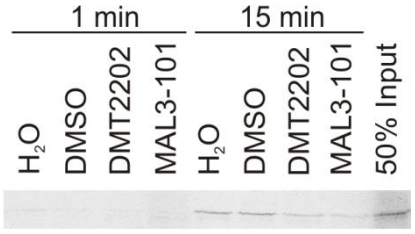
Due to its transmembrane region Fis1 is a hydrophobic protein that can easily aggregate in an aqueous environment like the cytosol. As Fis1 inserts into its membrane in a post-translational manner, the protein has to be kept in a non-aggregated state upon its release from the ribosome. The aforementioned results suggest that Pex19 might stabilize newly synthesized Fis1 molecules but this chaperone is not crucial for the biogenesis and / or function of Fis1. Chaperones of the Hsc70/Hsp40 family are alternative candidates for factors that keep Fis1 in an import competent conformation. To investigate a possible involvement of Hsc70/Hsp40 chaperones in the *in vitro* import of Fis1, import experiments with small molecule Hsp-inhibitors, Mal3-101 or DMT0022200, were performed. These inhibitors were shown to affect the involvement of Hsc70/Hsp40 chaperones in the membrane integration of ER TA proteins (Rabu et al 2008). The ^{35}S-Fis-TMC containing rabbit reticulocyte lysate was treated with one of the two Hsp-inhibitors and its import efficiency was monitored. As controls *in vitro* reaction with Aac that was reported to depend on Hsc70/Hsp40 (Young et al. 2003) and with pSU9-DHFR that is not known to require Hsc70/Hsp40 were performed under the same conditions. Of note, the import effiency of Fis1-TMC was decreased upon treatment with Hsp-inhibitors to about 70-60% in the case of Mal3-101 and to about 80% in the case of DMT0022200 (Fig. 3.7 A, D and E). As expected, import efficiency of Aac was reduced by 40% upon treatment with Hsp-inhibitors, while import with pSU9-DHFR was not affected by the presence of Mal3-101 and only slightly reduced to around 90% in the presence of DMT0022200 Fig. 3.7 B, C and D). These results show that import of Fis1 is hampered, but not completely inhibited by the absence of functional Hsc70/Hsp40-complexes. This might be due to residual functional Hsc70/Hsp40 after treatment with the inhibitors or other cytosolic factors can take over the role of the Hsc70/Hsc40 complexes. This would be consistent with the fact that Aac import is also only reduced to about 60% and not completely abolished, although its dependency on Hsc70/Hsp40 was shown before. Alternatively, it might well be that a sub-population of precursors of these proteins can keep their import competence also independently of the chaperone´s activity.

A



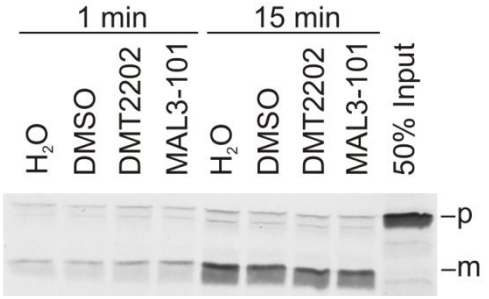
Fis1-TMC

B



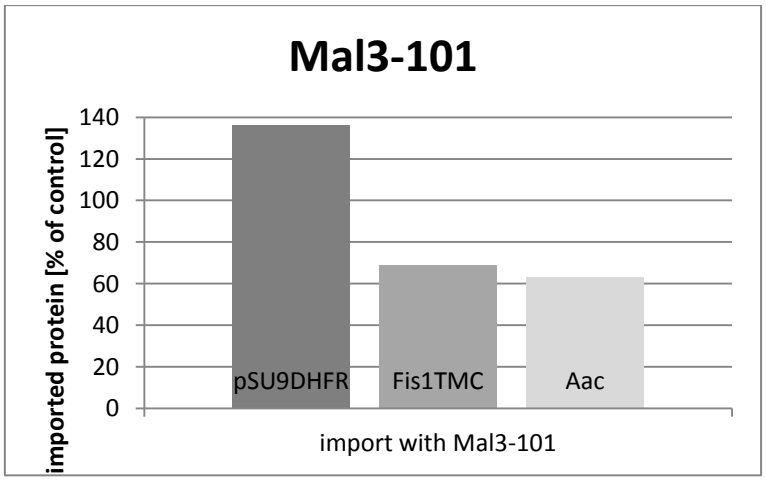
Aac

C



pSU9-DHFR

D



E

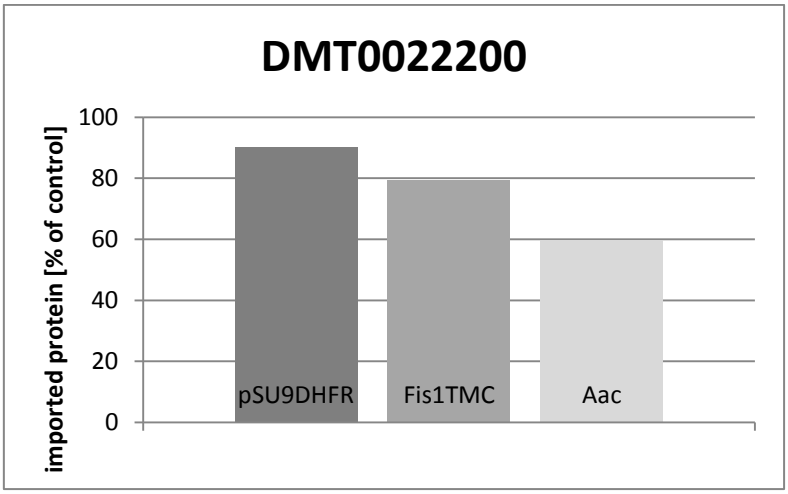


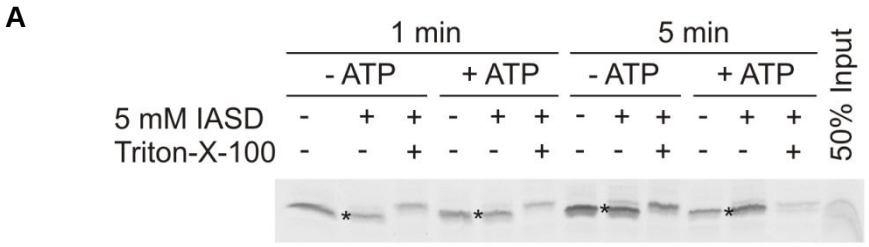
Fig. 3.7 *In vitro* import of Fis1-TMC is reduced in the absence of functional Hsc70/Hsp40 chaperones

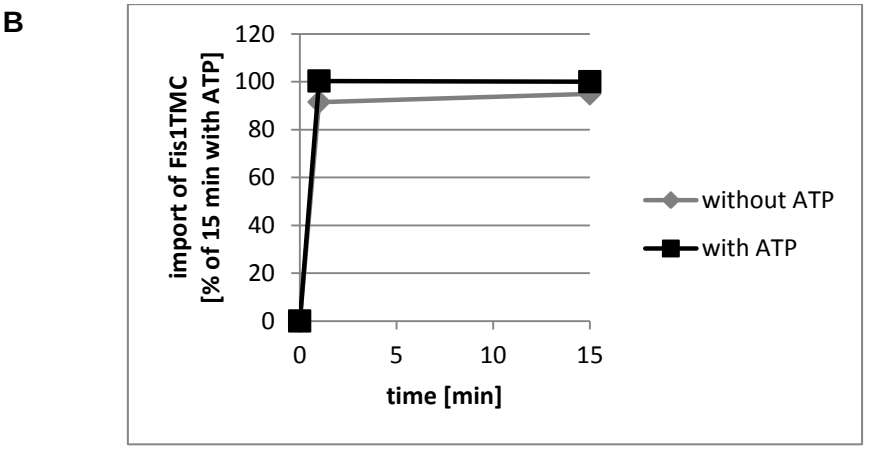
(A) Radiolabeled Fis1-TMC was imported into isolated WT mitochondria in the presence or absence of 250 µM small molecule Hsp-inhibitors or DMSO as a control followed by an IASD-assay, SDS-PAGE and autoradiography. Bands representing imported Fis1-TMC are marked by asterisks. (B) Radiolabeled Aac was imported into isolated WT mitochondria in the presence or absence of 250 µM small molecule Hsp-inhibitors, followed by Proteinase K treatment, SDS-PAGE and autoradiography. (C) Radiolabeled pSU9-DHFR was imported into isolated WT mitochondria in the presence or absence of 250 µM small molecule Hsp-inhibitors, followed by Proteinase K treatment, SDS-PAGE and autoradiography (p = precursor, m = mature protein).(D) Import efficiencies for pSU9-DHFR, Fis1-TMC and Aac in the presence of Mal3-101 in comparison to DMSO control. (E) Import efficiencies for pSU9-DHFR, Fis1-TMC and Aac in the presence of DMT0022200 in comparison to DMSO control.

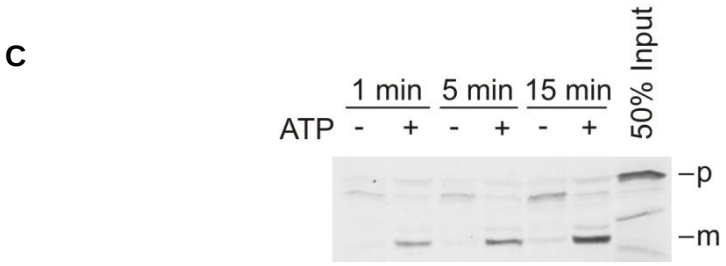
3.1.7 *In vitro* import of Fis1-TMC does not require ATP

Chaperones of the Hsp70 family are known to associate with their substrate protein in an ATP-dependent manner. It is currently unclear wether the import of Fis1 into mitochondria requires ATP. In this study this question was examined by *in vitro* import experiments. For this purpose radiolabeled Fis1-TMC was imported into isolated WT mitochondria in the presence or absence of ATP. To deplete ATP, mitochondria and rabbit reticulocyte lysate were treated with apyrase before the import reaction. The import efficiency of Fis1-TMC in the absence of ATP was comparable to its import with ATP (Fig. 3.8 A and B). ^{35}S-pSU9-DHFR was used as a control precursor protein since it is known that the import of this precursor protein is strongly dependent on ATP because of utilizing the ATP-dependent import motor at the inner mitochondrial membrane. Indeed import of pSU9-DHFR was strongly reduced in the absence of ATP

(Fig. 3.8 C and D). These findings show that Fis1-TMC can be efficiently imported into mitochondria in absence of considerable amounts of ATP. However, it cannot be excluded that minor amounts of ATP are left after apyrase treatment and that this minor amounts might be sufficient for Fis1 import, for example for the release of Fis1-TMC from chaperones that keep it in an import competent state.







D

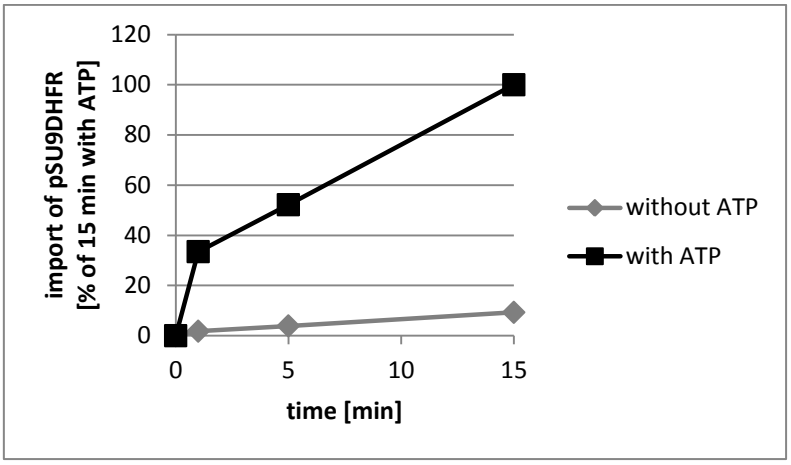


Fig. 3.8 *In vitro* import of Fis1-TMC is independent of ATP

(A) Fis1-TMC was imported into isolated WT mitochondria in the presence or absence of ATP followed by an IASD-assay, SDS-PAGE analysis and autoradiography. Bands resulting from imported Fis1TMC are marked by asterisks. (B) Bands of imported material from representative experiments like the one presented in part A were quantified. The intensity of bands representing imported material into mitochondria for the longest time period in the presence of ATP was set as 100% (C) pSU9-DHFR was imported into isolated WT mitochondria in the presence or absence of ATP, followed by Proteinase K treatment, SDS-PAGE analysis and autoradiography (p = precursor, m = mature protein). (D) Bands representing mature protein from representative experiments like the one presented in part C were quantified. The intensity of bands representing imported material into mitochondria for the longest time period in the presence of ATP was set as 100%.

3.1.8 *In vivo* site-directed photo-crosslinking of Fis1 reveals interactions with Ssa2 and Porin1

So far nothing is known about cytosolic interaction partners of Fis1 that might assist its targeting to mitochondria. To address this issue the method of *in vivo* site-directed photo-crosslinking was applied. A Fis1 mutant was engineered that carries an amber stop codon (TAG) instead of codon 139. For eventual pulldown experiments an N-terminal 3xHA-tag was added. The position of the amber stop codon lies within the putative transmembrane region because the aim was to search for putative targeting factors that bind to the signal region of Fis1. This construct was transformed into a yeast strain expressing an amber suppressor tRNA and its cognate aminoacyl-tRNA synthetase which charges the tRNA with Bpa. In this system a 3xHA-Fis1 with a Bpa residue in position 139 can be expressed and cross-linked to putative interaction partners. Before starting the crosslink experiments the functionality of the modified Fis1

was tested by fluorescence microscopy. A *fis1Δ* strain that was transformed with the tRNA system, the 3xHA-Fis1-139Bpa and an mtRFP (to stain mitochondria) showed wildtype-like mitochondrial tubular morphology (Fig. 3.9). Thus, the modified Fis1 can rescue the *fis1Δ* phenotype and is therefore functional and correctly localized.

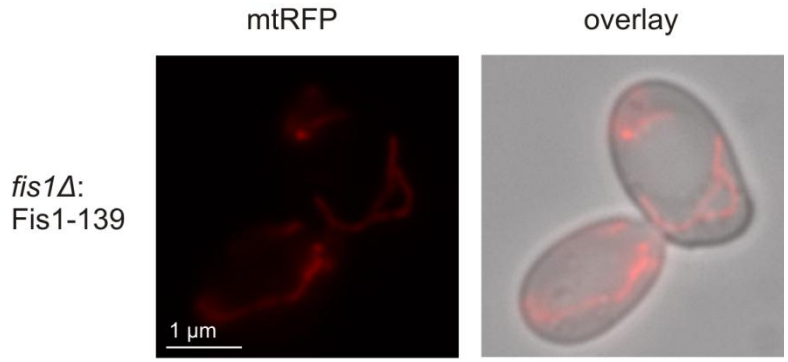


Fig. 3.9 3xHA-Fis1-139 can rescue the *fis1Δ* phenotype

fis1Δ cells expressing 3xHA-Fis139-Bpa were transformed with a plasmid for expression of mtRFP. Mitochondrial morphology was analyzed by fluorescence microscopy.

Next, cells were illuminated with UV-light and whole cell lysate from illuminated and non-treated cells was prepared. The lysate was then subjected to a pulldown via the 3xHA-tag to enrich cross-linked species and to separate them from other proteins. Samples containing the bound proteins were analyzed by SDS-PAGE and Western blotting or silver staining (Fig. 3.10). The Western blot with an antibody against Fis1 revealed a reproducible pattern of crosslink bands at higher molecular weight. The silver stain gel showed a high background of unspecific bands (similar bands in –UV and +UV lanes). Specific bands corresponding to those bands observed by the immunodecoration could not be observed probably because they were too weak to be detected. To overcome this problem the positions of the bands were estimated according to the Western blot and they were cut out from the silver gel and their protein content was analyzed by mass spectrometry (in collaboration with the Proteome Centre Tübingen). Among the proteins that were detected only in the UV-illuminated samples were Ssa1/2 and Porin1. Ssa1/2 are members of the cytosolic Hsp70 chaperones family whereas Porin1 is a very abundant general metabolite transporter in the outer mitochondrial membrane. The Hsp70 family proteins Ssa1 and Ssa2 share sequence

identity of 98%. The high sequence similarity between the two proteins does not allow a unique identification of the cross-linked adduct. Until now no interactions between Fis1 and either Ssa2 or Porin1 were reported.

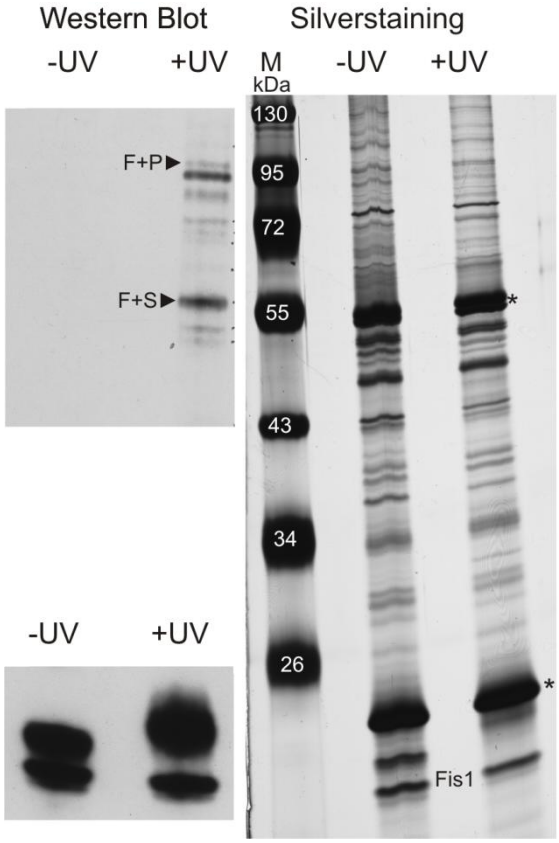


Fig. 3.10 Pulldown of 3xHA-Fis1-139 and cross-linked species

3xHA-Fis139 was expressed in WT cells and covalently cross-linked to putative interaction partners upon irradiation with UV-light. Control cells were kept in the dark without UV-light. Whole cell lysate of irradiated and control cells was prepared and proteins were pulled down via resin loaded with HA antibody. Eluted proteins were subjected to SDS-PAGE and analyzed by Western blotting with a Fis1 antibody or by silver staining of the gel. Bands resulting from cross-linked Porin and Fis1 (F+P) and cross-linked Ssa2 and Fis1 (F+S) are marked with arrowheads. Bands resulting from the heavy and light chains of the HA-antibody in the silver stained are marked by asterisks.

3.1.9 Steady-state levels of Fis1 are reduced in the absence of Ssa family chaperones

The aforementioned pulldown experiments showed a possible interaction of Fis1 with Ssa1 and/or Ssa2. Ssa1 and 2 are two of four Ssa proteins in yeast. Whereas both are constitutively expressed the other two memembers in the group, Ssa3 and Ssa4 are mainly expressed as part of a stress response (SGD Database). To investigate the role of Ssa proteins in the biogenesis of Fis1 the steady-state levels of Fis1 in cells mutated in *SSA1-4* were checked. As a knockout of all four Ssa proteins is lethal, a strain with a chromosomal deletion for *SSA2-4* and a thermosensitive allele of *SSA1* was used (kind gift from O. Pines). Cells were grown under permissive (24°C) and non-permissive (37°C) conditions for three hours and crude mitochondria were isolated from both strains and analyzed by SDS-PAGE and Western blotting (Fig. 3.11). Under permissive conditions the steady-state levels for Fis1 are similar in both strains. Thus one functional Ssa protein, in this case Ssa1, is enough to maintain the biogenesis of Fis1. Under non-permissive conditions at 37°C the steady-state levels of Fis1 were elevated in both strains in comparison to 24°C (Fig. 3.11). This is probably due to an increased need for Fis1 under stress conditions as mitochondria tend to fragment in stressed yeast cells. Importantly the steady-state levels of Fis1 at the non-permissive temperature are reduced in the strain containing the ts allele of *SSA1* in comparison to the control under these conditions. This indicates that at least one Ssa protein is required for optimal biogenesis of Fis1. Of note, residual levels of Fis1 are observed in the mutated cells suggesting that Ssa proteins are not absolutely required for Fis1 biogenesis. These results are in line with the observation that inhibition of Hsc70/Hsp40 complexes hinders the import of Fis1 into mitochondria under *in vitro* conditions. Taken together, these results support the idea of an involvement of Ssa chaperones in the biogenesis of Fis1.

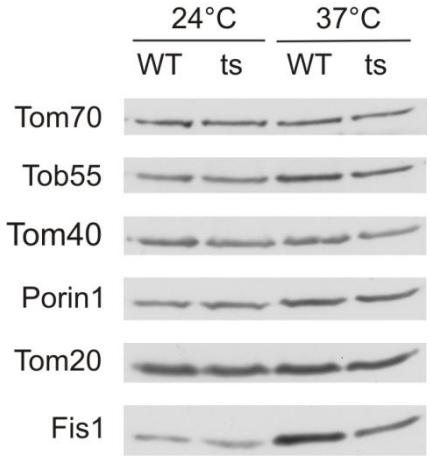


Fig. 3.11 Ssa family proteins are involved in the biogenesis of Fis1

ssa2-4Δ strains harboring either a native or thermosensitive allele of *SSA1* were grown under permissive (24°C) or non-permissive conditions (37°C). Crude mitochondria were isolated and subjected to SDS-PAGE and immunodetection with the indicated antibodies.

3.1.10 The interaction of Fis1 with Porin1 does not influence the biogenesis of Fis1

Next, I intended to verify the possible interaction of Fis1 and Porin1 that was indicated by the pulldown experiments and the mass spectrometry. To this goal 3xHA-Fis1-139 was expressed and cross-linked in WT, *por1Δ* and WT cells expressing 3xFlag-Por1. A strong crosslink adduct at about 53 kDa could be detected with a Fis1 antibody in the WT cells (Fig. 3.12). The size of the bands fits nicely to the calculated size of a crosslink adduct of Fis1 with Por1 (23 + 30 kDa). Moreover this specific band is not detected in cells lacking Porin1. Further supporting the identity of the adduct is the observation that in cells expressing 3xFlag-Porin1 the crosslink band was shifted to app. 60 kDa and was detected by antibodies either against Fis1 or the Flag-tag. These findings prove that Porin1 can indeed be cross-linked *in vivo* to Fis1.

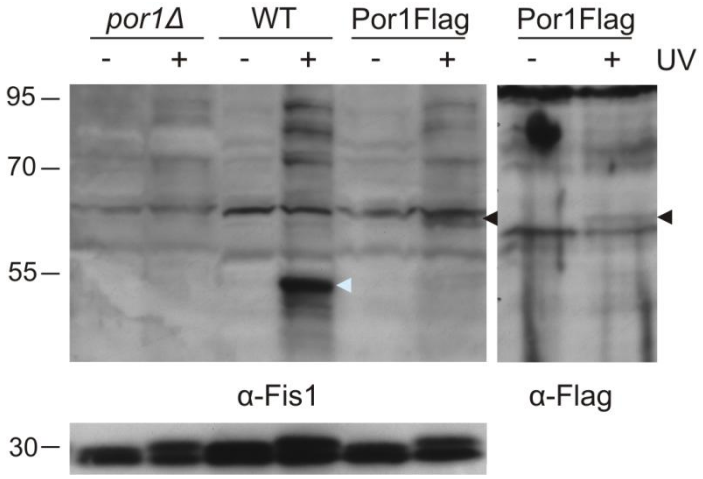


Fig. 3.12 Porin1 can be cross-linked to Fis1 *in vivo*

3xHA-Fis1-139 was expressed in WT, *por1Δ* and WT cells expressing Flag-tagged Porin1. After UV-crosslinking whole cell lysate was prepared from cross-linked and control cells and subjected to SDS-PAGE. Proteins were analyzed by immunodecoration with antibodies against Fis1 and Flag. The cross-linking adduct of Fis1 and Porin1 is marked with a white arrowhead and the adduct of Fis1 with Flag-Por1 with a black arrowhead.

To further investigate the physiological importance of Porin1 to Fis1 biogenesis steady-state levels of Fis1 in *por1Δ* and *por1Δpor2Δ* cells were compared to their corresponding WT strains. In the M3 background Fis1 was reduced in *por1Δ* mitochondria and the effect was even more severe in the double deletion strain (Fig. 3.13). However these results could not be confirmed in the W303α background which was used for the majority of the experiments in this work (data not shown).

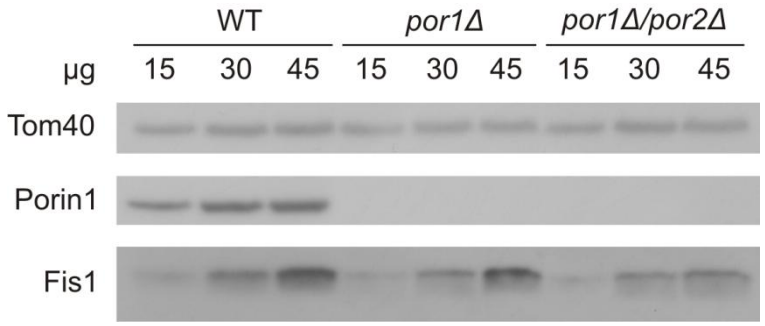


Fig. 3.13 Fis1 is reduced in *por1Δ* and *por1Δpor2Δ* in M3 background

Pure mitochondria were isolated from WT (M3), *por1Δ* and *por1Δpor2Δ* cells. The indicated amounts of proteins were analyzed by SDS-PAGE, Western Blotting and immunodetection.

To further study the potential role of Porin1 in import of Fis1 *in vitro* import experiments were performed with mitochondria isolated from WT, *por1Δ* and *por1Δpor2Δ*. ^{35}S-labeled Fis1-TMC and pSU9-DHFR (as control) were imported into the isolated mitochondria (Fig. 3.14). Deletion of Porin1 or both Porin1 and Porin2 did not decrease significantly the import efficiency of Fis1-TMC. Interestingly the import of pSU9-DHFR was largely reduced in the Porin1 deletion strains. This is probably due to a disturbed membrane potential of the inner membrane in these cells.

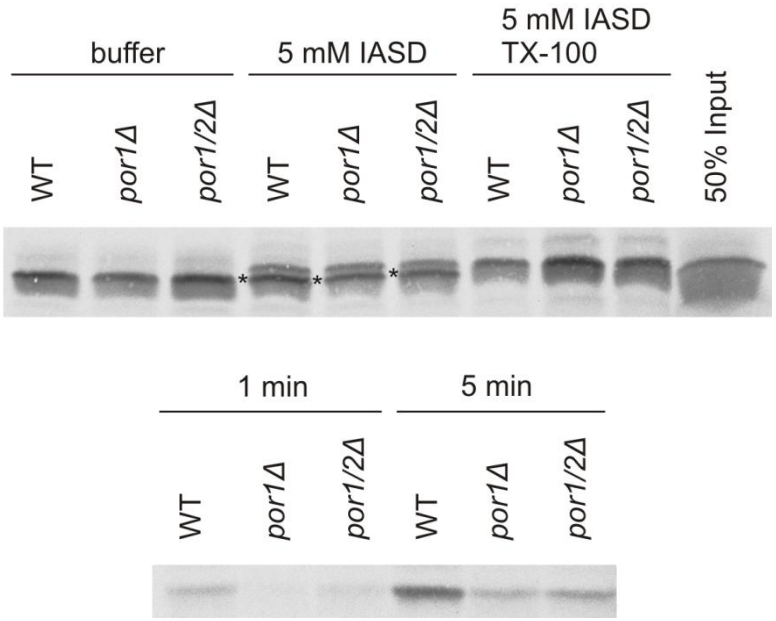


Fig. 3.14 Deletion of Porin does not influence the *in vitro* import of Fis1-TMC

(A) Radiolabeled Fis1-TMC was imported into isolated W303α, *por1Δ* and *por1Δpor2Δ* mitochondria, followed by an IASD-assay, SDS-PAGE analysis and autoradiography. Bands resulting from imported Fis1-TMC are marked by asterisks. (B) Radiolabeled pSU9-DHFR was imported into mitochondria as in part, followed by Proteinase K treatment, SDS-PAGE and autoradiography.

Taken together, a physiological role of the potential interaction between Fis1 and Porin1 could not be determined in this study. It cannot be excluded that the interaction of Fis1 and Porin1 as observed in the cross-link experiments results from the overexpression of Fis1 and the fact that Porin1 is the most abundant protein in the MOM.

3.2 The lipid composition of the MOM is sufficient as recognition criterion for tail-anchored proteins

3.2.1 Deletion of the P-type ATPase Spf1 causes mis-localization of mitochondrial TA-proteins to ER

A genetic screen performed by the lab of Maya Schuldiner at the Weizmann Institute of Science (Rehovot, Israel) revealed that the GFP-tagged mitochondrial TA-protein Gem1 is mis-localized to ER structures in a *spf1Δ* strain. Spf1 is a P-type ATPase resident in the ER membrane with functions in calcium homeostasis and ergosterol biosynthesis (Cronin et al. 2000, Cronin et al. 2002). In this study it was investigated if in addition to Gem1 also Fis1 as another TA-protein is affected. For this purpose a BY4741 WT and a *spf1Δ* deletion strain were transformed with Cherry-Fis1 and mtGFP and analyzed by fluorescence microscopy (Fig. 3.15). The wildtype strain showed co-localization of the signals for Cherry-Fis1 and mtGFP indicating that Fis1 is solely localized to mitochondria. In contrast, Cherry-Fis1 was not completely co-localized with mtGFP in the deletion strain but rather was also located to mitochondrial and ER structures. These findings confirm that Fis1 is partially mis-localized to ER structures upon deletion of Spf1.

The mis-localization of Fis1 could also be detected by biochemical methods. WT (W303α) and *spf1Δ* cells were subjected to subcellular fractionation and isolated mitochondria and ER were analyzed by SDS-PAGE and Western blotting (Fig. 3.16). Whereas in ER from wildtype cells no Fis1 signal was observed, a clear Fis1 signal was visible in ER form *spf1Δ* cells. The absence of the abundant mitochondrial marker protein Porin (Por1) in the ER fraction excludes the possibility that microsomes from the *spf1Δ* cells were contaminated by mitochondrial proteins. These results are consistent with the observations with the fluorescence microscopy and indicate that the deletion of Spf1 has a general effect on the localization of mitochondrial TA-proteins.

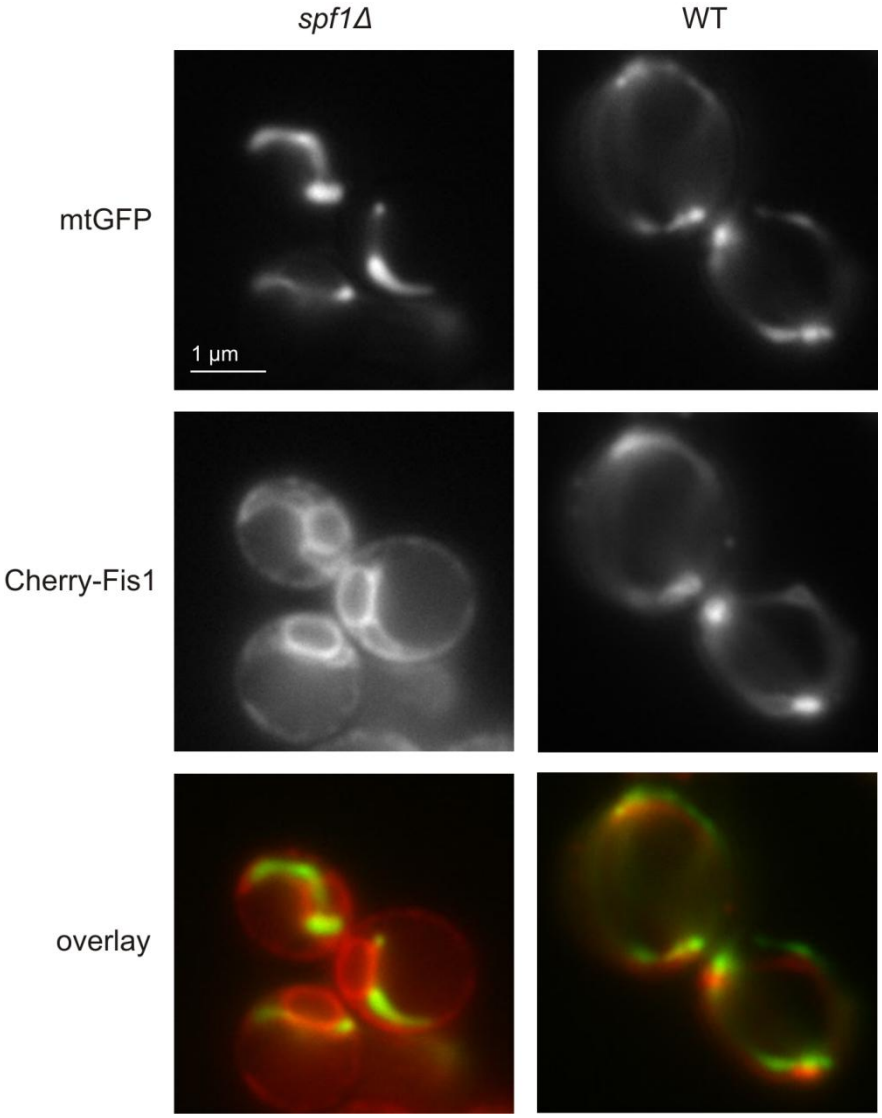


Fig. 3.15 Fis1 is mis-localized to ER structures in *spf1Δ*

WT and *spf1Δ* cells were transformed with plasmids encoding Cherry-Fis1 and mtGFP. The cellular localization of Cherry-Fis1 was visualized by fluorescence microscopy.

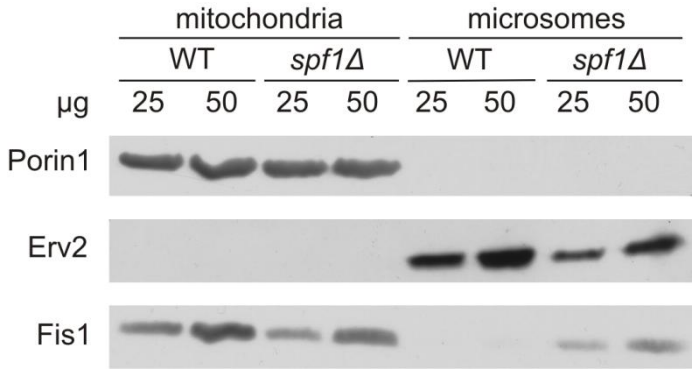
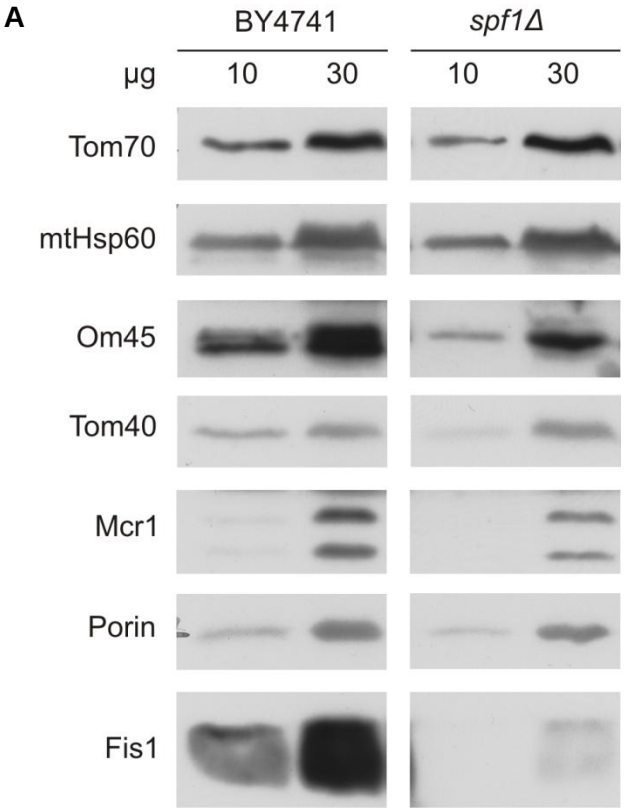


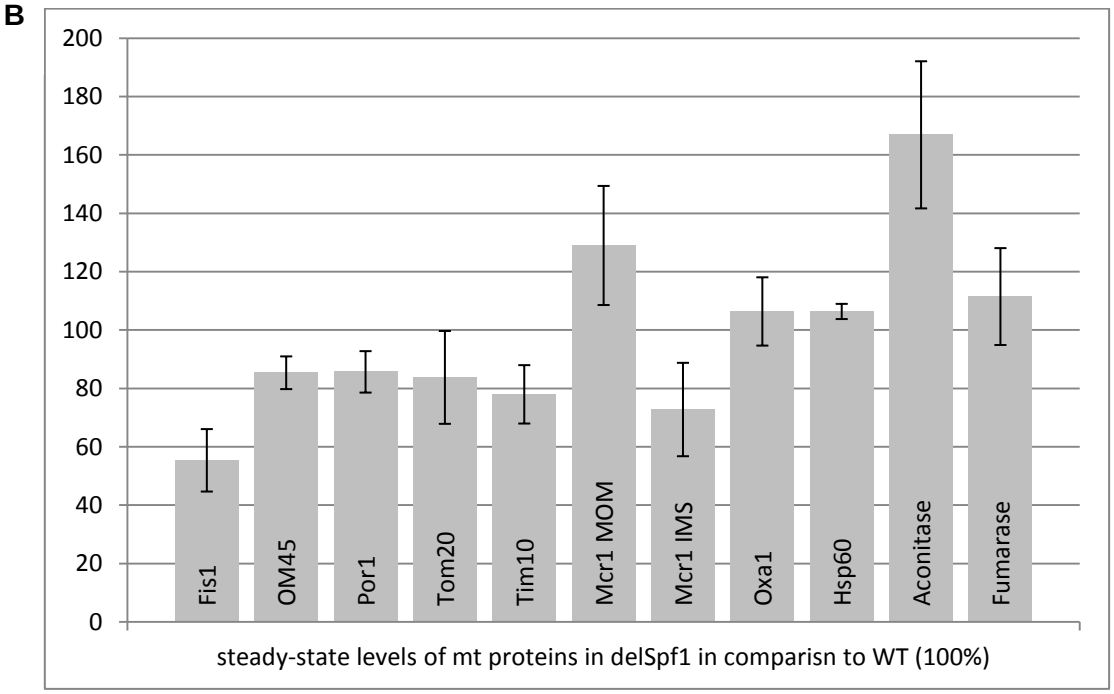
Fig. 3.16 Fis1 can be detected in the ER fraction of *spf1Δ* cells

Mitochondria and microsomes were isolated from WT (W303α) and *spf1Δ* cells and subjected to SDS-PAGE and Western blotting with the indicated antibodies.

3.2.2 Fis1 and OM45 steady-state levels are reduced in *spf1Δ* mitochondria

To further investigate the effect of Spf1 deletion on mitochondrial protein biogenesis, mitochondria from two *spf1Δ* strains and their corresponding wildtype strains, BY4741 and W303α were isolated and steady-state levels of several mitochondrial proteins were analyzed by SDS-PAGE and Western blotting (Fig. 3.17). The steady-state levels of most of the examined mitochondrial proteins were not significantly altered upon the deletion of Spf1 in both backgrounds. Some proteins showed increased or decreased levels in *spf1Δ* mitochondria only in one background. Importantly, only Fis1 and Om45 showed a major decrease in their steady-state levels in *spf1Δ* mitochondria of both backgrounds. Also the IMS form of Mcr1 was decreased in both backgrounds, but the reduction was not as pronounced as for Fis1 and OM45. These effects were most pronounced for mitochondria isolated from cells in the stationary growth phase (Fig. 3.17). Reduction of OM45 steady state levels showed that the deletion of Spf1 not only affects mitochondrial TA-proteins but also signal-anchored proteins.


A
BY4741
spf1Δ
µg
10
30
10
30
Tom70
mtHsp60
Om45
Tom40
Mcr1
Porin
Fis1


B
200
180
160
140
120
100
80
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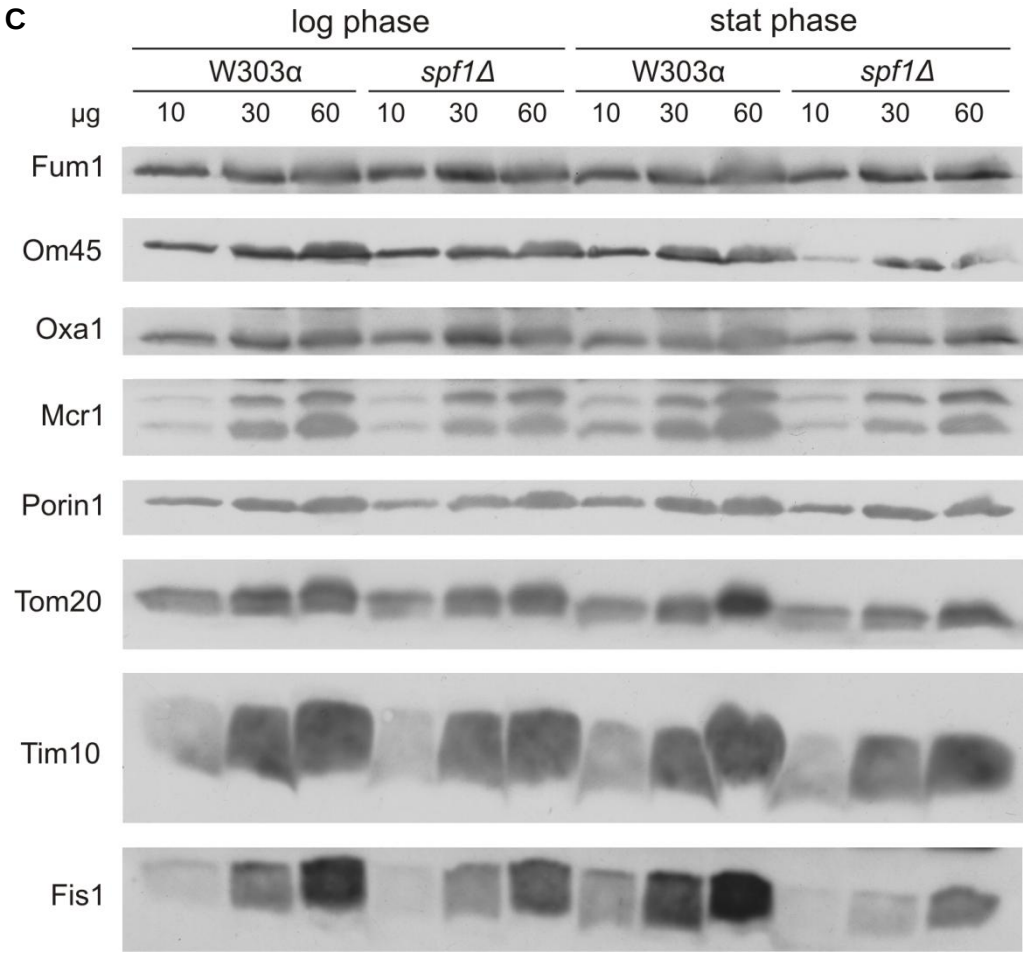
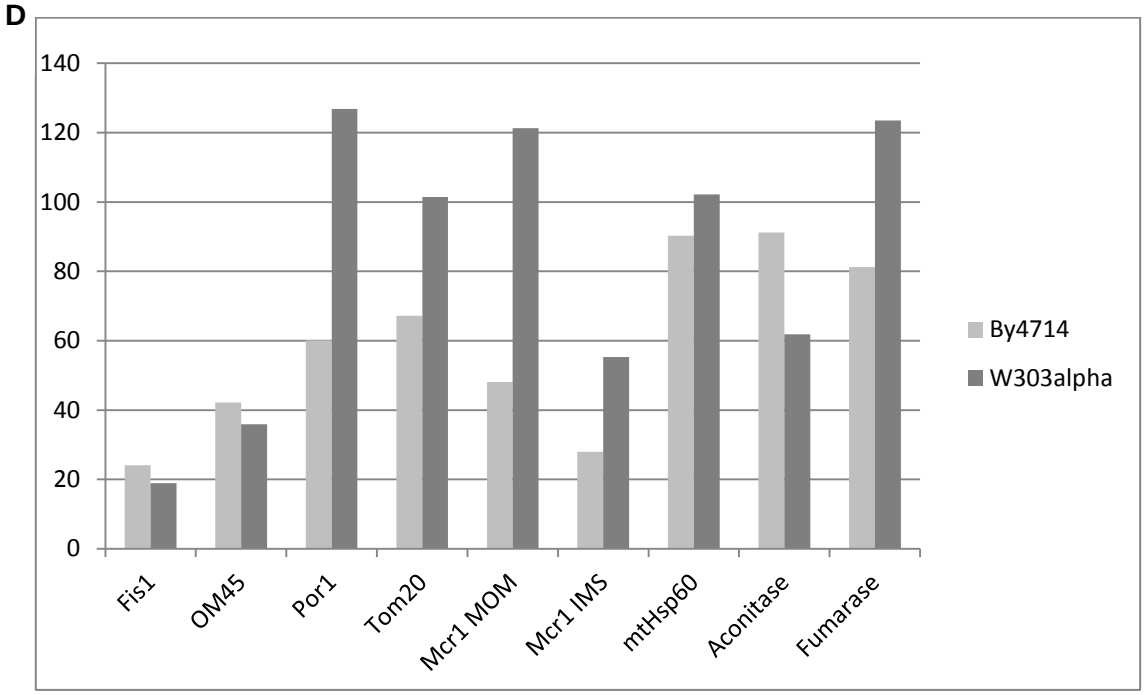
40
20
0
Fis1
OM45
Por1
Tom20
Tim10
Mcr1 MOM
Mcr1 IMS
Oxa1
Hsp60
Aconitase
Fumarase
steady-state levels of mt proteins in delSpf1 in comparisn to WT (100%)


C
log phase
stat phase
W303α
spf1Δ
W303α
spf1Δ
µg
10 30 60 10 30 60 10 30 60 10 30 60
Fum1
Om45
Oxa1
Mcr1
Porin1
Tom20
Tim10
Fis1


D
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120
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80
60
40
20
0
Fis1
OM45
Por1
Tom20
Mcr1 MOM
Mcr1 IMS
mtHsp60
Aconitase
Fumarase
By4714
W303alpha

Fig. 3.17 Steady-state levels of mitochondrial proteins in *spf1Δ*

(A) Steady-state levels of mitochondrial proteins in *spf1Δ* in BY4741 background. Mitochondria from early stationary BY4741 and *spf1Δ* cells were isolated and subjected to SDS-PAGE and Western blotting with the indicated antibodies. (B) Steady-state levels of mitochondrial proteins of *spf1Δ* in BY4741 background. Values are based on at least three independent experiments for each protein. Levels in WT were set to 100%. (C) Steady-state levels of mitochondrial proteins in *spf1Δ* in W303α background. Mitochondria from W303α and *spf1Δ* cells grown to logarhythmic or early stationary phase were isolated and subjected to SDS-PAGE and Western blotting with the indicated antibodies. (D) Comparison of steady-state levels of mitochondrial proteins in *spf1Δ* in the BY4741 and W303α background.

3.2.3 OM45 is partially mis-localized to ER structures in *spf1Δ* cells

Reduced steady-state levels of OM45 in *spf1Δ* mitochondria gave a hint that besides mitochondrial TA-proteins also the signal-anchored protein OM45 could be mis-localized to ER structures in a Spf1 deletion strain. To investigate this issue, *spf1Δ* cells were transformed with GFP-OM45 and mtRFP.

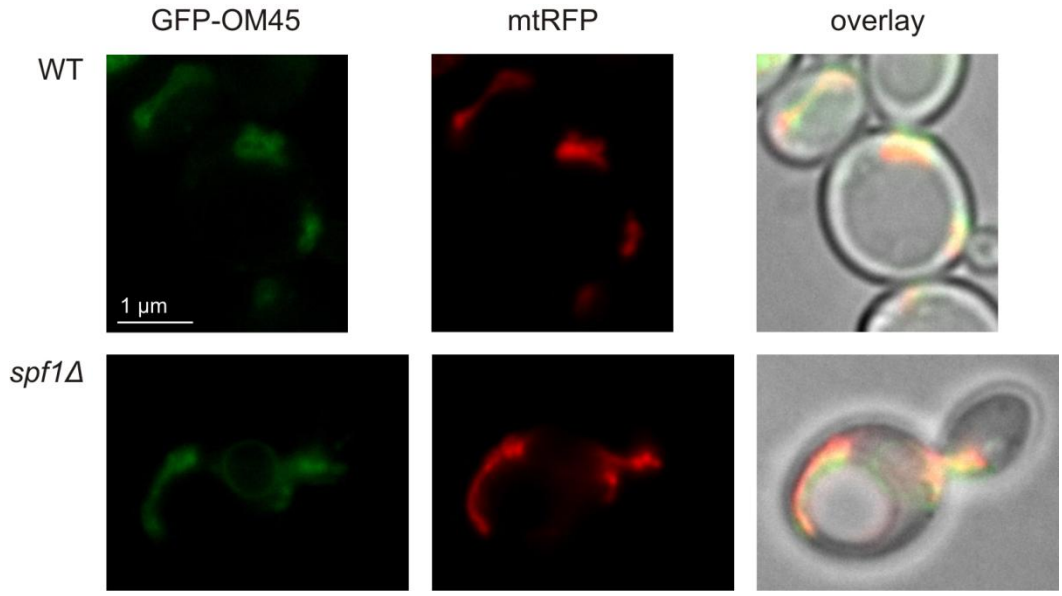

Fig. 3.18 The mitochondrial signal-anchored protein OM45 is mis-localized to ER structures in *spf1Δ*

WT (W303α) and *spf1Δ* cells were transformed with plasmids encoding GFP-OM45 and mtRFP. The cellular localization of OM45 was visualized by fluorescence microscopy.

Fluorescence microscopy showed that GFP-OM45 is located exclusively in mitochondria in WT cells. In contrast, in cells deleted of Spf1 the protein is not only co-localized with mtRFP in mitochondrial structures but also localized to ring-like ER structures (Fig. 3.18). This observation indicates that both TA- and signal-anchored proteins are partially mis-localized to ER structures in the absence of Spf1.

3.2.4 Fis1 is an integral membrane protein of mitochondria and ER in *spf1Δ* cells

Fluorescence microscopy and biochemical methods indicated that Fis1 is located to mitochondria and ER in *spf1Δ* cells. To further investigate whether Fis1 is indeed integrated to the membranes of these two compartments a carbonate extraction was performed (Fig. 3.19). The results indicated that Fis1 cannot be extracted from mitochondrial membranes upon carbonate extraction. Similarly, the vast majority of ER-located Fis1 behaved as a membrane embedded protein. The small amount of Fis1 molecules in the supernatant fraction from ER of *spf1Δ* cells might hint that the integration of Fis1 into the ER membrane is not as efficient as its integration into the MOM. Collectively, these findings suggest that Spf1 is not required for the membrane integration of Fis1 into both types of compartments.

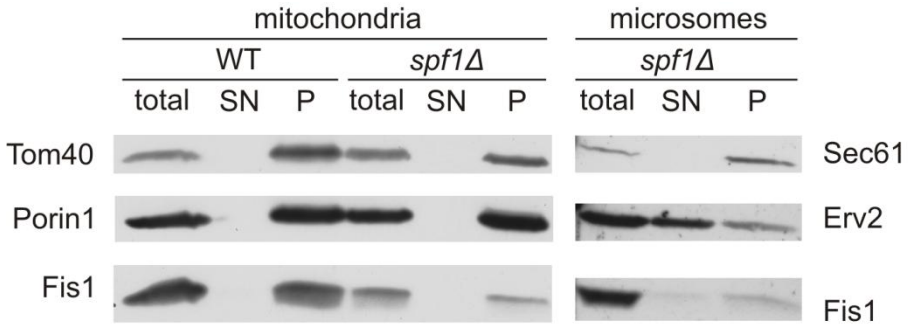

Fig. 3.19 Fis1 is an integral membrane protein of ER and mitochondria in *spf1Δ* cells

Mitochondria from WT and *spf1Δ* cells and microsomes from *spf1Δ* cells were isolated and subjected to a carbonate extraction. Untreated samples (total), the soluble protein fraction (SN) and the membrane protein fraction in the pellet (P) were loaded on SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

3.2.5 *In vitro* import of Fis1 into mitochondria and microsomes is not altered upon deletion of Spf1

To investigate the influence of Spf1 on the import of mitochondrial TA-proteins in more detail *in vitro* import experiments with mitochondria and microsomes from wildtype and ΔSpf1 cells were performed. Radiolabeled Fis1-TMC was imported into mitochondria or microsomes isolated from WT and *spf1Δ* cells (Fig. 3.20). Autoradiography results showed that for both strains Fis1-TMC was more efficiently imported into mitochondria than into microsomes. However, there was no significant difference in import efficiency into mitochondria from WT cells as compared to that into organelles from mutated cells (Fig. 3.20).

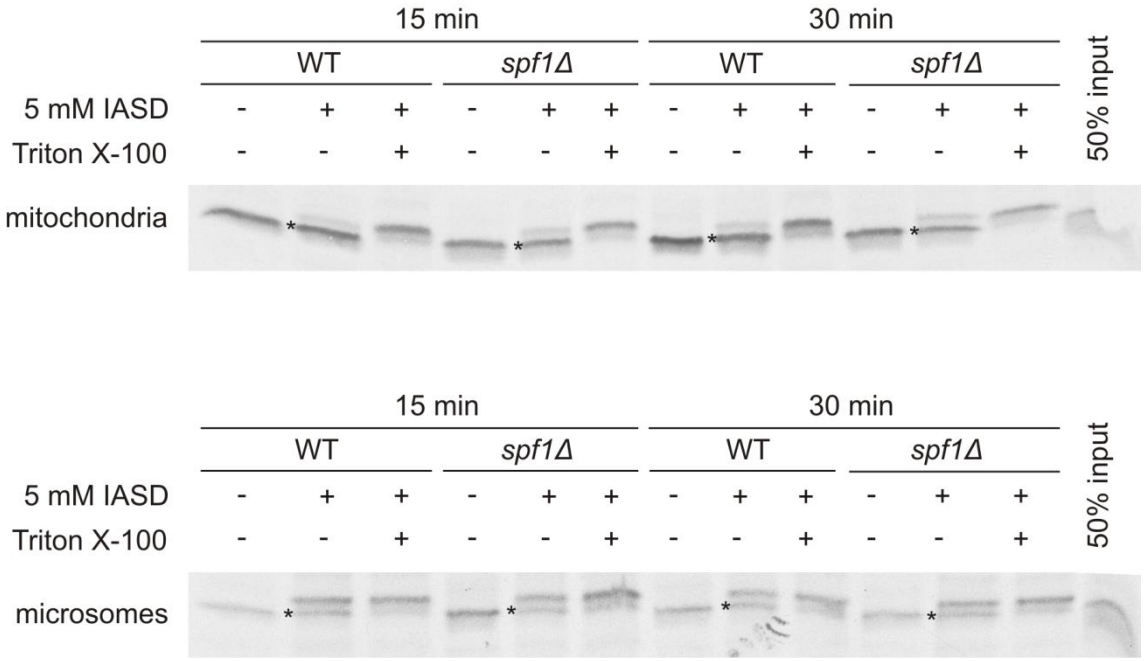

Fig. 3.20 *In vitro* import of Fis1 into mitochondria and microsomes is not altered upon deletion of Spf1

Radiolabeled Fis1-TMC was imported into isolated mitochondria and microsomes from WT and *spf1Δ* cells. Import of Fis1 was monitored by an IASD-assay, analysis by SDS-PAGE and autoradiography. Bands resulting from imported Fis1 are marked with asterisks.

Microsomes from mutated cells exhibited slightly higher import capacity than microsomes from WT cells. These results indicate that at least under *in vitro* conditions the mitochondrial import and membrane insertion of Fis1-TMC is not significantly

altered by the absence of Spf1. The lower import efficiency for ER compared to mitochondria is consistent with the results of the carbonate extraction that also showed that integration of Fis1 into the ER membrane is not as efficient as integration into the MOM.

The results so far indicate that a direct interaction between Fis1 and Spf1 is very unlikely. Hence, deletion of Spf1 might change other cellular conditions which lead to the mis-localization of mitochondrial TA- and signal-anchored proteins. Next, various cellular conditions that might be influenced by Spf1 were tested for their potential role in targeting of TA-proteins to mitochondria.

3.2.6 Deletion of Spf1 does not influence the localization of Fis1 via altered calcium levels

Spf1 is an ER resident Ca^{2+} pump, thus deletion of Spf1 might lead to higher cytoplasmic calcium levels which in turn can interfere with correct targeting of mitochondrial TA-proteins. To test this hypothesis, WT cells expressing Cherry-Fis1 and mtGFP were grown in media containing different calcium concentrations. The localization of Cherry-Fis1 was monitored by fluorescence microscopy (Fig. 3.21). Cherry-Fis1 was co-localized with mtGFP in mitochondrial structures under all conditions (media with 0-100 µM extra calcium). Of note, the actual cytosolic Ca^{2+} concentrations were not measured during this experiment. These results indicate that addition of calcium to the medium cannot mimic the *spf1Δ* Fis1 phenotype.

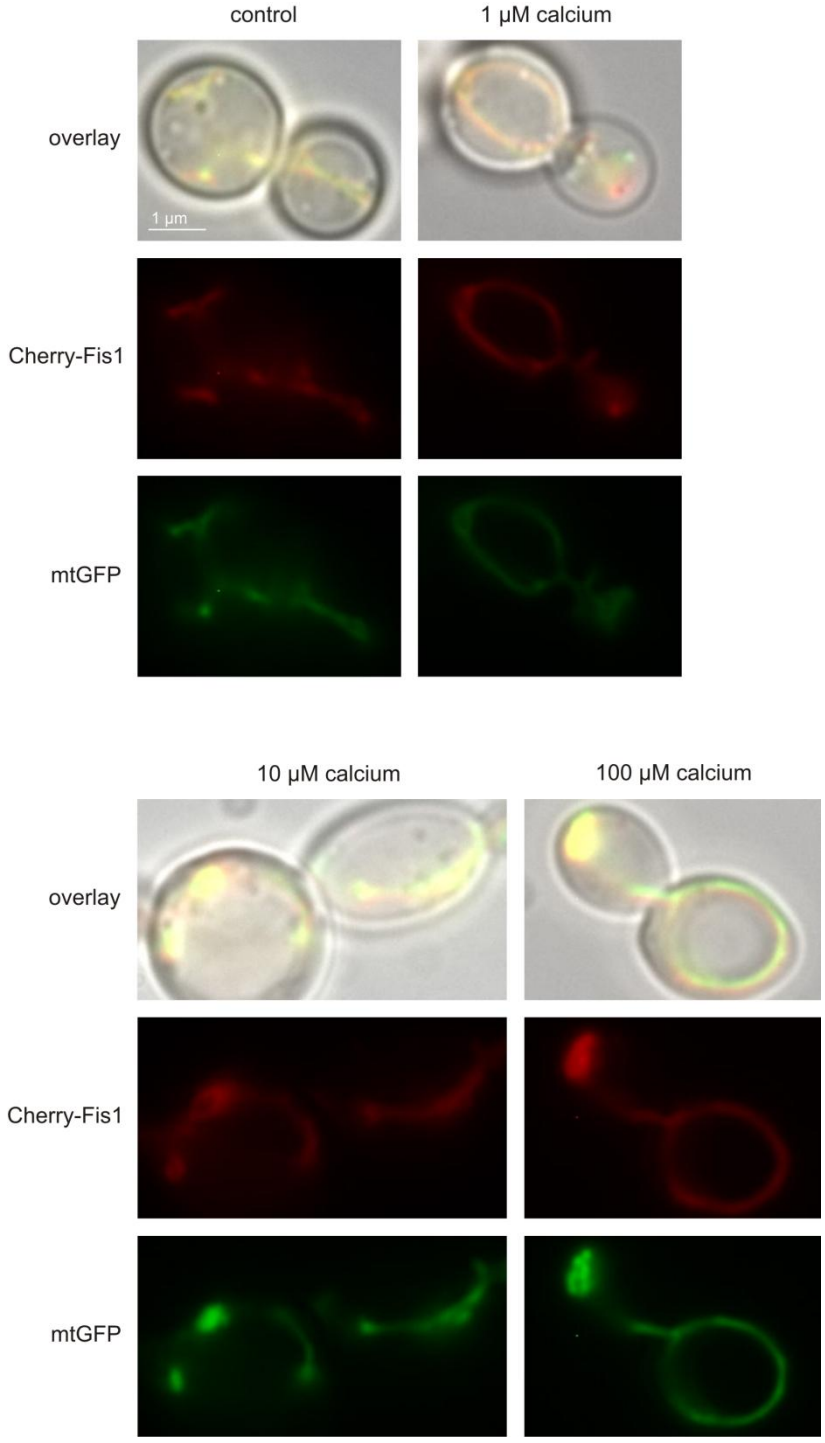

Fig. 3.21 Elevated external calcium levels do not influence the localization of Fis1

WT cells were transformed with plasmids encoding Cherry-Fis1 and mtGFP. The cells were grown in medium supplemented with 0, 1, 10 or 100µM extra calcium. The localization of Fis1 was analyzed by fluorescence microscopy.

3.2.7 Increased Get3 levels do not lead to a mis-targeting of mitochondrial TA-proteins

Get3 is the major import chaperone for ER tail-anchored proteins. Hence, a possible explanation for the effect of Spf1 on targeting of Fis1 might be that Spf1 influences the location and / or the levels of Get3. To test this possibility, subcellular fractionation of WT and *spf1Δ* cells was performed (Fig 3.22). The results showed that the steady-state levels of Get3 are slightly increased in the mutant cells. The majority of Get3 molecules in both cell types is localized in the cytosol. A smaller fraction of Get3 was also found in the microsomes fraction and even in mitochondria from WT cells (Fig. 3.22).

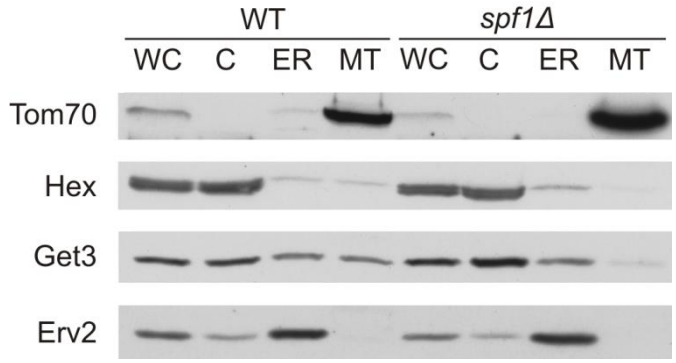

Fig. 3.22 Get3 levels are slightly increased in ΔSpf1 cells

Whole cell lysate (WC), cytosol (C), ER and mitochondria (MT) fractions were prepared from WT and *spf1Δ* cells and analyzed by SDS-PAGE and Western blotting with the indicated antibodies.

The slight increase in the levels of Get3 in the mutant cells might affect Fis1 targeting. To investigate the role of Get3 in the targeting of Fis1 in WT cells in more detail, these cells were transformed with plasmids encoding Cherry-Fis1 and an overexpression plasmid for Get3 (Fig. 3.23 A). Fluorescence microscopy revealed that even strong overexpression of Get3 did not cause any mis-targeting of Fis1 to the ER (Fig. 3.23 B). These findings indicate that Get3 has indeed a very specific role for ER tail-anchored proteins and does not affect mitochondrial TA-proteins.

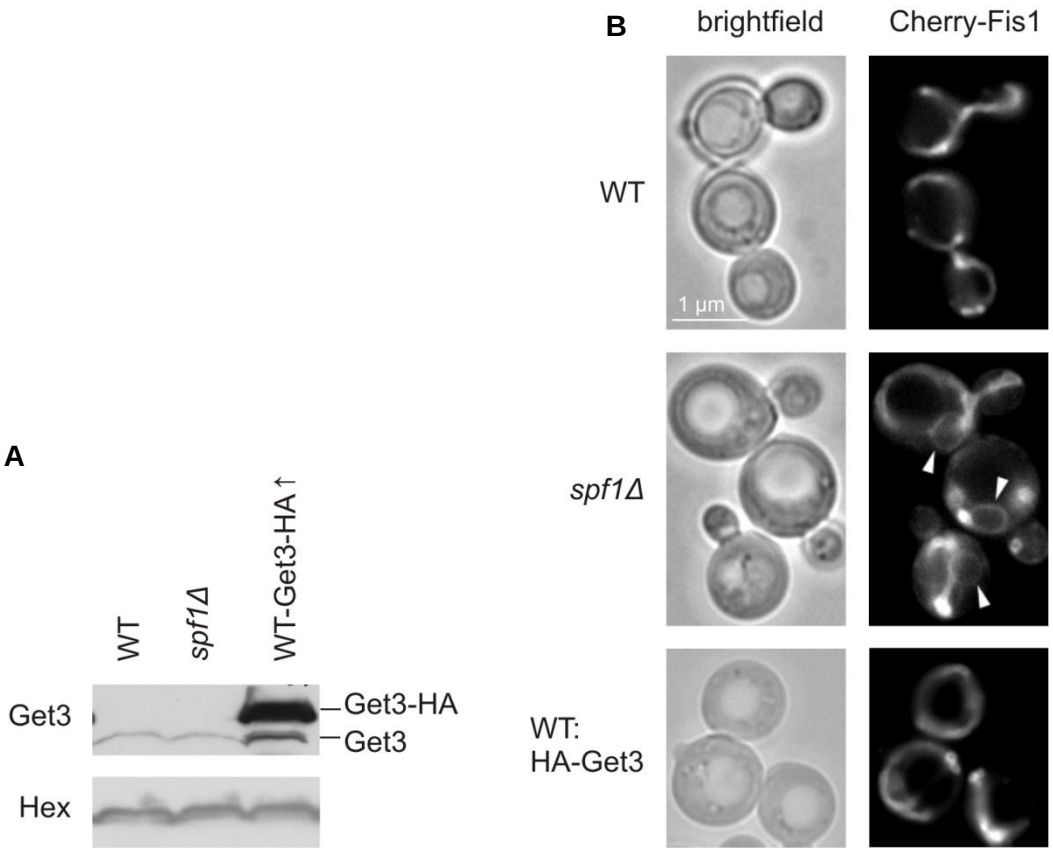

Fig. 3.23 Overexpression of Get3 does not influence the targeting of Fis1

(A) Whole cell extracts of WT, *spf1Δ* and WT cells over-expressing HA-Get3 were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. (B) Cells described in part (A) were transformed with a plasmid encoding Cherry-Fis1 and the location of Cherry-Fis1 in the cells was analyzed by fluorescence microscopy. ER structures stained by Cherry-Fis1 in *spf1Δ* cells are marked with arrowheads.

3.2.8 Mitochondria and ER from *spf1Δ* cells possess lower ergosterol levels than WT mitochondria

It was previously reported by Cronin et al. (2000) that Spf1 is required to control Hmg-CoA reductase (Hmg2) degradation in yeast. Hmg2 is a key enzyme of the mevalonate pathway and thus in the sterols biosynthesis pathway. The levels of Hmg2 are normally regulated by signals derived from farnesyl pyrophosphate (FPP) that by itself is a product of the mevalonate pathway. In the absence of Spf1 regulation of Hmg2 is uncoupled from FPP-derived signals and undergoes constant degradation.

Hence, Spf1 might indirectly influence ergosterol levels in yeast. To investigate this issue, mitochondria and ER were isolated from WT and *spf1Δ* cells. Lipids were extracted from the samples and were analyzed by mass spectrometry. Ergosterol levels and the total amount of phospholipids were determined. The lipid analysis by mass spectrometry was performed by the lab of Britta Brügger in Heidelberg. Analysis of samples from six different subcellular fractionations showed that mitochondria and ER from *spf1Δ* cells contain slightly lower ergosterol levels than wildtype mitochondria (Fig. 3.24). In agreement with previous reports (Zinser et al. 1991; Schneiter et al. 1999), we found that in control cells, mitochondrial ergosterol levels were lower than those in the ER (p value = 0.04). Remarkably, in *spf1Δ* cells, ergosterol content of both compartments did not show any significant difference. The change of ergosterol levels in the *spf1Δ* strain indicates that in these cells also ER membranes became ergosterol-poor. Given that the low amount of ergosterol in the MOM probably serves as a recognition signal for Fis1 and other mitochondrial TA-proteins, the deletion of Spf1 may lead to conditions which make it impossible for Fis1 and other single-span proteins to distinguish between ER and mitochondria anymore because both membranes are similarly ergosterol-poor. Therefore, mitochondrial TA-proteins are no longer hindered in insertion by higher ergosterol levels in ERand thus also integrate themselves into ER membranes.

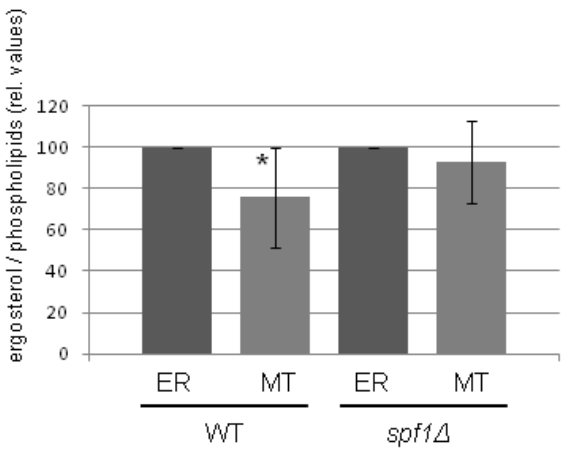

Fig. 3.24 Reduced ergosterol levels in *spf1Δ* cells are sufficient to cause ER localization of MOM TA-proteins

Lipids were extracted from mitochondria and microsomes isolated from control and *spf1Δ* cells. Equal amounts of lipids (according to phospholipid-determination) were analyzed for their ergosterol levels by gas chromatography / mass spectrometry. The results represent six independent experiments. The star represents statistical significance ($p<0.05$).

4 DISCUSSION

Tail-anchored proteins form a distinct group of membrane proteins, characterized by a single transmembrane domain close to the C-terminus. This TMD and its flanking regions contain the targeting information in such proteins (Borgese et al. 2003). TA-proteins are ubiquitously present in all kingdoms of life in nearly all membranes abutting from the cytosol. They fulfill various cellular functions including vesicle trafficking, apoptosis and organelle morphology (Borgese et al. 2007; Chen and Scheller 2001; Cory and Adams 2002). Due to their topology, the only possible mode of targeting and membrane insertion is posttranslational because the targeting signal at the C-terminus will emerge from the ribosome only upon complete translation of the protein (Johnson and van Waes 1999; Kutay et al. 1995).

Mitochondria harbor tail-anchored proteins in the outer membrane. Although the biogenesis of mitochondrial proteins and the functions of the mitochondrial import machineries have been elucidated in the last decades, the biogenesis of mitochondrial tail-anchored proteins is only poorly characterized. In contrast, the recent discovery of the mammalian TRC40 complex (Favaloro et al. 2008; Stefanovic and Hedge 2007) and its yeast homologue the GET machinery (Schuldiner et al. 2005 and 2008) shed new light on the mechanisms of biogenesis of ER-resident tail-anchored proteins. No such machinery has been identified so far for mitochondrial tail-anchored proteins. Due to the lack of any known proteinaceous component involved in recognition of mitochondrial TA-proteins it can be suggested that the distinct lipid composition of the MOM serves as a major specifity criterion.

This study focused on several questions regarding the biogenesis of mitochondrial tail-anchored proteins using Fis1 as a model protein. Two major issues were addressed and will be discussed in the following sections: (i) how to assure specific targeting of mitochondrial tail-anchored proteins? And (ii) what is the role of cytosolic factors in the biogenesis process?

4.1 The role of Spf1 in the targeting of mitochondrial tail-anchored proteins

Mitochondrial tail-anchored proteins possess a relatively short transmembrane domain of moderate hydrophobicity flanked by positively charged residues. However, they share this features also with some ER TA-proteins (Borgese et al. 2001; Horie et al. 2002; Isenmann et al. 1998; Kuroda et al. 1998). An open question is how these mitochondrial proteins assure their specific targeting to the MOM.

A high-throughput genetic screen on mitochondrial TA-protein localization that was performed by the laboratory of M. Schuldiner revealed mis-localization of the mitochondrial TA-protein Gem1 in only one strain among the complete yeast deletion library. In this strain Spf1, also known as Cod1, was deleted. Spf1 is an ER-resident calcium pump from the family of the P-type ATPases. The protein is widely conserved among metazoans. Spf1 was reported to control cellular calcium homeostasis together with another P-type ATPase, Pmr1. Both enzymes play important not redundant roles in ER function, maintenance of glycoprotein processing and ER quality control (Cronin et al. 2002). The group of M. Schuldiner could show that the mis-localization of mitochondrial TA-proteins is direct effect of the loss of enzymatically functional Spf1. Spf1 was expressed under control of the inducible *GAL* promotor. Upon growth on glucose mitochondrial TA-proteins were mis-localized while a shift to growth on galactose restored correct localization of these proteins. Furthermore ATPase dead mutants of Spf1 caused also a mis-localization of mitochondrial TA-proteins (M. Schuldiner, personal communication). This shows that the deletion of Spf1 is the direct cause for MOM TA-protein mis-localization. In this study it could be shown that not only Gem1 but also Fis1 as another tail-anchored protein and the signal-anchored protein OM45 are mis-located to ER structures in the absence of Spf1. These findings indicate that Spf1 has a general effect on the targeting of mitochondrial signal- and tail-anchored membrane proteins.

The ER-localization of Spf1 and the known information about the protein suggest that a direct interaction between Spf1 and mitochondrial TA-proteins seems to be very unlikely. Therefore this study addressed the question of the mechanism by which Spf1 might influence the targeting of mitochondrial TA-proteins.

It was reported that Spf1 is involved in cellular calcium homeostasis (Cronin et al. 2002). Therefore the effect of altered external calcium levels on the localization of Fis1 was investigated. No mis-localization of a Cherry-tagged Fis1 could be observed up to a concentration of 100 µM calcium in the growth medium. However, these results are difficult to interpret since the major Golgi membrane Ca^{2+} P-type ATPase, Pmr1 is also involved in calcium homeostasis and only upon double deletion of both genes the intracellular calcium levels were changed significantly (Vahist et al. 2002). Therefore the presence of Pmr1 in ΔSpf1 cells might be sufficient to maintain the normal cellular calcium homeostasis. Of note, deleting *PMR1* did not cause any mis-localization of Cherry-Fis1. Despite these limitations in the interpretation of the results of this experiment may suggest that the mis-localization of MOM TA-proteins is probably not due to any major changes of intracellular calcium levels.

Get3 is the major cytosolic targeting factor for ER-resident TA-proteins that utilize the GET machinery (Jonikas et al. 2009; Schuldiner et al. 2008). A potential explanation for the *spf1Δ* phenotype can be an overexpression of Get3 that in turn might falsely direct mitochondrial TA-proteins to the ER. The partial overlapping of the targeting information for ER and mitochondrial TA-proteins can support such a putative scenario. However, the results argue against such a possibility as Fis1 was exclusively localized to mitochondria in cells over-expressing Get3. Moreover, deletion of *GET3* in a *spf1Δ* background does not suppress the mis-localization of mitochondrial TA-proteins (M. Schuldiner, personal communication). This suggests that the GET machinery is not involved in the mis-localization of these proteins in a *spf1Δ* background and that Get3 has a high specifity for its substrates. Besides Get3 also Get4 and Get5 are needed for the recognition of newly synthesized ER TA-proteins. The Get4/5 complex first binds to the substrate TA-protein before Get3 is recruited. Thus the decision whether a TA-protein will be recruited to the GET-pathway or not is probably defined before it interacts with Get3. Therefore the interplay of three cytosolic factors of the GET machinery is an efficient system to avoid the binding and targeting of non-ER TA-proteins.

Besides its functions in calcium homeostasis Spf1 was also reported to be involved in the regulation of the mevalonate pathway for the synthesis of precursors for the sterol biosynthesis (Cronin et al. 2000). The consequences of Spf1 deletion are constantly

low levels of Hmg2 irrespective of the need for active enzyme in the pathway. Such low levels might lead to a lack of precursors for the sterol biosynthesis. It was shown before that even minor alterations in the ergosterol content of cellular membranes can lead to major effects on cellular processes (Stuven et al. 2003). However, deletion of *HMG2* did not phenocopy the situation in the absence of Spf1 (M. Schuldiner, personal communication).

A possible role of the ergosterol content of membranes in the targeting of mitochondrial TA-proteins has been shown *in vitro*. Lipid vesicles with higher levels of ergosterol were severely hampered in their integration capacity of TA-proteins (Kemper et al. 2008). Similar inhibitory effect of higher ergosterol levels could also be shown for the signal-anchored protein OM45 (Merklinger et al. 2012). Conversely, when ergosterol was depleted from microsomes ER TA-proteins, which can insert themselves in an unassisted manner, became inserted into those microsomes in higher efficiency (Brambillasca et al. 2005). All these findings suggest that the lipid composition and the ergosterol content of cellular membranes plays a major role in the targeting of mitochondrial tail-anchored proteins.

In wildtype yeast cells the mitochondrial outer membrane is the ergosterol poorest membrane of the cell, while the plasma membrane has a high content of ergosterol and the endoplasmic reticulum has average ergosterol content. To investigate if this difference is also kept in *spf1Δ* cells, the ergosterol levels of ER microsomes and mitochondria of *spf1Δ* cells were analyzed and compared to the levels in wildtype cells. The ergosterol contents of both microsomes and mitochondria of *spf1Δ* cells were lower than that of wildtype mitochondria. Moreover, no significant difference in ergosterol content between microsomes and mitochondria was observed in the *spf1Δ* cells. These facts show that the deletion of Spf1 alters the ergosterol contents of ER and mitochondrial membranes. Given that mitochondrial tail-anchored proteins do not need any proteinaceous receptors to interact with their target membrane, the specified lipid composition of the outer mitochondrial membrane could serve as recognition criterion. In *spf1Δ* cells mitochondrial tail-anchored proteins might not longer be able to distinguish between mitochondrial and ER membranes because they are both relatively ergosterol poor. In wildtype cells the higher ergosterol content of the ER and other membranes would prevent mis-targeting. In support to this hypothesis it has been

shown that the down-regulation of Erg9 expression that results in lowered cellular ergosterol levels, causes mis-localization of Fis1 to ER structures (M. Schuldiner, personal communication). Thus the results of this study suggest that the low ergosterol content of the outer mitochondrial membrane might be a sufficient recognition criterion for mitochondrial TA-proteins.

The same might be true for mitochondrial signal-anchored proteins, as it could be shown by this study that also signal-anchored proteins mis-localize to ER structures in *spf1Δ* cells. As for TA-proteins, no proteinaceous component for recognition of mitochondrial signal-anchored proteins was identified so far (Meineke et al. 2008; Merklinger et al. 2012). Moreover it was shown by this study that the targeting signals of signal- and tail-anchored proteins are interchangeable. The transmembrane domain of Tom70 in reversed order can serve as tail-anchored targeting signal that efficiently guides the chimera protein to the mitochondrial outer membrane. Thus also the membrane recognition criteria might be similar meaning that also signal-anchored protein targeting to the MOM might rely on the low ergosterol content of the membrane.

Membranes with low sterol contents are more fluid. A high fluidity of a membrane would facilitate the insertion of a transmembrane domain much better than a rigid sterol-rich membrane. The low ergosterol content does not have to characterize the complete surface of the MOM. Equally possible is a situation where ergosterol-poor microdomains in the outer mitochondrial membrane might provide an insertion side for single-span proteins. In the last few years it became obvious that lipids fulfill various roles in the biogenesis of membrane proteins. The lipid composition of a membrane influences a great variety of membrane parameters that are important for the function of membrane proteins or protein complexes (Schneiter and Toulmay 2007). Furthermore it has been shown that various proteins are dependent on a distinct lipid composition for correct targeting (Malinska et al. 2003; Umebayashi and Nakano 2003). For instance, sterols play an important role in protein sorting along the exocytic pathway (Bagnat and Simons 2002; Proszynski et al. 2005). All these findings support the idea that mitochondrial tail-anchored proteins are targeted independently of any proteinacious receptors but rather find their target membrane by its unique lipid composition.

In this study it could be shown that Fis1 can be cross-linked *in-vivo* to Porin1. Porin1 is the most abundant protein of the MOM that forms an unspecific transporter for small metabolites. Despite the cross-linking results no evidence for a physiological role of this interaction could be found and no effect of alteration of the Porin1 expression levels on the import and / or stability of Fis1 could be observed. Thus, it could also be that the high abundance of Porin1 in the MOM led to false positive results by the cross-linking approach. These results further point to a reliance of mitochondrial TA-protein targeting on the lipid composition of the membrane.

4.2 Cytosolic targeting factors

Tail-anchored proteins are synthesized on cytosolic ribosomes and inserted into their target membranes posttranslationally. Thus the fully synthesized protein has to travel through the aqueous cytosol. Tail-anchored proteins, like all membrane proteins are prone to aggregation in an aqueous environment. Therefore it is very likely that the proteins interact with cytosolic chaperones that prevent their aggregation and keep the proteins in an import competent state. The great variety of cellular locations of TA-proteins might imply also the involvement of a cytosolic sorting step. For many mitochondrial proteins it has been shown that their mRNAs are associated with mitochondria to enable a translation in the vicinity of the target organelle or even a co-translational import (Marc et al. 2002; Sylvestre et al. 2003). Most of these proteins harbor an N-terminal mitochondrial targeting sequence (MTS) that can interact with the Tom20 receptor upon release from the ribosome. Furthermore the 3´-UTR plays a crucial role in mRNA targeting which can for example be recognized by Puf3, an RNA binding protein (Corral-Debrinski et al. 2000; Eliyahu et al. 2010; Garcia-Rodriguez et al. 2007; Margeot et al. 2002; Saint-Georges et al. 2008) . It has been shown that both Tom20 and Puf3 are essential for mRNA targeting of a subset of mitochondrial proteins (Eliyahu et al. 2010). However, no such mRNA localization has been observed for tail-anchored proteins so far. *FIS1* mRNA shows comparably weak association with mitochondria (Gadir et al. 2011). This indicates that the targeting of mitochondrial tail-anchored proteins does not take place on the level of the mRNA but rather on the level of the protein.

The targeting of Fis1 includes also the challenge of a dual localization in mitochondria and peroxisomes. The membrane recognition mechanism might be the same for both types of membranes because also peroxisomal membranes are relatively ergosterol poor (Zinser et al. 1990). However, the fate of a Fis1 molecule can be determined by cytosolic targeting factors.

ER tail-anchored proteins can follow different cytosolic routes to the ER membrane. One is the GET or TRC40 pathway in yeast and mammals, respectively (Favaloro et al. 2008; Schuldiner et al. 2005; Stefanovic and Hedge 2007). Some of the ER TA-proteins are recognized by SRP in a posttranslational mode (reviewed in Rabu et al. 2009); while a subset of ER TA proteins interacts with an Hsc70/Hsp40 complex. This Hsc70/Hsp40 pathway is for example used by Sec61β and other TA proteins with moderately hydrophobic TMDs. It has been shown that the Hsc70/Hsp40 complex alone is sufficient for membrane insertion of the substrate TA-protein (Abell et al. 2007; Colombo et al. 2009; Rabu et al. 2008).

In this study it was found that the Fis1 TMD interacts with the Hsp70 family chaperones Ssa1 and / or Ssa2. These two proteins share high sequence identity and therefore a clear identification on the basis of mass spectrometry is not possible. Furthermore it could be shown that the deletion of functional Ssa proteins in yeast hampered the biogenesis of Fis1. *In vitro* import experiments as part of this study revealed a decrease in import efficiency of Fis1 into isolated mitochondria upon inhibition of Hsc70 and Hsp40 molecules in the reticulocyte lysate. These findings indicate that Ssa proteins, most probably Ssa1 or Ssa2 maybe together with a so far uncharacterized Hsp40 partner might be necessary for the biogenesis of Fis1. Due to the high homology and redundancy in function it could not be exactly determined whether Ssa1 or Ssa2 binds to Fis1. It is also conceivable that both proteins can take over functions of the other one if necessary. The chaperone activity of such an Hsp70/Hps40 complex would keep the protein in an import-competent state and would avoid aggregation during its journey through the cytosol. Fis1 harbors a transmembrane domain of moderate hydrophobicity like the ER TA-proteins that interact with an Hsc70/Hsp40 complex during their biogenesis. The involvement of an Hsc70/Hsp40 complex in both ER and mitochondrial targeting of TA-proteins raises the question how specifity is achieved. One possible scenario would be an additional cytosolic factor binding

specifically to chaperone complexes loaded with ER or mitochondrial TA-proteins. Such a factor was not found in the aforementioned genetic screen for targeting factors for mitochondrial TA-proteins (M. Schuldiner, personal communication). But it cannot be excluded that such a factor exists at least for the ER TA-proteins. Given that ER TA-protein / chaperone complexes are guided to the ER by an additional factor, mitochondrial TA-protein / chaperone complexes are somehow guided to mitochondria in a kind of default pathway due to the absence of additional targeting factors. Support for this idea is given by reports that ER TA-proteins in the absence of a functional GET pathway and some peroxisomal membrane proteins in the absence of Pex19 are mis-localized to the MOM (Jones et al. 2004; Schuldiner et al. 2008). The determination of specific Hsp40 co-factors and the identification of possible further interaction factors would be an interesting future issue as it would lead to a more detailed understanding of the cellular sorting mechanism of TA-proteins. The final step of membrane insertion is then determined by the specific lipid composition of the mitochondrial membrane. False unassisted integration of mitochondrial TA-proteins into other cellular membranes could be prevented by higher ergosterol levels in these membranes.

But it cannot be excluded that also other specific features of the MOM are contributing to assure the correct insertion of mitochondrial TA-proteins. It has been shown before that the ER-resident TA-protein cytochrome b5 is able of unassisted insertion but is partly dependent on the presence of functional Hsc70/Hsp40 complexes (Borgese et al. 2007). Like cytochrome b5 also Fis1 has a transmembrane domain of moderate hydrophobicity and it is likely that it can insert itself into the mitochondrial outer membrane unassisted.

It has been shown before and by this study that also the biogenesis of mitochondrial signal-anchored proteins depends on the MOM´s lipid composition and its ergosterol content. It would be of future interest to investigate whether mitochondrial tail-anchored and signal-anchored proteins share more features of their biogenesis, like the dependency on cytosolic chaperones.

A certain portion of Fis1 is not localized at the MOM but is rather found at the peroxisomal membrane where it functions in the peroxisomal fission. Obviously, the cell must be able to achieve this dual targeting by interaction of Fis1 with different

targeting factors for mitochondria and peroxisomes. It has been shown for the mammalian system that hFis1 contains a recognition site for Pex19, the major cytosolic import chaperone for peroxisomal membrane proteins and shows binding to Pex19 *in vivo* (Hallbach et al. 2006; Delille et al. 2008). Moreover mammalian Pex19 is able to increase the half-life of newly synthesized peroxisomal membrane proteins (PMPs) (Jones et al. 2004). In this study it could be revealed that Fis1 is stabilized by an overexpression of Pex19 in the yeast system. This indicates that also yeast Fis1 might be targeted to the peroxisomes with the help of Pex19. However, deletion of Pex19 did not affect Fis1, suggesting that in this case other factors like an Ssa/Hsp40 complex are able and sufficient to stabilize Fis1 in the cytosol and to keep it in an import-competent state that avoids degradation.

4.3 Model for the biogenesis of Fis1

Given the results of this work and the current knowledge on the biogenesis of mitochondrial tail-anchored proteins, the following model for biogenesis and targeting of the mitochondrial tail-anchored protein Fis1 is proposed: The Fis1 mRNA is translated on cytosolic ribosomes and as soon as the C-terminal transmembrane domain has left the ribosome and is exposed to the cytosol chaperones will bind to the protein. In the case of Fis1 molecules that are delivered to the mitochondria an Ssa protein, probably Ssa1 or Ssa2, binds to Fis1. It is very likely that also a so far unidentified Hsp40 co-chaperone is involved in the delivery of Fis1 to the mitochondria. Also other yet unknown factors might play a role in targeting Fis1. The recognition of the correct target membrane is accomplished by the membrane´s lipid composition. Low ergosterol content will serve as recognition criterion and will support the unassisted insertion of Fis1 into the membrane. Insertion into other membranes is avoided by a higher ergosterol content of these membranes. The targeting of fully synthesized Fis1 to the peroxisomal membrane might be facilitated by Pex19. This chaperone binds the cytosolic Fis1 and guides it to the peroxisomal membrane where Fis1 is inserted into the lipid bilayer. The membrane recognition and insertion mechanism might be similar to that in mitochondria as peroxisomal membranes also exhibit low ergosterol content. It is still not clear how the distribution of Fis1 between

mitochondria and peroxisomes is regulated. Pex19 and the Ssa/Hsp40 complex might compete for binding of Fis1 but the factors that dictate the distribution between the two compartments are still elusive.

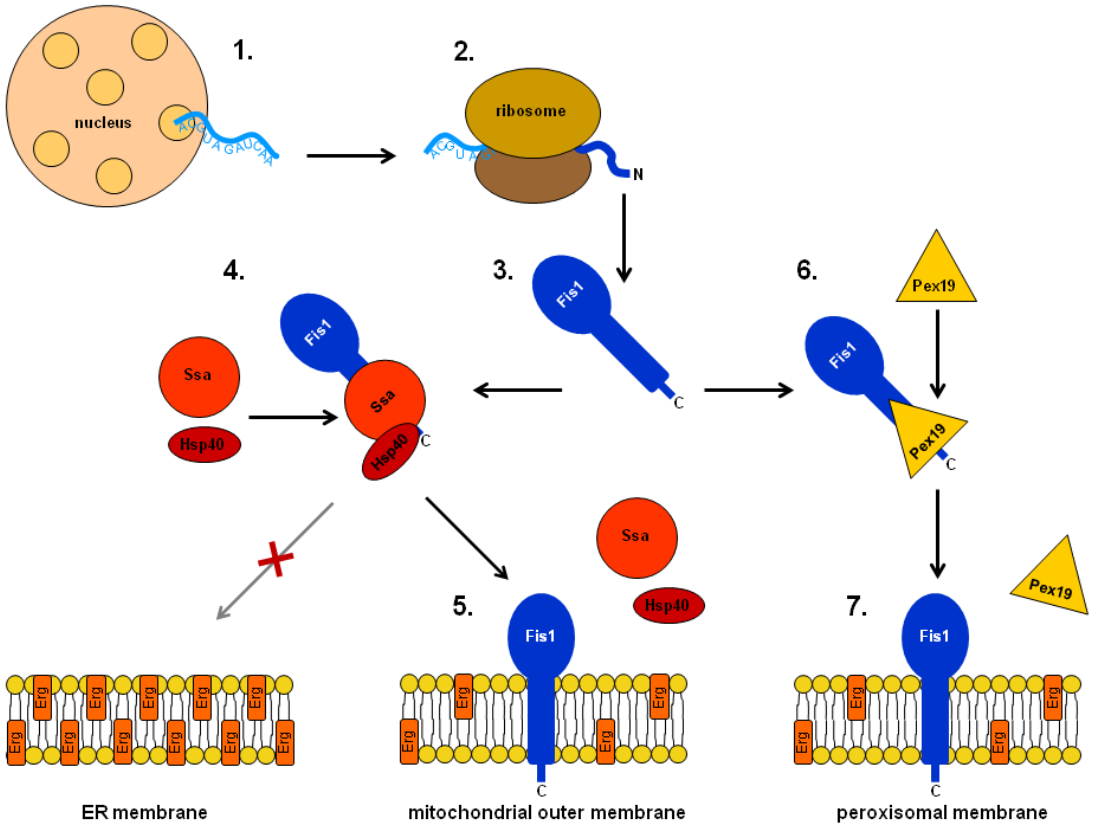

Fig. 4.1 Model for the biogenesis of Fis1

Fis1 is encoded in the nucleus (1) and translated on cytosolic ribosomes (2). After completed translation (3) the C-terminal region of Fis1 is recognized by cytosolic chaperones. Binding of an Ssa / Hsp40 complex (4) guides Fis1 to the outer mitochondrial membrane (5). Alternatively, interaction with Pex19 (6) leads to insertion of Fis1 into the peroxisomal membrane (7). Mis-location to other compartments, like the ER, is avoided by higher ergosterol content of their membranes.

5 SUMMARY

The mitochondrial outer membrane harbors proteins of different topologies. Among them are the tail-anchored proteins that are characterized by a single transmembrane domain very close to the C-terminus. The N-terminal part is protruding into the cytosol. The targeting information is enclosed in the transmembrane domain and its flanking regions. Therefore tail-anchored proteins have to be inserted into their target membranes in a posttranslational manner. Previous reports suggest that mitochondrial tail-anchor proteins do not utilize any known import components for their membrane integration.

This work addressed several questions concerning the biogenesis of mitochondrial tail-anchored proteins in *Saccharomyces cerevisiae*. The results show that upon deletion of the ER-resident P-type ATPase Spf1 the mitochondrial tail-anchored protein Fis1 and the signal-anchored protein OM45 are partially mis-localized to ER membranes. It was shown that this mis-localization is due to altered ergosterol contents in the mitochondrial and ER membranes. Moreover a site-directed *in vivo* cross-linking approach revealed an interaction between the transmembrane domain of Fis1 and Ssa1/2. It could be further shown that Ssa proteins play a role in the biogenesis of Fis1. Pex19, the major import receptor for peroxisomal membrane proteins was identified as a stabilizing factor for Fis1.

Based on the results of this study and previous reports a model for the biogenesis of mitochondrial tail-anchored protein Fis1 can be proposed. Fis1 is synthesized on cytosolic ribosomes and subsequently associates with cytosolic chaperones. Ssa proteins bind to the transmembrane region of Fis1 and guide it to the mitochondria. The targeting factor for peroxisomal localized Fis1 might probably be Pex19. The final integration into the target membrane occurs in both cases in an unassisted manner and is facilitated by the low ergosterol content of the membranes. Taken together, the biogenesis of mitochondrial tail-anchored protein relies on cytosolic chaperones and a distinct lipid composition of the target membrane.

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7 APPENDIX

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