Novel Insights into Biogenesis of Multi-Span Proteins of the Mitochondrial Outer Membrane

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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aus Split

Tübingen

2012

Tag der mündlichen Qualifikation:

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1 List of abbreviations

AAC	ADP/ATP carrier
ATP	Adenosine triphosphate
BN-PAGE	Blue native polyacrylamide gel electrophoresis
C-	Carboxyl-
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
HA	Haemagglutinin
Hsp	Heat shock protein
IMM	Inner mitochondrial membrane
IMP	Inner membrane peptidase
IMS	Intermembrane space
kDa	Kilodalton
MIA	Mitochondrial IMS import and assembly
MOM	Mitochondrial outer membrane
MPP	Mitochondrial processing peptidase
N-	Amino-
N. crassa	*Neurospora crassa*
PAGE	Polyacrylamide gel electrophoresis
Phe	Phenylalanine
PK	Proteinase K
POTRA	Polypeptide-transport-associated
RT-PCR	Reverse transcriptase polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SAM	Sorting and assembly machinery
SILAC	Stable isotope labeling with amino acids in cell culture
SDS	Sodium dodecyl sulfate
TA	Tail-anchored
TIM	Translocase of the inner mitochondrial membrane
TMD	Transmembrane domain
TMS	Transmembrane segment
TOB	Topogenesis of outer membrane β-barrel proteins
TOM	Translocase of the outer mitochondrial membrane
TX-100	Triton X-100

2 Summary

The vast majority of mitochondrial proteins are nuclear encoded and after their synthesis in the cytoplasm need to be imported into the organelle. The import pathway that a protein follows depends on its target destination within the mitochondria and the protein´s topology. In spite of significant progress in understanding of import pathways of mitochondrial protein precursors, a great deal of crucial information on these processes is still missing. This is especially the case with proteins of the mitochondrial outer membrane (MOM). In the work presented here I investigated the biogenesis of two groups of yeast MOM proteins: α-helical multispan and β-barrel protein precursors. The MOM protein Mim1 was identified as a key factor in import of MOM multispan helical proteins. Furthermore, in agreement with previous findings in mammalian systems, the Tom70 receptor was found to be involved in the initial recognition of these proteins. It was further discovered that the protein conducting pore of the TOM complex and components of the mitochondrial intermembrane space do not play a role in this process. Moreover, we identified Mim2 as another protein that together with Mim1 participates in the MIM complex. Mim2 is a single-span MOM protein that plays a crucial role in the assembly and stability of the MIM complex and hence also in the biogenesis of α-helical multispan proteins. In our work on the biogenesis of β-barrel proteins we were interested in finding out if β-barrel proteins from bacterial outer membrane can be recognized as import substrates by mitochondria. We observed that these heterologous proteins are successfully targeted to and assembled within mitochondria. Apparently, signals that govern biogenesis of β-barrel proteins in bacteria are conserved and can be interpreted by a eukaryotic system. In contrast to this similarity in the biogenesis process, mitochondrial β-barrel precursors lost their N-terminal signal sequence in the course of evolution. To investigate potential reasons for this loss we expressed bacterial β-barrel precursors with signal sequence in yeast cells. These proteins were successfully targeted to MOM with their signal sequence intact, albeit with considerably reduced efficiency. Interestingly, a portion of the bacterial proteins was targeted to the endoplasmic reticulum and underwent glycosylation there. Hence, this mis-localization was most likely the selective pressure that led to the loss of signal sequences during evolution of β-barrel precursors in eukaryotes.

3 List of publications contained in this thesis

Walther, D.M., D. Papic, M.P. Bos, J. Tommassen, and D. Rapaport. 2009. Signals in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria. *Proc Natl Acad Sci USA*. 106:2531-2536.

Muller, J.E., D. Papic, T. Ulrich, I. Grin, M. Schutz, P. Oberhettinger, J. Tommassen, D. Linke, K.S. Dimmer, I.B. Autenrieth, and D. Rapaport. 2011. Mitochondria can recognize and assemble fragments of a beta-barrel structure. *Mol Biol Cell*. 22:1638-1647.

Papic, D., K. Krumpe, J. Dukanovic, K.S. Dimmer, and D. Rapaport. 2011. Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent import pathway. *J Cell Biol*. 194:397-405.

Dimmer, K.S., D. Papic, B. Schumann, D. Sperl, K. Krumpe, D.M. Walther, and D. Rapaport. 2012. A crucial role of Mim2 in the biogenesis of mitochondrial outer membrane proteins. *J Cell Sci*.

4 Personal contribution to the publications contained in this thesis

Walther, D.M., D. Papic, M.P. Bos, J. Tommassen, and D. Rapaport. 2009. Signals in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria. *Proc Natl Acad Sci USA*. 106:2531-2536.

I performed carbonate extraction and proteinase protection assays with mitochondria from PhoE-low-expressing strain (Fig. 3A) and urea treatments with mitochondria from both low- and high PhoE-expressing strains with or without terminal Phe (Fig. 3B and 4A, respectively). Also, I tested the impact of expression of bacterial outer membrane β-barrel proteins OmpA, OmpC and Omp85 on steady state levels of other mitochondrial proteins (Fig. S3 (B)).

Muller, J.E., D. Papic, T. Ulrich, I. Grin, M. Schutz, P. Oberhettinger, J. Tommassen, D. Linke, K.S. Dimmer, I.B. Autenrieth, and D. Rapaport. 2011. Mitochondria can recognize and assemble fragments of a beta-barrel structure. *Mol Biol Cell*. 22:1638-1647.

I isolated mitochondria from yeast strains expressing PhoE with and without signal sequence and analyzed steady state levels of the proteins in them (Fig. 1A), conducted chase experiments with use of cycloheximide to monitor stability of the expressed PhoE with and without signal sequence (Fig. 1C). Furthermore, I performed alkaline extraction (Fig. 1D) and analysis of protein modifications by using Endoglycosidaze H_f (Fig. 1E). Also, I did subcellular fractionation (Fig. 1F) and analysis of the status of heterologous PhoE with and without signal sequence in *imp1*Δ strain (Fig. 1F and 1G, respectively).

Papic, D., K. Krumpe, J. Dukanovic, K.S. Dimmer, and D. Rapaport. 2011. Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent import pathway. *J Cell Biol*. 194:397-405.

I developed a novel assay to study *in vitro* import of radiolabeled Ugo1 precursors (Fig. 1), and performed experiments with import of radiolabeled Ugo1 precursor protein to investigate ATP requirement for the biogenesis of Ugo1 (Fig. 2). I conducted experiments

with pretrypsinization of mitochondria and subsequent import assay (Fig. 3A), isolated mitochondria from strains deleted for TOM receptors and performed the import assay (Fig. 3 B and C), or did BN-PAGE analysis of radiolabeled precursors´ import. I also determined steady-state levels of Ugo1 and other proteins in strains deleted for TOM receptors (Fig. 3E). Similarly, I isolated mitochondria from *mim1*Δ strain and performed import assay or used BN-PAGE as above (Fig. 4 A and B, respectively). Moreover, I perfomed BN-PAGE and SDS-PAGE analysis of steady state levels of proteins in *mim1*Δ mitochondria (Fig 4 C and D, respectively), as well as alkaline extraction (Fig. 4F). I further isolated mitochondria and analyzed their steady state protein levels via SDS-PAGE and immunodecoration in *mim1*Δ strains overexpressing full length Mim1 or N-terminally truncated Mim1 (Fig. S1), performed competitive import assays with pSu9-DHFR (Fig. S2 A) and radiolabeled precursor import experiments with swollen mitochondria followed by BN-PAGE analysis (Fig. S2 B). Also, I did import assays with mitochondria from *tim8/13*Δ strain (Fig. S2 C), as well as steady state proteins´ levels analysis of mitochondria from that and *mas37*Δ strain (Fig. S2 D and S3 B, respectively).

Dimmer, K.S., D. Papic, B. Schumann, D. Sperl, K. Krumpe, D.M. Walther, and D. Rapaport. 2012. A crucial role of Mim2 in the biogenesis of mitochondrial outer membrane proteins. *J Cell Sci*.

I performed experiments aimed at analysis of MIM complex in strains with *mim1Δ* and *mim2Δ* background with or without overexpression of Mim1 or Mim2-HA (Fig. 2C, S4 and S5), conducted antibody-shift experiments (Fig. 2D) and analyzed migration behavior of the MIM complex containing GFP-tagged Mim2 (Fig. 2F). Furthermore, I investigated steady state levels of the TOM complex in strains with *mim1Δ* and *mim2Δ* single and double deltions background with and without overexpression of Mim2-HA (Fig. S7 C, S8 B and 5B) or performed import of radioactive Tom40 precursors and monitored kynetics of TOM complex assembly (Fig. 6C). Furthermore, I conducted import experiments with radiolabeled Ugo1 in the presence of antibodies to Mim2 and monitored migration beahvior of Ugo1 and MIM complexes (Fig. 7). In all experiments I used BN-PAGE as a method to address the issue at hand.

5 Introduction

5.1 Intracellular protein trafficking

Eukaryotic cells appeared around 1.5 billion years ago. As compared to the prokaryotic cells they were larger and structurally far more complex containing a sophisticated system of internal compartments. Since biochemical processes are accomplished by the action of proteins, this means that each organelle needs to contain a set of specific proteins to perform its function properly. Given that the vast majority of the proteins are nuclear encoded and synthesized in the cytosol (apart from few proteins encoded by mitochondrial and plastid genomes), this further means that most of the proteins need to be targeted to their destined compartment where they perform their function (Neupert and Herrmann, 2007; Soll and Schleiff, 2004). To achieve such a specific sorting newly synthesized proteins contain targeting signals that direct them towards the specific organelles, which expose receptors at their surfaces that recognize distinct targeting signals. In close proximity to such receptors are oligomeric membrane protein complexes termed translocases. They mediate protein translocation across or integration into the membrane (Schnell and Hebert, 2003). Hence, understanding the mechanisms by which protein trafficking occurs is crucial for our ability to grasp the biology of the cell and the biogenesis of various organelles. Likewise, this knowledge could also lead to understanding of at least some of the physiological malfunctions that can occur in cells.

5.2 Origin, structure and function of the mitochondria

Mitochondria are one of the membrane-bounded organelles, which are typically present in the vast majority of eukaryotic cells. According to the endosymbiont hypothesis they are derived from an aerobic bacterium that was taken up through endocytosis by an anaerobic eukaryotic cell. In this way the anaerobic eukaryotic cell bettered its chances of survival in an increasingly oxygen-rich environment by profiting from the bacterium´s ability to produce ATP at the expense of oxidization of nutritients (Cavalier-Smith, 1987). This event was dated 1.5-2 billion years ago, although it remains unclear if all eukaryotic mitochondria originated from one single endosymbiotic event. In the course of symbiosis a transfer of the mitochondrial genes to the nucleus occurred. In this way the eukaryotic cell achieved better regulation of the important processes, which now resided in the mitochondria. Gaining integrated control was obviously of great importance since the whole process of functional

gene transfer is quite complex and still about 99% of mitochondrial proteins are now nuclear encoded. The gene transfer includes not only assimilation of the gene into the nuclear genome, but also its functional transcription, often acquiring of a targeting sequence that can lead the protein to mitochondria and adequate control of expression in order to achieve same level of fitness as in a mitochondrial gene (Blanchard and Lynch, 2000). Nowadays in yeast there are around 1000 different mitochondrial proteins (out of 6000 cellular proteins), and only 8 of these are synthesized in the organelle itself (Sickmann et al., 2003). While the reasons for nuclear localization of mitochondrial genes seem straightforward, at the moment there are no generally accepted explanations as to why the few mitochondrial proteins need to be synthesized in the organelle rather than in the cytosol.

Structurally and functionally one can distinguish between four different compartments in each mitochondrion: outer and inner mitochondrial membrane with matrix space engulfed by the inner membrane and the intermembrane space (IMS) between the outer and inner mitochondrial membranes. The outer membrane does not contain that many different proteins (so far 38 have been discovered in yeast), but is, however, very rich in the β-barrel channel-forming protein porin. Its diameter is large enough to enable free passage of molecules with a molecular weight of less than 5 kDa. This makes the intermembrane space practically chemically equivalent in terms of small molecules composition with the neighboring cytoplasm. Besides its mainly transport function outer membrane also contains enzymes involved in mitochondrial lipid homeostasis (Alberts B, 2002). Unlike the outer membrane, which has a smooth surface, the inner mitochondrial membrane forms numerous infoldings, termed christae, that protrude deep into the matrix. Furthermore, the inner mitochondrial membrane is generally impermeable – especially for small ions – a feature of vital importance for maintenance of electrochemical gradient. Proteins that carry out inner membrane key functions include subunits of the respiratory chain complexes and many transport proteins. Inner mitochondrial membrane is extremely protein-rich: it contains 1-1.5 μg proteins/μg phospholipids, a ratio which is 5 times higher than that of the outer mitochondrial membrane. Moreover, it contains significant amounts of cardiolipin – a phospholipid - which is a reminiscence of mitochondrial prokaryotic lineage, since it is found only in mitochondria and membranes of bacteria (De Kroon et al., 1997; Schneiter et al., 1999; Zinser et al., 1991). The matrix is similarly a protein-rich compartment. Citric acid cycle, oxidization of pyruvate and fatty acids take place here and it also houses several copies of mitochondrial DNA, as well as mitochondrial translational machinery. Although the aforementioned structure is shared by all mitochondria this does not mean that their morphology is unison or static. They are very

plastic organelles whose appearance may significantly vary from one cell type to another and it may also be a reflection of physiological state of the cells. Their shape ranges from kidney-like individual forms to elongated structures participating in a dynamic network made of physically connected mitochondria.

Given the fact that mitochondria are an organelle of symbiotic origin in the eukaryotic cell it is somewhat unexpected that in the course of the evolution the cell has come to depend on them in respect to so many functions. By means of oxidative phosphorylation they are the powerhouses of the cell. In addition, they harbor various enzymes involved in crucial steps in a variety of metabolic pathways such as fatty-acid metabolism, the citric acid cycle, and urea cycle. Mitochondria are also the site for biosynthesis of phospholipids, nucleotides, aminoacids, heme and many coenzymes. Likewise, synthesis of iron-sulfur clusters, important co-factors of many proteins, takes place here (Lill and Muhlenhoff, 2008). Mitochondria are involved in apoptosis, ageing and the process of carcinogenesis (Chan, 2006; Galonek and Hardwick, 2006; Youle, 2007; Youle and Karbowski, 2005). Recently, the discovery of components that tether ER to mitochondria has attracted special attention indicating an important role of mitochondria in Ca^{2+} homeostasis and phospholipid biogenesis (Hayashi et al., 2009; Kornmann et al., 2009). Also, mitochondria participate in response to viral infections (Seth et al., 2005). Considering all these functions it is not surprising that an array of disorders has been linked to mutations in mitochondrial proteins (Scheffler, 2001).

5.3 Overview of targeting and import of mitochondrial proteins

During their biogenesis mitochondrial proteins are synthesized in the cytosol, and since they have not reached their final conformation yet, and thus might be prone to aggregation, they are most likely stabilized through interaction with cytosolic factors (Neupert and Herrmann, 2007). The ones identified so far include cytosolic chaperones from the Hsp70 and Hsp90 families (Mihara and Omura, 1996; Young et al., 2003). Upon this initial interaction precursor proteins are targeted to mitochondria. Successful targeting requires a correct ´address´, i.e. signal within the protein that could be recognized by targeting factors and decoded by mitochondria. Mitochondrial proteins generally use two kinds of signals. Most of the matrix proteins, but also many inner membrane and intermembrane space proteins contain an N-terminal cleavable stretch of 10-80 amino acids termed presequence (Prokisch et al., 2006). The presequence guides the protein through both membranes and intermembrane space into the matrix, where it is cleaved by mitochondrial matrix processing peptidase (MPP)

(Neupert and Herrmann, 2007). Alternatively, proteins can lack any form of cleavable presequence, as it is the case with all outer membrane proteins and some inner membrane and IMS proteins. Here, the targeting signal is retained in the mature form of the protein. Recognition of the precursor proteins occurs through interaction with the translocase of the outer membrane (TOM) complex located in the mitochondrial outer membrane (MOM) (Bolender et al., 2008). This is why the TOM complex has been dubbed ´The Mitochondria´s Portal´ (Chacinska et al., 2009). Upon this interaction and depending on their signal proteins are relayed to one of the remaining five major translocation machineries in the outer and inner mitochondrial membrane, which help proteins reach their target location. Besides TOM complex there are two additional translocases in the outer membrane: TOB complex (topogenesis of mitochondrial outer membrane β-barrel proteins) also named SAM complex (sorting and assembly machinery) and MIM (mitochondrial import) complex. TOB/SAM complex (Figure 1, pathway e) is - as its name suggests - involved in biogenesis of β-barrel proteins, whereas MIM complex (Figure 1, pathway a) has been found to assist the import of most α-helical single-span proteins of the TOM complex. The inner mitochondrial membrane also houses three import machineries: TIM22 - translocase of the inner mitochondrial membrane carrier proteins (Figure 1, pathway b), TIM23, which contains a protein conducting channel and is involved in import of proteins that have a presequence (Figure 1, pathway c), and OXA1 (oxidase assembly 1) complex, insertase dedicated to inserting mitochondrially encoded, matrically synthesized proteins into the mitochondrial inner membrane (Figure 1, pathway f). OXA1 is also utilized by some nuclear encoded proteins, which are first delivered from the cytosol to the mitochondrial matrix before they are inserted into the membrane (Bonnefoy et al., 2009; Neupert and Herrmann, 2007). Functioning of these main import machineries is facilitated by supporting systems. For example, Tim9-Tim10 and Tim8-Tim13 chaperone complexes reside in the IMS and bind to the β-barrel and multispan carrier precursors en route from TOM complex to TOB and TIM22, respectively; different mitochondrial processing peptidases (MPPα/β, Oct1, IMP) contribute to protein biogenesis by their specific cleavage, MIA complex forms intramolecular disulfide bridges in proteins in the IMS, thus trapping them there (Figure 1, pathway d) (Hell, 2008; Herrmann et al., 2009). Similarly, various chaperones support folding of the proteins in the matrix (Hsp70, Mdj1, Hsp60/Hsp10, Hsp78, Zim17).

It should be noted that the elements of mitochondrial protein biogenesis presented so far do not cover all the varieties of import routes. For example, Fis1, Bcl-X_L, Bak and Omp25 – all of them MOM proteins - do not seem to require TOM complex, or any kind of

proteinaceous membrane structure for their import (Kemper et al., 2008; Setoguchi et al., 2006). Likewise, the predicted presequence in some matrix proteins (chaperonin 10) is not cleaved upon import, or is cleavable, but it is located at the C-terminus, such is the case with DNA helicase Hmi1 (Jarvis et al., 1995; Lee et al., 1999). Furthermore, the typical scenario implies that the import happens after the protein is synthesized in the cytoplasm - that is – posttranslationally. However, there is ample evidence that co-translational import also occurs (Ahmed and Fisher, 2009; Eliyahu et al., 2012).

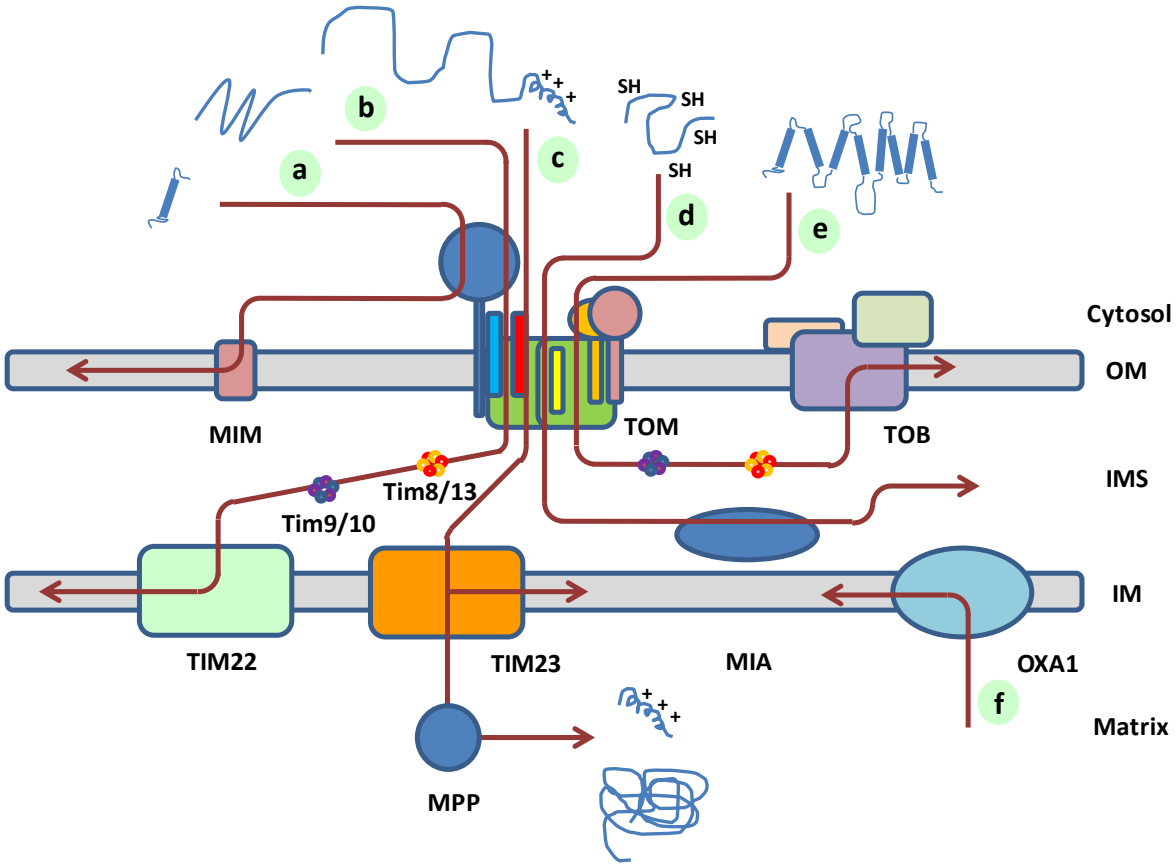

Figure 1. Translocation machineries in mitochondria. After interaction with TOM complex mitochondrial precursor proteins take different pathways to their final destination: singlespan proteins of the TOM complex are inserted into the MOM with the help of the MIM complex (a); multispan proteins of the IMM interact with Tim chaperones in the IMS and are then integrated into the inner mitochondrial membrane through TIM22 complex (b); presequence containing proteins reach inner mitochondrial membrane or matrix through TIM23 complex in the inner membrane, where MPP protease cleaves off the presequence (c); cysteine enriched proteins of the IMS are trapped there by formation of disulfide bonds in reaction catalyzed by the MIA complex (d); β-barrel precursors interact with Tim chaperones in the IMS and are integrated into the MOM through TOB complex (e). Proteins synthesized in the matrix are directly inserted into the inner mitochondrial membrane by OXA1 complex (f).

5.4 Biogenesis of mitochondrial outer membrane proteins

5.4.1 Topology of mitochondrial outer membrane proteins

Integral proteins of the mitochondrial outer membrane can generally be divided into those that span the membrane with their α-helical segments or, alternatively, as β-barrel structures (Figure 2, colored and in gray, respectively). Further, those that are anchored in the membrane via helical structures can do so with one or more α-helices. The single-spanning α-helical proteins can have different orientations. The so-called signal-anchored proteins have their α-helix in the N-terminal region (Figure 2, green). They expose the bulk of the C-terminal part of the protein to the cytosol and only a small N-terminal region protrudes into the IMS. They were named signal-anchored, since the targeting signal is formed by the transmembrane, anchoring segment. TOM complex receptors Tom20 and Tom70, as well as OM45 and a membrane isoform of Mcr1 are members of this group.

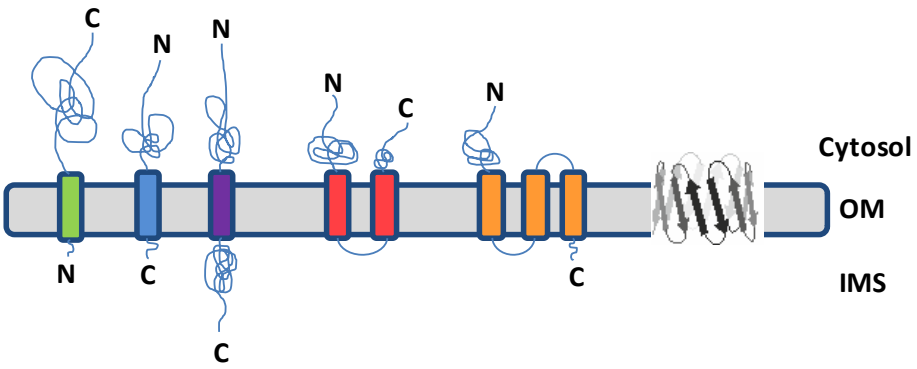

Figure 2. Topologies of mitochondrial outer membrane proteins. Signal-anchored (green), tail-anchored (blue), single-span with soluble IMS and cysolic domains (violet), multi-span proteins (red and orange), and β-barrel proteins (gray) are presented. (Adopted from (Walther and Rapaport, 2009))

Tail-anchored proteins such as small components of the TOM complex Tom5, Tom6, Tom7 and protein involved in membrane fission Fis1 appear similar to signal-anchored proteins. However, they have an opposite orientation - they are anchored into the membrane with an α-helix that is at the C-terminus (Figure 2, blue). The third group of single-spanning proteins has the same orientation like the tail-anchored ones, but here there is also considerable portion of the protein at the C-terminus protruding into the IMS (Figure 2, violet). TOM receptor Tom22, Mim1 and protein involved in initiation of mitophagy, ATG32, are members of this class. Fzo1 and Ugo1 are involved in mitochondrial fusion and span the membrane twice and three times, respectively (Figure 2, red and orange respectively). Similar

to them Tom40, Tob55, porin and Mdm10 also span the membrane several times, although not with α-helices, but with amphipathic β-strands.

5.4.2 Import machineries of the outer mitochondrial membrane

5.4.2.1 The TOM complex

For most of the nuclear encoded mitochondrial proteins the TOM complex is the point of initial interaction with mitochondria on their way to their target destination within the organelle (Figure 1). The central component of the complex is Tom40, which is in all likelihood a β-barrel protein (Ahting et al., 2001; Hill et al., 1998; Mannella et al., 1996). The exact stoichiometry of the complex is still not resolved, but it was estimated that two or three Tom40 copies participate in each complex (Rapaport et al., 1998b). The complex contains three receptors: Tom70, Tom20 and Tom22 (Figure 3). Whereas Tom20 is an initial recognition site for preproteins with presquence, but also β-barrel proteins (Krimmer et al., 2001; Saitoh et al., 2007), Tom70 recognizes polytopic inner membrane proteins such as metabolite carrier proteins (Brix et al., 1999). However, if one of the two receptors is genomically deleted, the other one can complement the function of the deleted receptor. Tom22 is a central receptor to which the precursor proteins are relayed before being inserted into the conducting pore (Chacinska et al., 2009). The C-terminal domain of Tom22 binds to the substrate proteins after they passed through the conducting pore, playing a role in their release to the IMS (Dukanovic and Rapaport, 2011). Also, it exhibits overlapping substrate specificity with Tom20 (Yamano et al., 2008). Structural investigations of the TOM complex using electron microscopy, import of rigid compounds and ion conductance showed that the channel of the TOM complex is 1.6 – 2.6 nm wide (Hill et al., 1998; Künkele et al., 1998; Schwartz and Matouschek, 1999). This is enough to accommodate two α–helices. The protein conducting pore is mainly made of Tom40. It provides an environment in which proteins can partially unfold and their folding

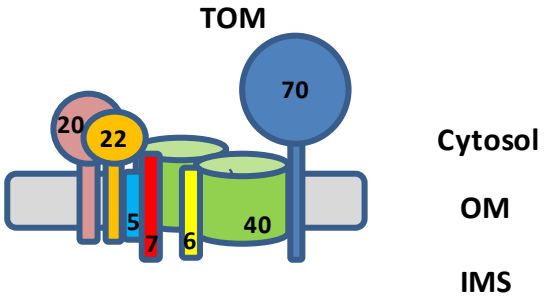

Figure 3. Composition of the TOM complex. The complex is composed of β-barrel protein Tom40, and α-helical proteins Tom5, Tom6 and Tom7 (membrane embedded small Toms), as well as MOM α-helical surface receptors Tom22, Tom20 and Tom70. All the helical components are predicted to be anchored to the membrane by a single TMD.

ability is maintained by the pore´s chaperoning activity (Esaki et al., 2003). The other, smaller units of the TOM complex are Tom5, Tom6 and Tom7. They are α-helical proteins and together with Tom22 and Tom40 form the TOM core complex with molecular mass of 450-500 kDa. Tom5 was proposed to participate in the transfer of preproteins from Tom22 receptor to the channel and stabilize the complex, as well as take part in the early stage of assembly of newly synthesized Tom40 molecules into preexisting TOM complexes (Dietmeier et al., 1997; Model et al., 2001a; Schmitt et al., 2005; Wiedemann et al., 2003). Tom6 and Tom7 regulate stability of the TOM complex. Tom6 together with the transmembrane domain of the Tom22 contributes to complex´s stability, probably by linking Tom22 and Tom40 (Alconada et al., 1995; Dembowski et al., 2001). On the other hand Tom7 facilitates disassembly and thus influences the dynamics of the TOM complex (Hönlinger et al., 1996).

5.4.2.2 The TOB complex

The TOB complex is another translocation machinery of the outer membrane that has a β-barrel protein at its core. Tob55 is the largest protein in this complex and has a predicted membrane-integrated β-barrel structure located in its C-terminal region (Figure 4). The N-terminal hydrophilic region is exposed to the intermembrane space (Paschen et al., 2005). Like Tom40, Tob55 is also an essential protein in yeast and has homologues throughout eukaryotic domain, but also in prokaryotes. In chloroplasts and bacteria, these are Toc75 and Omp85/BamA/YaeT, respectively (Eckart et al., 2002; Voulhoux and Tommassen, 2004). Electron microscopy investigations have shown that the pore of the TOB complex is 7-8 nm wide (Paschen et al., 2003). Like with the TOM complex pore, the structure of the TOB pore is still not quite clear. If one is to assume that the pore is Tob55 itself, release of the folded β-barrel protein would happen either through an unlikely event of lateral opening of Tob55´s β-barrel structure or through vertical release into the IMS (or cytosol, for that matter) followed by re-insertion, which also does not seem probable. Alternatively, if the assembling of the β-barrel structure happens among several Tob55 molecules, release would happen through rearrangement of these units (Chacinska et al., 2009).

Tob38 together with Tob55 makes the TOB core complex. Like Tob55, it is essential and its reduced levels lead to lower amounts of β-barrel proteins (Milenkovic et al., 2004; Waizenegger et al., 2004). It is, however, not integral protein of the mitochondrial outer membrane. Both of its termini face the cytosol and the bulk of the protein is embedded in proteinaceous environment (Kutik et al., 2008). It performs a receptor-like function by binding precursors to the TOB complex in cooperation with Tob55 (Chacinska et al., 2009; Habib et al., 2005). Metaxin 2 in mammals is considered to be its paralog in spite of low homology between the two (Kozjak-Pavlovic et al., 2007). Like Tob38, Mas37, the third member of the TOB complex is not an integral but rather a peripheral membrane protein (Gratzer et al., 1995; Wiedemann et al., 2003). Unlike the previous two TOB complex constituents, it is not essential in yeast, but rather required for growth at elevated temperatures (Gratzer et al., 1995). In the absence of Mas37 one can observe reduced insertion and assembly of Tom40 (Wiedemann et al., 2003). This in turn has further effects on import of β-barrel proteins and morphology of mitochondria. These effects stem from its function in releasing of precursor proteins from the TOB complex (Chan and Lithgow, 2008; Dukanovic et al., 2009). In mammals, the homolog of Mas37, Metaxin 1, seems to form a complex only with Metaxin 2, and not with Tob55. It does, however, play an important role in the biogenesis of β-barrel proteins (Kozjak-Pavlovic et al., 2007).

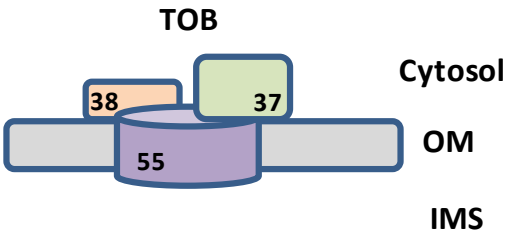

Figure 4. Composition of the TOB complex. The central component of the complex is the β-barrel protein Tob55. Further components are Tob38 and Mas37, which are attached to Tob55 peripherally, from the cytosolic side of the membrane.

Mdm10, another β-barrel protein, was originally identified as factor involved in mitochondrial distribution and morphology, and hence its abbreviated name (Sogo and Yaffe, 1994). Subsequent investigations showed that it associates with the TOB complex and promotes stepwise assembly of Tom40 with receptor proteins like Tom22 (Meisinger et al., 2004). It was proposed that this happens through its role in releasing Tom40 precursors from the TOB complex in cooperation with Tom7 (Yamano et al., 2010). In *Neurospora crassa* Mdm10 seems to participate in the biogenesis of porin as well (Wideman et al., 2010). Mdm10 has probably a dual function as it is also a part of the complex tethering ER and mitochondria (Kornmann et al., 2009). Finally, Mim1, a protein implicated in biogenesis of most single span α-helical proteins of the TOM complex was found to associate with the TOB complex, though transiently. It was proposed to modulate the TOB complex in a way that

supports assembly of the Tom40 with several α-helical proteins of the TOM complex (Becker et al., 2008).

5.4.2.3 The MIM complex

Mim1 was initially discovered in a high throughput screen for yeast mutants that accumulate mitochondrial precursor proteins (Mnaimneh et al., 2004). It is a rather small integral protein of the mitochondrial outer membrane (13 kDa) with considerable portions of the protein at the C- and N- terminus protruding into IMS and cytosol, respectively (Lueder and Lithgow, 2009; Waizenegger et al., 2005). It was demonstrated that its deletion affects biogenesis of single span α-helical proteins of the TOM complex - Tom20, Tom70 - and small TOM proteins: Tom5, Tom6, Tom7 (Becker et al., 2008; Hulett et al., 2008; Popov-Celeketic et al., 2008; Waizenegger et al., 2005). It also plays a role in the biogenesis of Tom40 through aforementioned interaction with TOB complex. Mim1 itself is found only in fungi, and there it exhibits a conserved amino acid sequence in the transmembrane domain of the protein (Waizenegger et al., 2005). It forms homooligomeric structures through its two helix dimerization GXXXG/A motifs, which are essential for its proper function (Popov-Celeketic et al., 2008). It is reported to be a component of a higher molecular weight complex of unknown composition with a size of 200-300 kDa as estimated by blue native PAGE (Becker et al., 2008). In contrast to the transmembrane domain, the cytosolic and intermembrane space segments are not conserved, and have initially been found expendable for the function of Mim1, since double, N- and C-terminally truncated Mim1 was able to rescue the phenotype of *mim1*Δ mutant (Popov-Celeketic et al., 2008).

5.4.3 Biogenesis of α-helical proteins of mitochondrial outer membrane

5.4.3.1 Signal-anchored proteins

The mitochondrial outer membrane proteins from this group include the TOM receptors Tom20 and Tom70, as well as OM45, a protein of high abundance, yet of an unknown function, and the 34 kDa isoform of the NADH-cytochrome *b5* reductase, Mcr1. All of them do not possess a cleavable presequence, but are targeted to mitochondria by the internal signal located at their N-terminus, which is also used as a structure that anchors them in the lipid bilayer. The signal-anchor domains of the above proteins do not share any sequence similarity, so the targeting information must be contained in the structural elements rather than in specific primary sequence (Rapaport, 2003). Key feature that is common to all of them

is moderate hydrophobicity of the transmembrane domain (TMD). Also, a net positive charge in the regions flanking the TMD enhances targeting and function of yeast signal-anchored proteins, but is not essential (Waizenegger et al., 2003). In mammalian cells, however, net positive charge in C-terminal flanking region of the TMD was reported to be crucial for mitochondrial targeting (Kanaji et al., 2000; Suzuki et al., 2002). Experiments with combining transmembrane segments of one protein with cytosolic domain of another within this group showed that signal anchors are functionally interchangeable (Waizenegger et al., 2003). In sharp contrast to the overwhelming majority of other mitochondrial proteins, signal anchored proteins do not require mitochondrial surface receptors for their import (Figure 5) (Ahting et al., 2005; Meineke et al., 2008; Merklinger et al., 2012; Schlossmann and Neupert, 1995; Schneider et al., 1991). The differences in biogenesis pathways exist also among the proteins of this group. Whereas biogenesis of Tom20 and Tom70 is affected by the loss of Mim1, this is not the case with OM45 and Mcr1 (Becker et al., 2008; Hulett et al., 2008; Meineke et al., 2008; Popov-Celeketic et al., 2008).

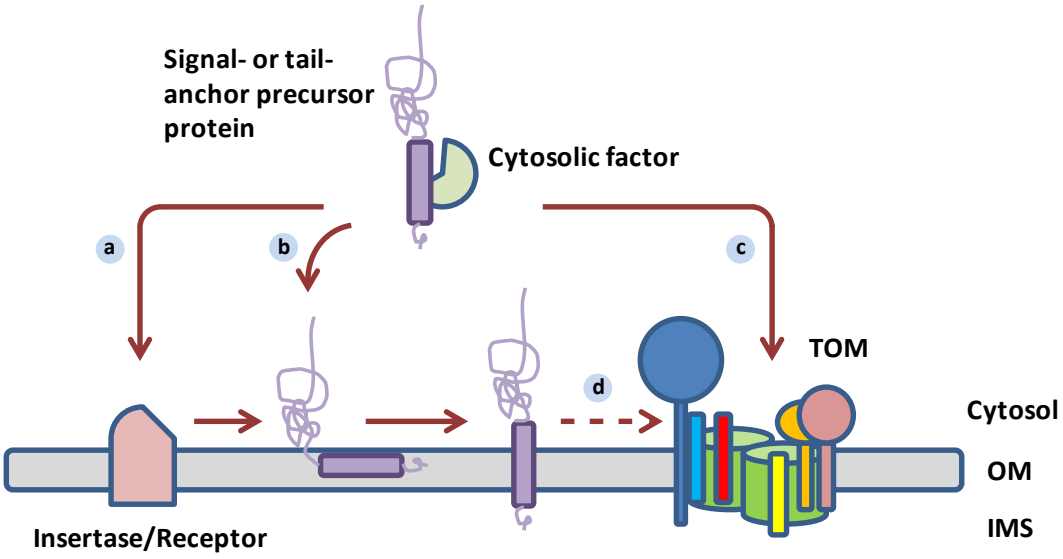

Figure 5. Possible import pathways for signal- and tail-anchored MOM proteins. The hydrophobic TMD of these proteins is likely bound to cytosolic chaperones. Insertion can be mediated by an insertase (a), or can occur in an unassisted manner (b). Signal- anchored Tom components require the presence of pre-existing TOM complexes for their assembly. The TOM complex can be involved in the initial steps of membrane integration (c), or alternatively its participation can be a post-insertional event, occurring after the initial insertion into the lipid bilayer (d). (Adopted from (Dukanovic and Rapaport, 2011))

5.4.3.2 Tail-anchored proteins

Tail-anchored proteins resemble signal-anchored proteins in several aspects. They look similar: they have one hydrophobic TMD functioning both as an anchor and a sorting signal located in this case at the C-terminus – hence the name tail-anchored. Again, IMS segment is very short, whereas the cytosolic domain is rather large. The TMD bears the same features as with signal-anchored proteins: it is of moderate hydrophobicity and flanked by positive charges, which were shown to be of great importance for the mitochondrial targeting of some proteins like mitochondrial isofrom of cytochorome b5 or VAMP-1B (Horie et al., 2002; Isenmann et al., 1998; Kuroda et al., 1998). Also, the transmembrane segments of mitochondrial TA proteins do not share any sequence similarities and are rather shorter than in their counterparts that are targeted to the ER (Horie et al., 2003; Isenmann et al., 1998). Given that tail-anchored proteins contain hydrophobic segments it is assumed that upon their synthesis they interact with yet un-indentified cytosolic factors, which prevent their aggregation and help their targeting to the mitochondrial outer membrane. The existence of such elements is supported by discoveries of various cytosolic chaperones that interact with peroxisomal and ER tail-anchored proteins (Abell et al., 2007; Dagley et al., 2009; Halbach et al., 2006; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). The pathway by which tail-anchored proteins are integrated into the MOM is currently unresolved (Figure 5). Whereas two of the small TOM proteins in *N. crassa* - Tom6 and Tom7 - require both the receptors of the TOM complex and the Tom40 protein for their biogenesis, the third small Tom, Tom5, needs only Tom40 (Dembowski et al., 2001; Horie et al., 2003). Furthermore, it seems that these proteins require Mas37, but not Tob55 and Tob38 from the TOB complex in the post-insertion steps of biogenesis (Stojanovski et al., 2007). Though, it is not clear if this is a direct effect, or a consequence of general disruption of β-barrel proteins biogenesis that includes Tom40. Unlike TOM-related tail-anchored proteins, insertion of Fis1 was found to be independent from exposed receptors and any known import components of the MOM (Kemper et al., 2008). Similarly, the integration of the mammalian tail-anchored proteins Bak, Bcl-XL and Omp25 was reported to be independent of known factors (Setoguchi et al., 2006). Thus, it seems that in this case the membrane properties dictated by lipid composition, and especially the low ergosterol levels of the MOM, play a significant role in import of these proteins.

5.4.3.3 Multi-span proteins

Multispan proteins comprise a distinct class of MOM proteins and are integrated into the lipid bilayer via multiple transmembrane segments (TMSs). Some of them, like Fzo1 in yeast (Mfn1/2 in mammals), cross the membrane twice, exposing N- and C-terminal domains toward the cytosol (Fritz et al., 2001; Rojo et al., 2002). Additional multispan MOM proteins with three or more TMSs are Ugo1 and OM14 in yeast and members of the Bcl-2 family and human peripheral benzodiazepine receptor (PBR) in higher eukaryotes (Burri et al., 2006; Chipuk et al., 2010; Coonrod et al., 2007; Hoppins et al., 2009; Otera et al., 2007). Previous research using mutants of Ugo1 and Mfn2 revealed that the domain that includes the predicted TMS harbors the information necessary for mitochondrial targeting, although additional targeting signals in other regions of the protein could not be excluded (Coonrod et al., 2007; Rojo et al., 2002). Experiments with Mfn2 indicate similarities between polytopic and tail-anchored (TA) proteins in terms of motifs and mechanisms responsible for their insertion into the MOM (Rojo et al., 2002). The idea that import pathways of TA and multispan proteins overlap (at least partially) is supported by import competition assays in which import of PBR was strongly inhibited by an excess amount of the TA protein Bak (Otera et al., 2007). However, in contrast to the biogenesis of TA proteins in which import receptors are probably not essential for the process (Kemper et al., 2008; Setoguchi et al., 2006), such receptors appear to play a role in the membrane integration of multispan proteins. Fzo1 requires protease-sensitive import receptors for its insertion into the MOM (Rapaport et al., 1998a), and later investigations revealed that import of mammalian polytopic proteins like PBR and Mfn2 require interaction with Tom70 but is independent of other TOM components (Becker et al., 2011; Otera et al., 2007; Papic et al., 2011; Yamano et al., 2008). The biogenesis of mammalian multispans apparently also depends on the unknown element from the IMS (Otera et al., 2007).

5.4.4 Biogenesis of β-barrel proteins of mitochondrial outer membrane

β-barrel proteins can be found in outer membranes of Gram-negative bacteria, mitochondria and chloroplasts, a distribution that echoes mitochondria´s and cloroplasts´ prokaryotic origin. In mitochondria this group is comprised of five members: Tom40, Tob55/Sam50, two isoforms of porin/VDAC, and Mdm10 (Neupert and Herrmann, 2007). They span the MOM with multiple β-strands, each of which is formed by 9-11 amino acid residues (Wimley, 2003). After their synthesis in the cytosol and assumed interaction with

chaperones that prevent their aggregation, β-barrel proteins are recognized at the surface of mitochondria mainly by Tom20 receptor of the TOM complex (Habib et al., 2005; Krimmer et al., 2001; Model et al., 2001; Rapaport and Neupert, 1999). Extensive investigations have led to conclusion that there is probably no linear signal sequence (Court et al., 1996; Rapaport and Neupert, 1999; Rapaport et al., 2001). Instead, it was proposed that signal by which β-barrel proteins are recognized as import substrates is contained in β-barrel-specific structural elements (Walther et al., 2009). After the initial recognition Tom20 directs the substrate further towards the protein conducting pore of the TOM complex (Model et al., 2001b; Paschen et al., 2003; Rapaport and Neupert, 1999; Wiedemann et al., 2003). Upon reaching the IMS side of the MOM, β-barrel proteins interact with small Tim chaperones, Tim9-Tim10 and Tim8-Tim13 complexes (Habib et al., 2005; Hoppins and Nargang, 2004; Wiedemann et al., 2004). The Tim chaperones participate in the relay of the proteins towards the TOB complex, where Tob38 recognizes C-terminal intra-mitochondrial sorting signal termed β-signal. This signal is composed of four conserved residues: a large polar residue (mostly lysine or glutamine), an invariant glycine and two large hydrophobic residues (Kutik et al., 2008). It is suggested that Tob38 widens the cavity of the TOB complex, enabling entrance and folding of the precursor (Kutik et al., 2008), upon which the precursor is released into the outer membrane, a step where Mas37 is involved (Chan and Lithgow, 2008; Dukanovic et al., 2009).

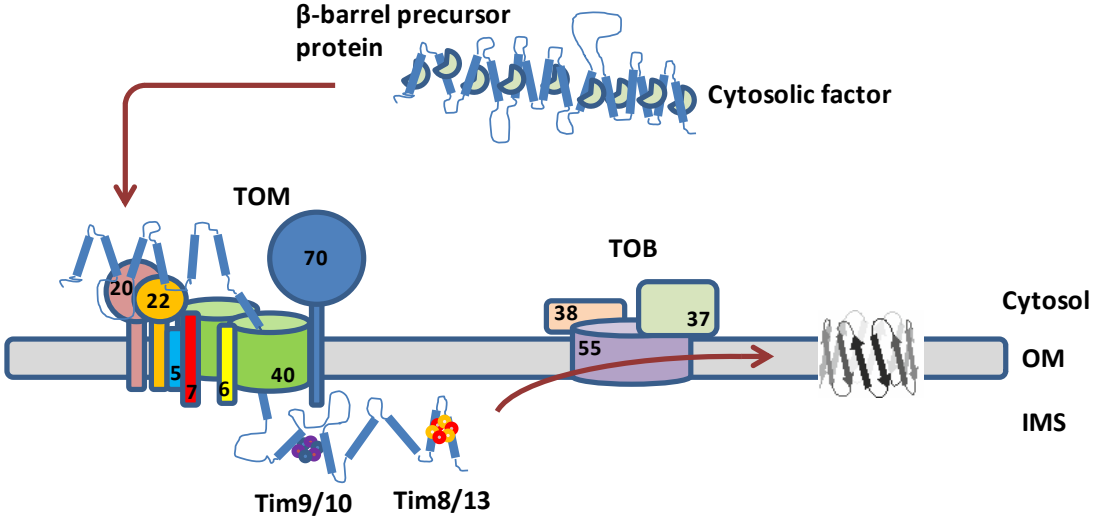

Figure 6. Biogenesis of β-barrel proteins. Cytosolic factors deliver β-barrel precursor proteins to the Tom receptors. Precursor proteins are then translocated through the import pore of the TOM complex and become exposed to the IMS. At this stage the β-barrel precursors bind to the small Tim chaperones. Finally, with help of the TOB complex β-barrel precursors are inserted and assembled into the MOM. (Adopted from (Dukanovic and Rapaport, 2011)

Here again, as with the TOM complex, it is more likely that the pore is formed by several Tob55 molecules than by a single Tob55 β-barrel. From such a central channel the precursor protein can be laterally released into the lipid phase (Kutik et al., 2008; Walther et al., 2009). It is important to emphasize that the processes happening at the TOM and TOB complex during β-barrel proteins insertion are highly coordinated and inter-dependent, although no physical interaction between TOB and TOM complexes has been observed yet (Habib et al., 2007; Paschen et al., 2003; Walther et al., 2009; Wiedemann et al., 2003). Other proteins like Mdm10 and Mim1 were shown to participate in Tom40-specific import pathway (Ishikawa et al., 2004; Meisinger et al., 2004; Waizenegger et al., 2005). Also, Mdm12 and Mmm1, proteins previously known for their involvement in maintenance of mitochondrial morphology seem to be implicated in the post-TOB steps of general β-barrel biogenesis. However, their function in this process remains unclear (Meisinger et al., 2007).

5.5 Biogenesis of β-barrel proteins in Gram-negative bacteria

Gram-negative bacteria have two cellular membranes: the inner and the outer one separated by periplasmic space. The two membranes are rather different. Whereas the inner membrane is a typical phospholipid bilayer with mainly α-helical proteins, the outer membrane is asymmetrical with phospholipids facing the periplasmic space and lipopolysaccharides in the outer leaflet. The outer membrane contains mainly β-barrel proteins and lipoproteins (Koebnik et al., 2000). β-barrel proteins of the bacterial outer membrane are synthesized as signal sequence containing precursors in the cytoplasm. There, they are kept in unaggregated state by SecB chaperones (Figure 7), with which they reach the Sec machinery located in the inner bacterial membrane. The Sec machinery translocates β-barrel proteins into the periplasm where the signal sequence is cleaved off (de Keyzer et al., 2003; Papanikou et al., 2007). After emerging on the periplasmic side of the inner membrane, β-barrel precursors interact again with chaperons: holding chaperone Skp and/or SurA bind to them and keep the precursors in the unfolded conformation (Chen and Henning, 1996; De Cock et al., 1999). Assembly of the outer membrane proteins is performed by β-barrel assembly machinery (BAM complex). Central component of this complex is BamA/Omp85, which is a bacterial homolog of mitochondrial Tob55. It is essential for viability and required for folding and assembly of all outer membrane proteins examined (Voulhoux et al., 2003). In *E. coli* BamA forms a complex with four lipoproteins termed BamB-E (Tashiro et al., 2008; Wu et al., 2005). It is suggested that the lipoproteins function as effectors and regulators of

the BAM complex, (Walther et al., 2009). Omp85 itself is presumed to have a β-barrel domain situated in the lipid bilayer and additional five polypeptide-transport-associated (POTRA) subdomains in the periplasmic space (Voulhoux et al., 2003), of which in some bacteria only the fifth one is essential (Bos et al., 2007). This fact renders a functional core of the Omp85 very similar to the mitochondrial Tob55 (Walther et al., 2009). The role of the POTRA domains is believed to be binding of accessory components of the BAM complex and possibly substrate binding and guidance towards the core of the BAM complex (Kim et al., 2007; Knowles et al., 2008). Interaction of Omp85 with substrates occurs through precursors´ C-terminus (Struyve et al., 1991). There is a conserved pattern in this region that includes 12 amino acid residues. The terminal phenylalanine with its aromatic nature seems to provide the strongest recognition signature, since its deletion significantly reduces substrate affinity (de Cock et al., 1997; Struyve et al., 1991).

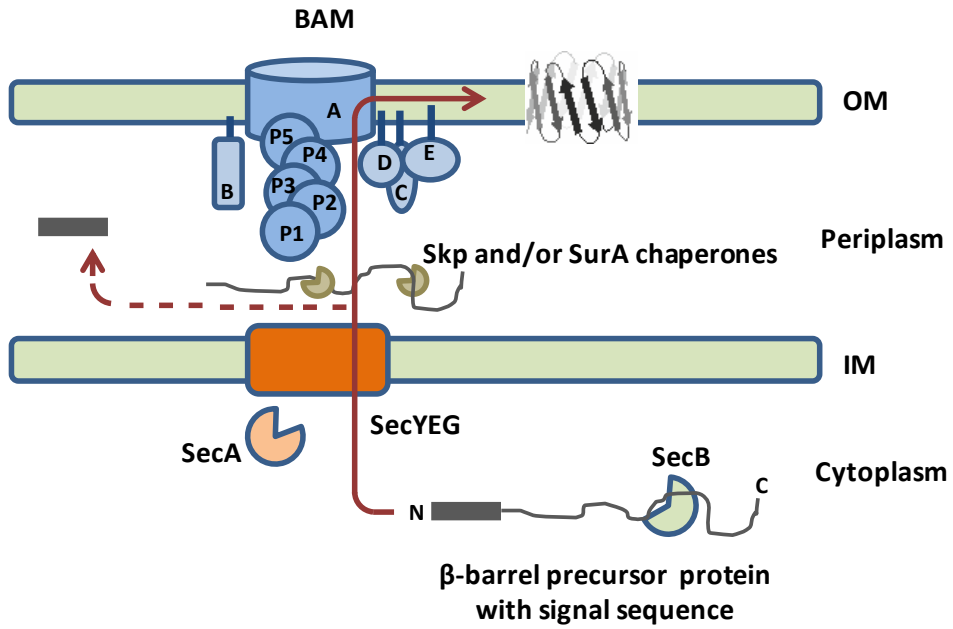

Figure 7. Biogenesis of bacterial OM β-barrel proteins. Bacterial β-barrel precursor proteins are synthesized in the cytoplasm with an N-terminal signal sequence. They are transported through the inner bacterial membrane into the periplasm by Sec machinery. Upon reaching the outer membrane the BAM machinery facilitates their insertion and assembly into the membrane. Throughout this pathway they remain in contact with chaperones. (Adopted and modified from (Rigel and Silhavy, 2012))

6 Aims of the study

Normal physiological operation of mitochondria depends on a distinct set of proteins specifically sequestered to this organelle. Given this, understanding the mechanisms of targeting and insertion of mitochondrial proteins is vital for understanding how mitochondria function. The work presented here focuses on the biogenesis of proteins that span the outer mitochondrial membrane several times.

Specifically, in the report *Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent import pathway* by Papić et al. one of the aims was to devise an assay for monitoring the *in vitro* import of Ugo1. This protein was used as a model protein in investigation of biogenesis of multi-span α-helical proteins. Furthermore, we sought to identify which of the known import factors are involved in the biogenesis of multi-span α-helical proteins. This study should shed new light on the import mechanism of this type of proteins. In the research article *A crucial role of Mim2 in the biogenesis of mitochondrial outer membrane proteins* by Dimmer et al. the overall goal was to elucidate the role of Mim2, a novel protein of the mitochondrial outer membrane. Specifically, its participation in the formation and function of the MIM complex, which was shown to be crucial for import of multispan α-helical proteins, was investigated. Such a detailed study should allow comprehensive understanding of the function of this complex in the biogenesis of the mitochondrial outer membrane. My goals were to provide additional evidence that Mim2 is indeed part of MIM complex and to investigate Mim2 involvement in the biogenesis of the TOM complex. Furthermore, I aimed to demonstrate direct interaction of Mim2 with substrate proteins and to test the ability of Mim2 to compensate for the lack of Mim1.

In the other two projects within this thesis our focus was on biogenesis of β-barrel proteins. We wanted to investigate if the signals that govern targeting of bacterial outer membrane β-barrel proteins can be recognized in the eukaryotic milieu and facilitate targeting of the precursors to mitochondria. In the article *Signals in bacterial beta-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria* by Walther et al. my goal was to demonstrate that the bacterial proteins are indeed imported into mitochondria and that their expression does not interfere with normal cellular functioning. In the continuation of this work (*Mitochondria can recognize and assemble fragments of a beta-barrel structure* by Müller et al.), I investigated the impact of the bacterial signal sequence on import of bacterial outer membrane β-barrels into mitochondria.

7 Summary of the results

7.1 *Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent pathway*

Study of import mechanisms of mitochondrial proteins relies in great part on *in vitro* experiments with radiolabeled protein precursors. By this approach it is possible to detect miniscule amounts of protein of interest imported into isolated mitochondria in a given period of time. However, inherent problem of such approach is that it alone can be insufficient to enable distinguishing between the precursors that were properly imported and those that were not. To address this problem in our experiments I developed a specific assay to monitor *in vitro* insertion of Ugo1. Previous experiments have demonstrated that Ugo1 is a protein with three TMDs with N-terminus exposed to cytosol and C-terminus in the IMS. Furthermore, it was found that trypsin treatment of mitochondria with C-terminally HA-tagged Ugo1 renders a 23 kDa C-terminal protease-resistant fragment (Coonrod et al., 2007; Hoppins et al., 2009). I imported radioactively labeled Ugo1 with an HA tag on its C-terminus into mitochondria from *ugo1*Δ strain overexpressing Ugo1-HA and was able to observe the ~23 kDa fragment when the reactions were analysed by an SDS-PAGE, followed by blotting and radiography (Fig 1B, upper panel). Decoration with an antibody against HA demonstrated that the radiography indeed detects C-terminal protease-protected fragment (Figure 1B. lower panel). Given that trypsin treatment degrades radioactive precursors that are not inserted, the 23 kDa radioactive signal obtained in the trypsin protection assay can be used to measure the levels of *in vitro* imported Ugo1. Through carbonate extraction it was demonstrated that the fragment is indeed membrane embedded and solubilization of mitochondria in Triton X-100 detergent excluded the possibility of protease protection artifact, which would have been membrane unrelated.

It was shown that various mitochondrial multispan proteins exhibit an overall dependence on ATP (Otera et al., 2007; Rapaport and Neupert, 1999; Wiedemann et al., 2001). This dependence can be linked to the Hsp70 and Hsp90 chaperones needed for protection against aggregation and misfolding (Young et al., 2003). In order to see if Ugo1 displays the same requirement, I performed the import assay with the reaction components that have been previously treated with apyrase, which degrades ATP. The treatment caused a strong reduction in import efficiency (Fig. 2A), similarly as with pSu9-DHFR (Fig 2B), matrix targeted precursor that was known to require ATP for efficient import.

Mitochondrial multispan proteins such as β-barrel precursors and inner membrane carrier-like proteins are recognized at the surface of the MOM by Tom70 and Tom20 receptors, respectively (Brix et al., 1999; Krimmer et al., 2001; Rapaport and Neupert, 1999; Yamano et al., 2008). I examined if removing exposed parts of mitochondrial surface receptors would affect import of Ugo1. Indeed, the import was reduced to levels comparable to those of Por1, a protein known to exhibit such dependence (Fig. 3A). To distinguish between Tom20 and Tom70 receptors´ contribution to this reduction, an import into mitochondria isolated from corresponding deletion strains was performed. Whereas import into *tom20*Δ mitochondria was even improved in respect to wt mitochondria, the import into *tom70*Δ*tom71*Δ mitochondria was reduced (Fig. 3 B and C). The similar effect was observed when the import reactions were analyzed by BN-PAGE (Fig. 3D). Next, I wanted to see if the *in vitro* findings correlate with the *in vivo* levels of Ugo1. To that end mitochondria were isolated from *tom20*Δ and *tom70*Δ*tom71*Δ strains and 10 µg and 30 µg were analyzed via SDS-PAGE. Again, *tom70*Δ*tom71*Δ mitochondria displayed lower amounts of Ugo1, while the levels of Ugo1 in *tom20*Δ mitochondria remained unchanged (Fig. 3E). Finally, to investigate interaction between Tom70 and Ugo1 Katrin Krumpe performed a binding assay. GST or a protein consisting of cytosolic domain of Tom70 receptor fused to GST was bound to glutathione beads and then incubated with radioactive precursors of Ugo1 (Fig. 3E). Radiolabeled precursors were found to bind to GST-Tom70 and not to GST alone demonstrating a direct Ugo1-Tom70 interaction.

Given that vast majority of proteins destined to mitochondria pass through TOM complex pore after interaction with the receptors of the TOM complex, I wanted to test if Ugo1 also shares that feature. To that end I performed import assay in the presence of various amounts of recombinant pSu9-DHFR, which is known to utilize TOM complex pore on its way to mitochondrial matrix (Fig S2 A). Import of Ugo1 was not strongly reduced even at highest amount of pSu9-DHFR present in the reaction, whereas Por1, a control protein, showed obvious decrease in import. Next, I probed the involvement of elements of IMS in the biogenesis of Ugo1: import of radiolabeled precursors was performed with mitochondria swollen through hypotonic pretreatment. In this way the mitochondrial outer membrane was transiently disturbed allowing the leakage of the IMS content into the surrounding solution. The mitochondria were subsequently analyzed by BN-PAGE (Fig. S2 B). In contrast to Tom40, whose import was severely affected, import of Ugo1 did not show major decrease even after 90 min. This result is in line with other experiments performed to address this issue. First, I wanted to know if Ugo1 requires Tim8/Tim13 and Tim9/Tim10 IMS chaperone

systems, which were shown to be involved in the biogenesis of multispan proteins with β-barrel structure (Habib et al., 2005; Hoppins and Nargang, 2004; Wiedemann et al., 2004). In the experiments where mitochondria from a deletion strain lacking Tim8/Tim13 chaperones were used import of Ugo1 was unaffected (Fig. S2 C), and neither were the steady-state levels (Fig. S2 D). Furthermore, mitochondria from a strain expressing a thermo-sensitive variant of Tim10 did not show major reduction in import capacity for Ugo1, as opposed to the one for a known Tim8/Tim10 substrate AAC1. Collectively, elements of IMS do not seem to be essential for the biogenesis of Ugo1.

TOB complex was reported to be crucial for insertion of multispan proteins with β-barrel structure (Gentle et al., 2004; Paschen et al., 2005; Pfanner et al., 2004). Thus, I wanted to test if it is also necessary for the biogenesis of Ugo1. For that purpose a strain lacking Mas37, which is a component of the TOB complex, was used. Import assay using *mas37*Δ mitochondria did not show any reduction in Ugo1 import (Fig. S3 A), and likewise the steady state levels of Ugo1 in the *mas37*Δ strain remained unchanged (Fig S3 B).

Mim1, a MOM protein, was demonstrated to be required for membrane integration of α-helical components of the TOM complex (Dimmer and Rapaport, 2009; Ishikawa et al., 2004; Lueder and Lithgow, 2009; Thornton et al., 2010; Waizenegger et al., 2005). It is conceivable that such a protein is also involved in the biogenesis of proteins consisting of several membrane-anchored α-helical proteins. The import assay with *mim1*Δ mitochondria showed a drastic reduction in imported Ugo1 (Fig. 4A). The same was observed when the import reactions were analyzed by BN-PAGE (Fig 4B). Furthermore, steady state levels of Ugo1 in *mim1*Δ mitochondria investigated both via SDS-PAGE and BN-PAGE reflected the *in vitro* situation (Fig. 4 C and D, respectively). Inspite of the major reduction in steady state levels, a little less than 50% of Ugo1 were still detectable. To test if these molecules are indeed membrane-integrated, an alkaline extraction was performed. This procedure partially disturbs integrity of mitochondrial membranes and segregates soluble and proteins loosely attached to mitochondrial membranes from membrane-anchored ones. Ugo1 like control membrane-anchored proteins appeared in the pellet fraction, whereas no Ugo1 was detectable in the supernatant (Fig. 4F). Finally, to probe the direct interaction between Mim1 and its substrate Ugo1 Katrin Krumpe performed a binding assay. MBP alone or Mim1 fused to MBP was bound to maltose beads and subsequently incubated with radiolabeled Ugo1 and analyzed via SDS-PAGE (Fig. 4E). Ugo1 precursors were found to bind to MBP-Mim1 and not to MBP alone demonstrating a direct Ugo1-Mim1 interaction.

7.2 *A crucial role for Mim2 in the biogenesis of mitochondrial outer membrane proteins*

Mim1 was reported to be a subunit of a higher molecular weight complex of unknown composition termed MIM complex (Becker et al., 2008; Dimmer et al., 2012; Ishikawa et al., 2004; Lueder and Lithgow, 2009; Popov-Celeketic et al., 2008; Waizenegger et al., 2005). In the experiments that combined immunoprecipitation with stable isotope labeling with amino acids in cell culture (SILAC) it was found that one of the proteins that potentially interacts with Mim1 is an ORF *YLR099W-A* (Dimmer et al., 2012). Because of its identification as an interaction partner of Mim1 the ORF was named *MIM2* (Dimmer et al., 2012). To investigate if Mim2 is a component of the MIM complex mitochondria from *mim1*Δ and *mim2*Δ strains harboring an empty plasmid or overexpressing either Mim1 or Mim2-HA were analyzed by BN-PAGE (Fig. 2C). After blotting the membrane was consecutively decorated against Mim1 and HA. Both Mim1 and Mim2-HA migrated as a complex of approximately 200 kDa indicating that the two proteins are components of the same oligomeric structure (Fig. 2C compare lanes 1, 3 and 12). The same experiment provided us with insights in respect to importance of Mim1 and Mim2 for the formation of the MIM complex. The absence of Mim2 rendered mitochondria without Mim1-containing complex (Fig. 2C, lane 4). This means that Mim2 is crucial for the biogenesis of Mim1 and formation of the MIM complex. The absence of Mim1 leads to a loss of a detectable Mim2-HA containing complex (Fig. 2C, lane 16), and if overexpressed in the *mim1*Δ background Mim2 can be observed as a non-assembled species (Fig. 2C, lane 18). To further support our claim that Mim1 and Mim2 are constituents of the same complex an antibody-shift assay with mitochondria from wt strain and *mim2*Δ strain overexpressing Mim2-HA was performed (Fig. 2D). Addition of HA antibody to *mim2*Δ + Mim2-HA mitochondria caused a major shift in gel migration of the entire complex, whereas no such shift was observable when the antibody was added to wt mitochondria.

Overexpression of Mim1 in the *mim2*Δ strain led to the formation of the MIM complex of apparently the same size as the one in the wt mitochondria (Fig. 2C, compare lanes 1 and 5). This observation suggested relatively small share of Mim2 in the overall complex. To explain such gel migration behavior mitochondria from strains overexpressing Mim2-GFP were analyzed via BN-PAGE (Fig. 2F). In comparison to the complex from the wt mitochondria, the MIM complex from Mim2-GFP containing mitochondria runs obviously at a less speed, however this difference is rather small. This confirms that although Mim2 is an integral component of the complex, it contributes much less to the complex´s mass than Mim1.

To investigate the function of Mim2 in the biogenesis of the TOM complex I analyzed its assembly status in the strains lacking Mim1 or/and Mim2 via BN-PAGE. Decoration with an antibody against Tom40 revealed that in the absence of Mim1 or/and Mim2 the TOM complex levels were reduced (Fig. 5B, left panel). Accordingly, an unassembled species of Tom40 was observable in those strains. Decoration with αTom22 showed how dramatic a decrease in fully assembled TOM complex is – no Tom22 was visible in all three mutated strains at the size corresponding to the fully assembled TOM complex (Fig. 5B, middle panel). At the same time TOB complex was unaffected by the lack of Mim1 or/and Mim2 (Fig. 5B, right panel). Similar defect in TOM complex biogenesis was visible when *in vitro* assembly of radiolabeled Tom40 in *mim2*Δ mitochondria was monitored (Fig. 6C). Likewise, when mitochondria from a strain with MIM2 under GAL promoter were analyzed, one could observe an increase in the amount of unassembled Tom40 species correlated with lack of galactose during cultivation (Fig. S8 B). The role of Mim2 in the biogenesis of TOM complex was also probed in an opposite approach. Here, mitochondria from a *mim1*Δ strain overexpressing Mim2-HA were analyzed (Fig. S7 C). The overexpression led to a reduction in the amount of the unassembled species of Tom40 observable in *mim1*Δ mitochondria. Collectively, Mim2 appears to be important factor in the biogenesis of the TOM complex.

Next, I wanted to probe the involvement of Mim2 in the import of MIM complex substrates. To that end an import of radiolabeled Ugo1 into mitochondria from *mim2*Δ strain overexpressing Mim2-HA followed by antibody shift assay with αHA was performed. Addition of HA antibody to mitochondria containing Mim2-HA caused a clear shift in radioactive signal of Ugo1, which was not observable in the reaction with wt mitochondria. This suggests that Mim2-containing MIM complex is directly involved with substrate during import process (Fig. 7, left panel). Accordingly, when the membrane was immunodecorated with antibody against Mim1, a size shift for the entire MIM complex was visible in mitochondria with HA- tagged Mim2 (Fig. 7, right panel).

7.3 *Signal in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria*

β-barrel proteins in yeast do not contain a cleavable presequence and so far no internal linear amino acid sequence that could serve as a targeting signal could be detected. They are thought to be recognized by mitochondria on the basis of their structure (Rapaport, 2003). In our study we investigated the ability of yeast cells to target bacterial outer membrane protein

PhoE to mitochondria, only cell structure that harbors such proteins in eukaryotes besides chloroplasts. Yeast´s capability to mitochondrially target bacterial proteins would be a proof of principle that it is indeed structure that serves as a signal, since bacterial and yeast β-barrel proteins do not share significant sequence similarities.

Subcellular fractionation of cells expressing PhoE demonstrated that the protein appears in the mitochondrial fraction (Walther et al., 2009). To test if PhoE is integrated into mitochondrial membrane or remains uninserted alkaline extraction was performed on mitochondria obtained from the PhoE low-expressing strain (Fig. 3A). This procedure disturbs the integrity of mitochondria releasing their soluble content and disrupting attachment of the proteins that are only loosely bound to mitochondrial membranes. Separation of the two protein populations is then achieved by centrifugation. PhoE was found in the pellet fraction together with other proteins from the inner and outer mitochondrial membranes suggesting that it is integrated into a membrane. To further substantiate this finding, a protease treatment was performed (Fig. 3A). Mitochondria were treated with proteinase K present at two different concentration without and with previous hyposmotic treatment. Hypoosmotic pretreatment makes IMS accessible to protease and was used to exclude the possibility that apparent protease resistance stems from IMS accumulation of the PhoE precursors. Membrane integrated mitochondrial β-barrel proteins are rather resistant to protease treatment and PhoE appeared to behave that way, just like MOM β-barrel protein porin. Next, to exclude the possibility that membrane localization is an artifact caused by aggregates of PhoE urea extraction was performed. Mitochondria from strains with high and low expression levels of PhoE were analyzed (Fig. 3B). A great deal of PhoE in mitochondria from the high-expression strain was found in the supernatant, suggesting high level of aggregation. Conversely, all the PhoE in the strain with lower expression level was found in the membrane fraction. Collectively, results indicate that PhoE is embedded into mitochondrial membrane in the strain with lower expression of PhoE.

To exclude the possibility that the observed targeting of PhoE to mitochondria is somehow a consequence of disturbed biogenesis of yeast cell´s own proteins three other bacterial β-barrel proteins, OmpA, OmpC and Omp85, were expressed in yeast and steady state levels of mitochondrial proteins were monitored (Fig. S3 B). Neither mitochondrial β-barrel proteins Tob55 and porin exhibited major changes in their levels, nor was the signal-anchored Tom20 affected.

Interaction of bacterial insertase for β-barrel precursors with its substrates is achieved through precursor´s C-terminus. The most important feature of this signature sequence is the

ultimate phenylalanine. To test the relevance of this sequence for sorting in yeast cells mitochondria from strains expressing PhoE without the terminal Phe were subjected to urea extraction. In the strain that had higher level of expression of the truncated construct greater part of PhoE was found in the urea-soluble fraction, while in the strain with lower expression levels all PhoE was found in this fraction (Fig. 4A). Since it was shown that PhoE without terminal Phe is targeted to mitochondria (Walther et al., 2009), findings of the urea extraction experiment suggest incorrect assembly into the MOM.

7.4 *Mitochondria can recognize and assemble fragments of a β-barrel structure*

Experiments with heterologous expression of bacterial β-barrel proteins have shown that yeast cells can recognize non-self β-barrel structures and target them to mitochondria (Walther et al., 2009). These findings were obtained by use of recombinant constructs devoid of bacterial cleavable signal sequence needed for targeting to the Sec machinery in the inner bacterial membrane. Since mitochondrial β-barrel proteins do not contain any form of cleavable presequence, it seemed interesting to investigate if bacterial β-barrel protein expressed in yeast cells with its signal sequence would still be targeted to mitochondria. After isolating mitochondria from strains harboring plasmids coding for either mature form of PhoE or its variant with the signal sequence, Sig-PhoE, levels of expressed proteins were compared (Fig. 1A). Sig-PhoE was indeed found in the mitochondria, however its levels were reduced in comparison to those of PhoE without signal sequence. To rule out the possibility that the difference arose as a consequence of reduced mRNA levels in the case of Sig-PhoE Kai S. Dimmer analyzed mRNA levels from both strains via RT-PCR (Fig. 1B). The results indicated presence of comparable amounts of mRNAs of interest in the two strains. Possible explanation of the observed difference might be enhanced degradation of the signal-sequence containing proteins. To test this option growing yeast cultures were supplemented with cycloheximide, which arrests protein synthesis, and crude mitochondria were isolated. Analysis of PhoE and Sig-PhoE levels detected a short-lived modified version of Sig-PhoE (Fig. 1C). Non-modified forms of both Sig-PhoE and PhoE, as well as control β-barrel protein porin, remained stable with comparable turnover rates. In contrast, Tom70, a MOM protein with large segment protruding into the cytosol, exhibited expected enhanced turnover in comparison to membrane embedded porin and PhoE. To test if the modified form of Sig-PhoE is indeed membrane embedded an alkaline extraction was performed. Results of this analysis showed that the modified versions of Sig-PhoE are mostly found in the supernatant together

with other soluble proteins such as matrix protein Hsp60 (Fig. 1D). Conversely, PhoE and the unmodified form of Sig-PhoE were sequestered to the pellet fraction along with the MOM protein porin. Thus, the modified forms are not integrated into cellular membranes. Next, to analyze the nature of the modification, isolated membrane fractions from the two strains were treated with recombinant endoglycosidase H, which removes oligosaccharides from N-linked glycoproteins. The treatment caused a disappearance of the modified Sig-PhoE and an equivalent increase in the amount of non-modified Sig-PhoE in (Fig 1E). This rendered PhoE signal intensities from the two strains equal suggesting equal expression levels. However, given that glycosylation within the cell occurs in the endoplasmic reticulum (ER), this also implies that a part of synthesized Sig-PhoE is targeted to this organelle. The reason for this is most likely similarity of the signal sequence of PhoE with the eukaryotic ones. However, when a subcellular fractionation was performed modified Sig-PhoE was not detectable in the light microsomal fraction (ER) (Fig. 1F). The reason for this is likely the aforementioned instability.

It was observed that Sig-PhoE migrates slower than PhoE when analyzed with SDS-PAGE. This suggested that the signal sequence is not processed in yeast. Yeast peptidase Imp shares several key features with bacterial leader peptidase that cleaves the signal sequence after translocation across inner bacterial membrane (Schneider et al., 1991). In order to test if the signal sequence in yeast remains intact PhoE and Sig-PhoE were expressed in a strain deleted for one of the subunits of the Imp peptidase. When mitochondria from these strains were analyzed, an identical migration pattern of Sig-PhoE was observed, suggesting lack of processing of bacterial signal sequence in yeast (Fig. 1G).

Collectively, results suggest that signal sequence in eukaryotic β-barrel proteins was lost because of its potential to interfere with proper, mitochondrial targeting of these proteins within the cell.

8 Discussion

In the present work I wanted to elucidate the biogenesis mechanism of mitochondrial α-helical multi-span proteins. Using Ugo1 as a model protein and a novel proteolytic assay I discovered that the import receptor Tom70 is involved in initial recognition of Ugo1 precursors. I could also identifiy Mim1, a single span protein with soluble segments in the cytosol and in the IMS, as a critical element in the insertion process of Ugo1. Conversely, the import pore of the TOM complex, elements of the intermembrane space and the TOB complex are not required for this process. The dependence of the biogenesis of MOM polytopic proteins on Tom70 was already demonstrated in mammals (Otera et al., 2007). Hence, the current identification of Tom70 is in line with this previous report and the fact that Tom70 is known to be a receptor with prevalent affinity to polytopic carrier proteins of the inner membrane (Brix et al., 1999). Moreover, it is concordant with the finding that the Tom70 receptor is a docking site for cytosolic chaperones, which are most likely necessary for prevention of misfolding and aggregation, given the hydrophobic nature of the multi-span proteins (Young et al., 2003). Multispan MOM precursors seem to by-pass the pore of the TOM complex after interaction with Tom70; they are relayed to the MIM complex and inserted into the membrane. It is noteworthy that both steady state levels of Ugo1 in *tom70*Δ*tom71*Δ cells and the efficiency of *in vitro* import of radiolabeled Ugo1 into mitochondria isolated from these mutated cells are reduced by around 50% (Papic et al., 2011). This means that Tom70 is not absolutely essential for import of Ugo1, but functions as an enhancer of this process. Lack of Mim1 causes a more severe reduction in levels of *in vitro* imported radiolabeled Ugo1. Accordingly, steady state amounts of Ugo1 in *mim1*Δ strain are also lowered. It is noteworthy that the lack of Mim1 apparently does not reduce the quality of import process, since the imported Ugo1 is indeed integrated in the outer membrane (Papic et al., 2011). Similar effects of *MIM1* deletion were shown also for other multispan proteins of the MOM in a work parallel to ours (Becker et al., 2011). It is interesting that even if the cytosolic domains of MOM proteins are digested by trypsin prior to *in vitro* import reaction, the import is still not completely abolished (Papic et al., 2011). A possible explanation to this observation might be provided by a previous report that overexpression of the transmembrane domain of Mim1 alone is able to rescue *mim1Δ* phenotype (Popov-Celeketic et al., 2008). Bearing this in mind, it sounds reasonable to speculate that the TMDs of Mim1 provide a platform that can facilitate import and insertion of multispan helical proteins that contain hydrophobic segments in the form of α-helices, be it one or more of them. Considering that

Mim1 contains also soluble portions protruding into the cytosol and IMS, it is feasible that the combination of hydrophilic cytosol-exposed segments and the membrane embedded moderately hydrophobic complexes is a right environment for handling the proteins that have complex structure with several α-helices connected by hydrophilic loops. The energy that would drive the process of insertion would be gained from stabilization of hydrophobic segments of the precursor protein amongst TMSs of the MIM complex. One additional aspect that is unknown, and could help us to understand the biophysics of protein insertion, is the specific architecture of the MIM complex within the lipid bilayer. It could be that MIM complex causes a favorable perturbation in its surrounding, which contributes to the overall insertion process. In this sense, Popov-Čeleketić suggested that ergosterol might bind to the TMS of Mim1 and function as an agent of this perturbation.

So far, Mim1 was found exclusively in fungi. It will be very interesting to see if any proteins with similar (or at least partially similar) function will be discovered in mammals in the future.

In conclusion, we propose that the integration of Ugo1 into the MOM occurs via a novel pathway. This pathway involves initial docking of chaperone-associated Ugo1 to the import receptor Tom70. Ugo1 precursor is then inserted into the membrane in a process that is facilitated by the membrane-embedded protein Mim1 (Fig. 8).

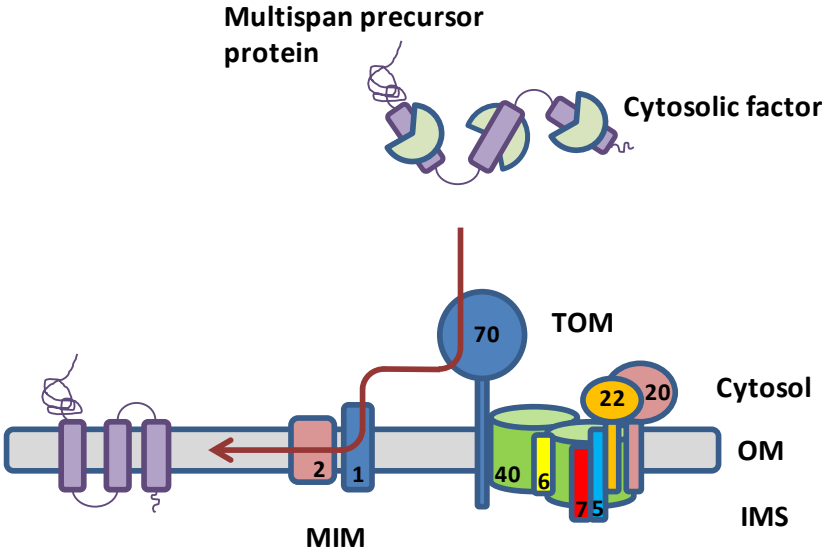

Figure 8. Biogenesis of multispan proteins. Precursor proteins likely interact with cytosolic factors whereupon they are delivered to the Tom70 receptor. Integration occurs immediately after this step through action of the MIM complex without involvement of the protein conducting pore of the TOM complex.

Mim1 was a protein surrounded with a fair share of unknown for quite a while, and yet at the same time its importance for biogenesis of several proteins - most of single-span proteins of TOM complex - was recognized (Becker et al., 2008; Hulett et al., 2008; Popov-Celeketic et al., 2008; Waizenegger et al., 2005). The results from our work on Mim1 and polytopic α-helical proteins of the MOM have provided valuable insights and enabled us to better understand the scope of the roles and significance of Mim1. Naturally, we aimed at learning more about this protein and looked for proteins that interact with Mim1. In the SILAC based immunoprecipitation experiment we found one protein that was enriched to a similar extent as the bait protein Mim1. Since the identified protein was until then classified as an ORF, and it interacted with Mim1, we named it Mim2 (Dimmer et al., 2012). At the same time the newly discovered protein was a good candidate for another factor that is involved in the biogenesis of α-helical proteins of the MOM. Existence of such a factor was postulated, since we observed that even in the absence of Mim1 we were able to see some import of the multispan α-helical proteins.

Further analysis of the novel protein revealed that Mim1 and Mim2 are members of the same complex (Dimmer et al., 2012). The absence of either of the two renders mitochondria without MIM complex. Given that in the absence of Mim2 there are only trace amounts of Mim1, one can conclude that Mim2 is vital for biogenesis of Mim1. However, the two proteins seem to behave differently within the complex: whereas the over-expressed Mim1 is still able to form MIM complex in the *mim2*Δ background, it does not work the other way around. Over-expressed Mim2 in the *mim1*Δ background does not form MIM complex, and is seen as unassembled species (Dimmer et al., 2012). Since our BN-PAGE analysis showed that regardless of the level of expression, the size of MIM complex remains the same, it led us to conclude that the MIM complex is made of fixed number of copies of Mim1 and Mim2. Also, it is noteworthy to observe that the size of the MIM complex did not change even if Mim2 was not present, which means that the complex is mainly composed of Mim1 molecules, with only very few Mim2 molecules. This is underscored by just a slight change in the migration behavior on BN-PAGE observed when the MIM complex from WT mitochondria was compared with the one from mitochondria expressing Mim2-GFP instead of Mim2. However, it seems that the interaction between Mim1 and Mim2 within the complex is quite tight, since we were able to see a size shift of the entire complex when we incubated Mim2-HA containing mitochondria with an antibody against HA-tag. Also, Mim2 remains an integral part of the complex throughout the process of substrate insertion as demonstrated in antibody-shift experiment with radioactively labeled precursors. This obvious

close interaction of the two proteins is reflected in our observations that the Mim2 deletion causes phenotypes that are very similar to those of Mim1 deletion. In both cases the mitochondria exhibit lower steady state levels of Mim1 substrates like Tom20 and polytopic proteins and have reduced stability of the TOM complex. Like Mim1, Mim2 is also a single-span protein with soluble C-terminal and N-terminal segments in the IMS and cytosol, respectively (Dimmer et al., 2012; Ishikawa et al., 2004; Lueder and Lithgow, 2009; Waizenegger et al., 2005) and both of them have so far been found only in fungi (Dimmer and Rapaport, 2009).

As stated previously, Mim2 is necessary for the insertion and/or stability of Mim1. Hence, the lack of Mim1 would produce all the listed effects of Mim2 deletion, rendering Mim2 a secondary factor. However, it seems that Mim2 plays a unique role in the insertion as its over-expression was able to reduce the amount of unassembled Tom40 in the cells lacking Mim1. On the other hand, it is tempting to speculate that stoichiometry of the MIM complex with less Mim2 than Mim1 molecules may indicate also that Mim2 functions also as some sort of an organizer of the MIM complex. At any rate, the discovery of Mim2 is a very important step in understanding the factors involved in the biogenesis of (multitopic) α-helical proteins of the MOM. However, further investigations which would look more closely into separate specific contributions of Mim1 and Mim2 to the insertion process might lead us to a better understanding of the mechanisms at play in the biogenesis of α-helical proteins of the MOM.

In the other two projects within this thesis our focus was on the biogenesis of β-barrel proteins. Like other proteins of the MOM they do not contain a presequence. Moreover, so far no internal linear amino acid sequence that could serve as a targeting signal was found in these proteins. Thus, there is an assumption that mitochondrial import machinery recognizes β-barrel specific structural elements (Rapaport, 2003). To test this hypothesis we used yeast strains that heterologously expressed bacterial β-barrel proteins, which had no significant sequence similarities with the endogenous mitochondrial β-barrel proteins. In this way these proteins were devoid of any potential targeting sequence, which could have evolved during the endosymbiotic evolution of mitochondria. We were able to show that the proteins were imported into mitochondria (Walther et al., 2009). Thus, it seems that the ability of a protein to adopt a membrane-embedded β-barrel structure is sufficient to ensure its specific targeting to mitochondria. This is concordant with previous research showing the necessity of partial folding for a β-barrel protein to be efficiently imported into mitochondria (Rapaport and

Neupert, 1999). On the other hand, this means that the mitochondrial β-barrel proteins, which most likely evolved from bacterial ancestors, did not need to develop targeting signals anew. Of note, the ability of bacterial proteins to be targeted to mitochondria is apparently not a general feature in eukaryotic systems. Müller et al. reported that non-pathogenic bacterial porins were not targeted to mitochondria of mammalian cells. The only bacterial β-barrel protein that was targeted to the mitochondria in their investigations was PorB from a pathogenic *Neisseria* species (Müller et al., 2002). It cannot be excluded that it is the unique virulence features that are responsible for the targeting of PorB to mitochondria, rather than general characteristics pertaining to its β-barrel structure.

Since we were able to see that bacterial β-barrel proteins can be recognized by mitochondria through Tom20 receptors (Walther et al., 2009) and subsequently successfully integrated into mitochondrial outer membrane, we were also interested if the substrate recognition is maintained in the steps downstream of translocation. Namely, bacterial β-barrels contain a C-terminal signature sequence that provides initial contact with the Omp85 (Robert et al., 2006). Phenylalanine at the ultimate C-terminal position of this sequence is its most important feature crucial for proper membrane integration and assembly (Struyve et al., 1991). The deletion of this phenylalanine in PhoE, our heterologously expressed bacterial protein, caused it to fail to be correctly imported into mitochondria, although the targeting itself was not affected. Collectively our results demonstrate that ancestral signals from bacterial β-barrel proteins can be recognized and correctly processed by mitochondrial assembly machinery.

The mitochondrial β-barrels of the MOM are considered to be reminiscent of mitochondria´s bacterial past. These and other MOM proteins do not contain any form of presequence, but are targeted to mitochondria with the help of their internal signals. The absence of an N-terminal signal sequence is a major difference between bacterial outer membrane and mitochondrial outer membrane β-barrels since the former contain an N-terminal cleavable signal sequence. This sequence guides them after their synthesis in the cytosol to the Sec machinery located in the bacterial inner membrane. After translocation through the inner membrane the presequence is cleaved off, and the precursor advances through the periplasmic space to the outer bacterial membrane where its integration occurs with support from BAM machinery. We wondered whether there is any substantial reason for mitochondrial β-barrel proteins to lose this signal. To address this point, I expressed in yeast cells a bacterial β-barrel protein with its signal sequence and observed that this protein was imported into mitochondria. However, the signal sequence-containing protein was imported to

a significantly lower level than the protein without the signal sequence. Furthermore, a population of unstable modified proteins with signal sequence was present. When combined, the two species of the protein made roughly the same amount as was observed to be mitochondrially localized in the case of the heterologously expressed bacterial β-barrel protein without signal sequence. We found the modified molecules to be glycosylated, a reaction that happens in the ER lumen. Given that bacterial β-barrel signal sequence is similar to the ER targeting sequence, it is conceivable that this signal competes with the mitochondrial targeting sequence contained in the β-barrel domain of the protein. Thus, while one part of the molecules is targeted towards the TOM complex and imported into mitochondria, the other molecules are directed to Sec machinery in the ER, which translocates them into the ER lumen. There these molecules are glycosylated and probably recognized as nonfunctional and eventually degraded. Obviously, this scenario provides a selective pressure that favors the disappearance of the signal sequence in mitochondrial β-barrel proteins, since their import and assembly would have been less efficient if the signal sequence had been retained.

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10 Acknowledgements

I did my PhD studies in the group of Prof. Dr. Doron Rapaport and I would like to express my gratitude to him for having me in his lab, for his generous teaching and constant support in my own scientific endeavors.

Also, I want to thank Prof. Dr. Ralf-Peter Jansen, Prof. Dr. Gabriele Dodt and Dr. Dirk Linke for their time and effort in being on my defence examination panel.

Further credits for some good years go to the persons who I have been sharing my lab everyday with.

To Jovana for her work on the Ugo1 project, as well as for being a patient instructor and a good friend. To Katrin for her amazing help with Ugo1 project when I needed it most. To Dirk for trusting me with his project. To Elena for her outstanding work and her great energy. To Gerti for sharing the right side of the lab with me. To Katharina for making everyday lab-life work. To Rhea, Elisa, Jonas, Thomas and Benni for being the greatest possible wrong side of the lab. To Anja for being a *spiritus movens* in matters of creativity. To Kai for his help on the Ugo1 project, nice cooperation on his project, his effort and sharing his knowledge. To Hiroki for his generous advice. To Tao for his good ways.

I would also like to thank my Tübingen friends Janani, Anna, Leonie, Orit, Agnia, Sarah, Markus, Alex and Franklin for being there for me. Finally, I would like to thank my Belgrade friends and family for their very long-term support *i svojim mami, tati i sestri što su mi porodica.*

11 Appendix – Accepted papers

Published August 8, 2011

Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent import pathway

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The mitochondrial outer membrane (MOM) harbors several multispan proteins that execute various functions. Despite their importance, the mechanisms by which these proteins are recognized and inserted into the outer membrane remain largely unclear. In this paper, we address this issue using yeast mitochondria and the multispan protein Ugo1. Using a specific insertion assay and analysis by native gel electrophoresis, we show that the import receptor Tom70, but not its partner Tom20, is involved in the initial recognition of the Ugo1 precursor. Surprisingly, the import pore formed by the translocase of the outer membrane complex appears not to be required for the insertion process. Conversely, the multifunctional outer membrane protein mitochondrial import 1 (Mim1) plays a central role in mediating the insertion of Ugo1. Collectively, these results suggest that Ugo1 is inserted into the MOM by a novel pathway in which Tom70 and Mim1 contribute to the efficiency and selectivity of the process.

Introduction

The mitochondrial outer membrane (MOM) contains a diverse set of proteins with various functions (Burri et al., 2006; Schmitt et al., 2006; Zahedi et al., 2006). All of these proteins, like the vast majority of mitochondrial proteins, are nuclear encoded, synthesized in the cytosol, and imported into the organelle (Neupert and Herrmann, 2007; Chacinska et al., 2009; Endo and Yamano, 2009; Walther and Rapaport, 2009). Multispan proteins comprise a distinct class of MOM proteins and are integrated into the lipid bilayer via multiple transmembrane segments (TMSs). Some of them, like Fzo1 in yeast (Mfn1/2 in mammals), cross the membrane twice, exposing N- and C-terminal domains toward the cytosol (Fritz et al., 2001; Rojo et al., 2002). Additional multispan MOM proteins with three or more TMSs are Ugo1 and OM14 in yeast and members of the Bcl-2 family and human peripheral benzodiazepine receptor (PBR) in higher eukaryotes (Burri et al., 2006; Coonrod et al., 2007; Otera et al., 2007; Hoppins et al., 2009; Chipuk et al., 2010). Previous research using mutants of Ugo1 and Mfn2 revealed that the domain that includes the predicted TMS harbors the information necessary for mitochondrial targeting, although additional targeting signals in other regions of the protein could not be excluded (Rojo et al., 2002; Coonrod et al., 2007). Experiments with Mfn2 indicate similarities between polytopic and tail-anchored (TA) proteins in terms of motifs and mechanisms responsible for their insertion into MOM (Rojo et al., 2002).

The idea that import pathways of TA and multispan proteins overlap (at least partially) is supported by import competition assays in which import of PBR was strongly inhibited by an excess amount of the TA protein Bak (Otera et al., 2007). However, in contrast to the biogenesis of TA proteins in which import receptors are probably not essential for the process (Setoguchi et al., 2006; Kemper et al., 2008), such receptors appear to play a role in the membrane integration of multispan proteins. Fzo1 requires protease-sensitive import receptors for its insertion into the MOM (Rapaport et al., 1998), and later investigations revealed that import of PBR and Mfn2 requires interaction with Tom70 but is independent of other translocase of the outer membrane (TOM) components (Otera et al., 2007; Yamano et al., 2008).

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Abbreviations used in this paper: AAC, ATP–ADP carrier; BN, blue native; DHFR, dihydrofolate reductase; IMS, intermembrane space; MBP, maltose-binding protein; MOM, mitochondrial outer membrane; PBR, peripheral benzodiazepine receptor; PK, proteinase K; TA, tail anchored; TMS, transmembrane segment; TOM, translocase of the outer membrane.

Supplemental Material can be found at:
http://jcb.rupress.org/content/suppl/2011/08/06/jcb.201102041.DC1.html

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 194 No. 3 397–405
www.jcb.org/cgi/doi/10.1083/jcb.201102041

Published August 8, 2011

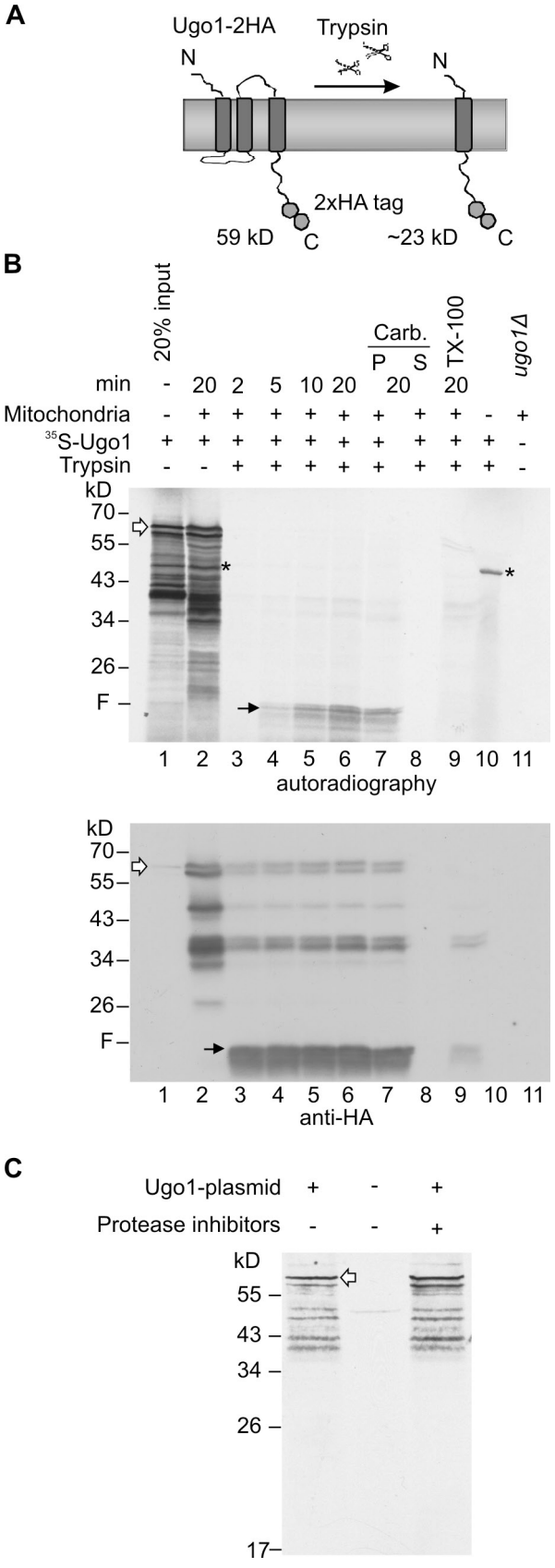

Figure 1. **A novel assay to study in vitro import of Ugo1.** (A) A schematic representation of 2HA-tagged Ugo1 (Ugo1-2HA) and Ugo1-2HA C-terminal 23-kD fragment protected from trypsin activity. The scissors represent the protease trypsin. (B) A proteolytic fragment of 23 kD is formed upon the correct insertion of Ugo1-2HA. Radiolabeled Ugo1-2HA was incubated for the indicated time periods with mitochondria isolated from *ugo1Δ* cells expressing plasmid-encoded Ugo1-2HA. Mitochondria were further incubated without (lane 2) or with trypsin (lanes 3–6) and pelleted. An additional sample was analyzed after import and trypsinization by carbonate extraction (Carb.), and pellet (P) and supernatant (S) fractions were loaded (lanes 7 and 8). In one sample, trypsin treatment was performed only after the mitochondria were solubilized with Triton X-100 (lane 9). As a control, 100% of input radiolabeled protein was treated with trypsin in the absence of mitochondria (lane 10). The proteolytic fragment (F) is indicated by black arrows, and full-length Ugo1 is indicated by white arrows. A nonspecific band resulting from preexisting mRNA in the reticulocyte lysate is indicated by the asterisks. In lane 11, mitochondria from the *ugo1Δ* strain harboring the empty plasmid were loaded as a control for the specificity of the HA antibody. All samples were analyzed by SDS-PAGE followed by autoradiography (top) and then immunodecorated with HA antibody (bottom). (C) The transcription–translation-coupled system was incubated with or without a plasmid encoding Ugo1-HA. In one sample, a commercial protease inhibitor cocktail was added. Samples were analyzed by SDS-PAGE and autoradiography. Full-length Ugo1 is indicated by a white arrow.

Despite the aforementioned progress, the mechanisms by which newly synthesized multispan proteins are recognized at the organelle surface and inserted into the MOM are still largely unresolved. It is especially unclear whether dedicated membrane insertion machinery for such proteins exists. To address these questions, we studied the membrane integration of the model multispan protein Ugo1. Our results suggest a novel insertion pathway in which the mitochondrial import receptor Tom70 and the outer membrane protein mitochondrial import 1 (Mim1) regulate recognition and insertion of Ugo1.

Results and discussion

A specific assay to monitor the in vitro insertion of Ugo1

A long-lasting problem in analyzing the integration of multispan proteins is the lack of a reliable assay for correct membrane integration. To overcome this problem, we developed a proteolytic assay based on previous experiments suggesting Ugo1 to have at least three TMSs (Coonrod et al., 2007; Hoppins et al., 2009). These earlier observations and our current results indicate that the addition of trypsin to mitochondria containing C-terminally HA-tagged Ugo1 resulted in the formation of a 23-kD C-terminal fragment probably as a result of a proteolytic cleavage site between putative TMS 2 and 3 (Fig. 1 A). Of note, C-terminally HA-tagged Ugo1 is fully functional and thus has a nativelike topology (Hoppins et al., 2009).

To allow for a comparison with the endogenous protein and to check whether the observed proteolytic fragment indeed represents a proper membrane insertion, we isolated mitochondria from *ugo1Δ* cells expressing plasmid-encoded Ugo1-HA. Next, we incubated these mitochondria with radiolabeled precursors of Ugo1-HA and, upon completion of the import reaction, performed the trypsin treatment. As expected, we observed a time-dependent formation of the anticipated C-terminal fragment in trypsin-treated mitochondria (Fig. 1 B, lanes 4–6, marked with F). This fragment behaved as a membrane-embedded polypeptide, as it remained in the membrane fraction of an alkaline extraction (Fig. 1 B, lanes 7 and 8). Furthermore, it disappeared

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upon solubilization of the organelle with detergent and was strictly dependent on the presence of mitochondria (Fig. 1 B, lanes 9 and 10). Thus, formation of the tryptic fragment requires an intact MOM and is not caused by aggregation. Immunodecoration of the same membrane with an anti-HA antibody demonstrated that the newly inserted Ugo1 molecules behave identically to preexisting endogenous Ugo1-HA (Fig. 1 B, bottom, lanes 3–9). Of note, the radiolabeled material contains several bands with smaller size that probably represent translation initiation on internal methionine residues (Fig. 1 B, lanes 1–2). We disfavor the possibility that these additional species result from proteolytic digestion of the protein because they were also observed upon synthesis of the protein in the presence of a mixture of protease inhibitors (Fig. 1 C). Collectively, the proteolytic treatment of newly synthesized molecules of Ugo1 provides a specific assay for membrane integration.

Import of Ugo1 depends on Tom70 but not on Tom20

Upon their synthesis in the cytosol, precursors of multispan proteins should be protected from aggregation. This task can be executed by cytosolic chaperones of the Hsp70 and Hsp90 families (Young et al., 2003). Accordingly, an overall dependency on ATP was reported for the import of various mitochondrial membrane proteins like Tom40, PBR, and the ATP–ADP carrier (AAC; Rapaport and Neupert, 1999; Wiedemann et al., 2001; Otera et al., 2007). We examined whether the import of Ugo1 shares this feature. When apyrase, which hydrolyzes ATP, was added to the import reaction, a dramatic reduction in the import of both Ugo1 and the control matrix–destined precursor pSu9–dihydrofolate reductase (DHFR) was observed (Fig. 2, A and B). We excluded the possibility that the apyrase sample contained protease contamination, as no proteolytic fragments were observed when only apyrase was added to the radiolabeled proteins (Fig. 2, A and B, last two lanes). As there are currently no indications that mitochondrial matrix chaperones are involved in the biogenesis of MOM proteins, we assume that the effect of apyrase on matrix ATP is irrelevant for the reduction in Ugo1 membrane integration. We propose that the ATP is required for the release of the hydrophobic multispan precursor proteins from the cytosolic chaperones to which they are associated.

The import of multispan proteins into mitochondria requires their recognition by the organelle. For example, the recognition of β-barrel proteins is mediated mainly by the import receptor Tom20 (Rapaport and Neupert, 1999; Krimmer et al., 2001; Yamano et al., 2008), whereas Tom70 preferentially recognizes precursors of the inner membrane carrier-like proteins and mammalian multispan outer membrane proteins like PBR (Schlossmann et al., 1994; Brix et al., 1999; Wiedemann et al., 2001; Young et al., 2003; Otera et al., 2007). We examined whether pretreatment of mitochondria with trypsin, which removes any exposed parts of surface receptors, can affect the insertion of Ugo1. A substantial reduction in the membrane integration of Ugo1 was observed upon such a treatment, similar to the expected effect on the membrane integration of porin (Fig. 3 A; Krimmer et al., 2001).

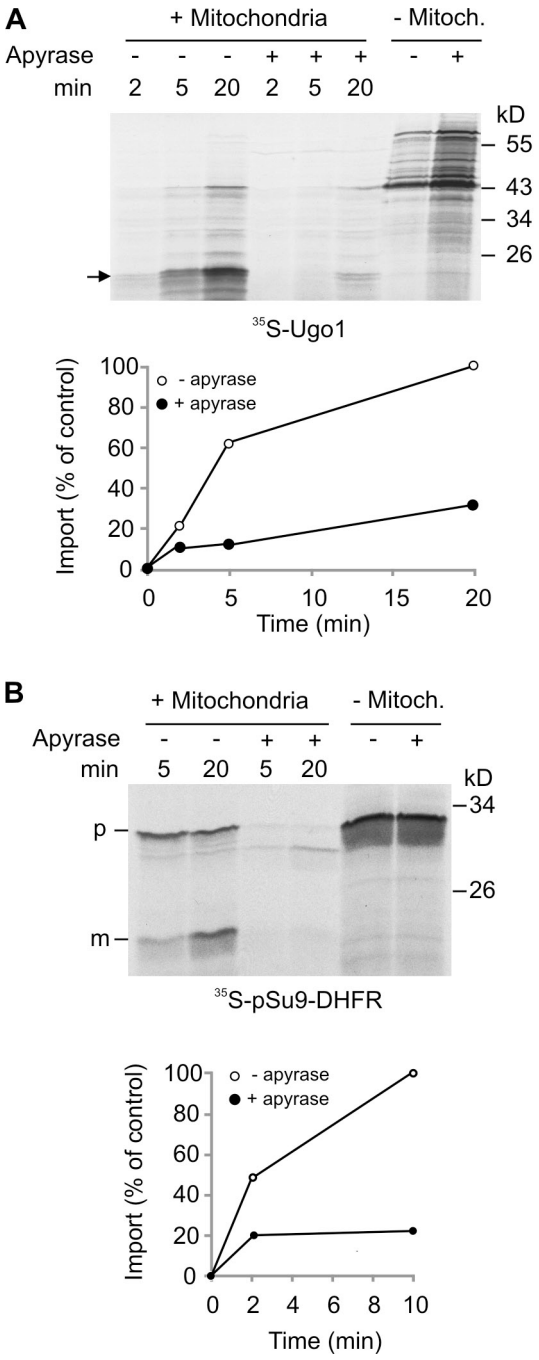

Figure 2. **Import of Ugo1 is strongly compromised by removal of ATP.** (A and B) Radiolabeled Ugo1 (A) or pSu9-DHFR (B) was incubated in import buffer with mitochondria for the indicated time periods in the presence or absence of apyrase. As a control, samples without mitochondria (− Mitoch.) were incubated in the presence or absence of apyrase and analyzed directly by SDS-PAGE. At the end of the import reactions, mitochondria were treated with either trypsin (Ugo1 import reactions) or proteinase K (PK; pSu9-DHFR import reactions) and reisolated. Imported proteins were analyzed by SDS-PAGE and autoradiography. The insertion of Ugo1 was quantified by analyzing the formation of the 23-kD fragment (indicated by an arrow), whereas for pSu9-DHFR, the protease-protected mature form (m) was quantified. The amount of proteins imported into untreated mitochondria for the longest time period was set to 100%. An experiment representative of three independent repeats is presented. p, precursor.

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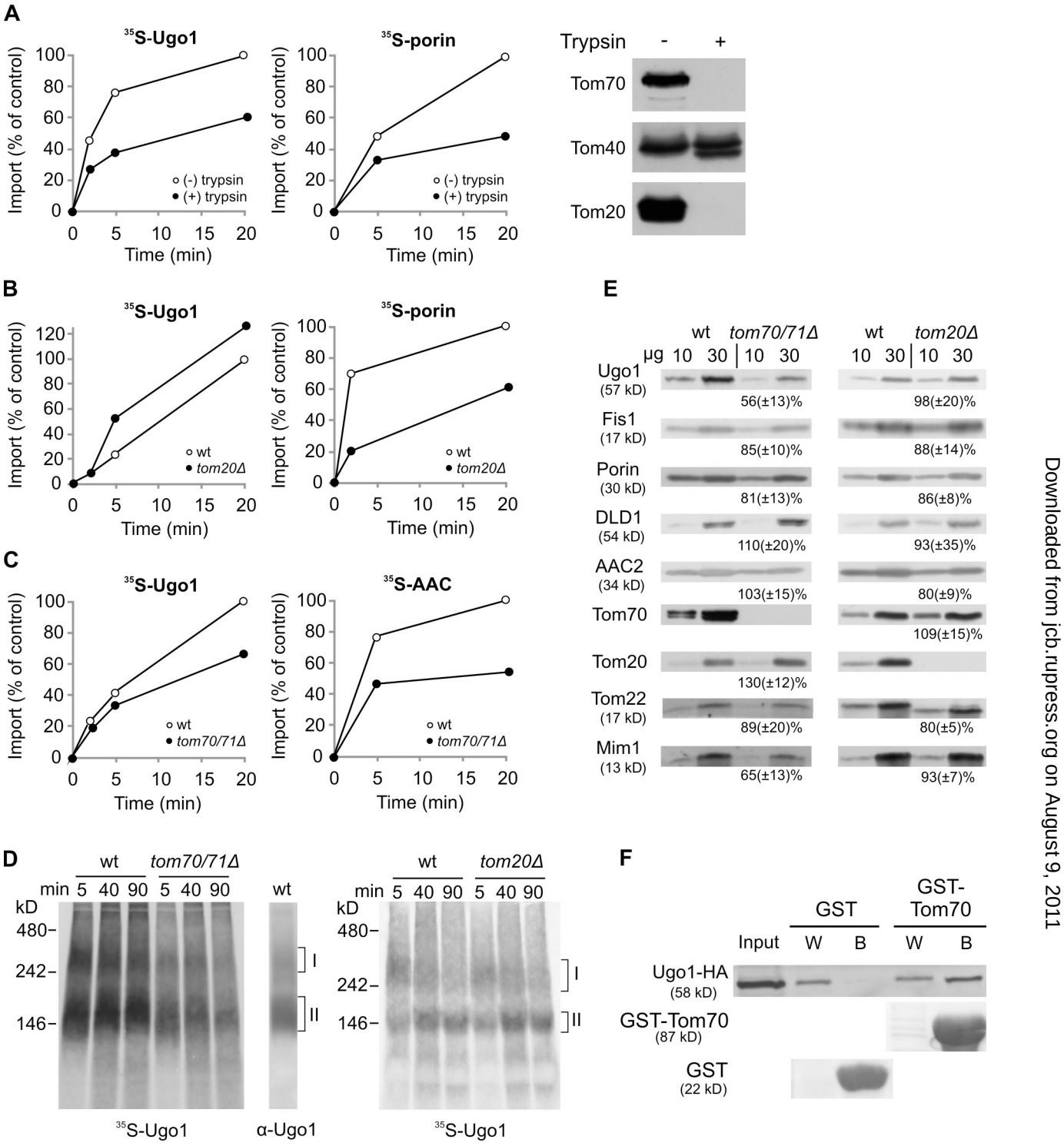

Figure 3. **Ugo1 requires the import receptor Tom70 for its import and assembly.** (A) Mitochondria were left intact or pretreated with trypsin followed by reisolation of the organelles. Aliquots of both trypsin-treated and intact mitochondria were removed, and the trypsin activity was monitored by immunodecoration with antibodies against Tom components (right). Next, radiolabeled Ugo1 and porin were incubated with the trypsin-treated or intact mitochondria for the indicated time periods. At the end of the import reactions, samples containing Ugo1 were treated again with trypsin, whereas to those harboring porin, PK was added. Proteins were analyzed by SDS-PAGE and autoradiography. The insertion of Ugo1 was quantified by analyzing the formation of the 23-kD fragment, whereas for porin, the PK-protected molecules were quantified. The amount of precursor proteins imported into intact mitochondria for 20 min was set to 100%. (B) Radiolabeled precursors were imported into mitochondria isolated from either wild-type (wt) or *tom20Δ* strains. Imported proteins were analyzed as described in A. (C) Radiolabeled precursors of Ugo1 and AAC were imported into mitochondria isolated from either wild-type or *tom70Δtom71Δ* strains. Imported proteins were analyzed by SDS-PAGE and radiography. The insertion of Ugo1 was quantified as described in A, whereas the PK-protected molecules of AAC were quantified. (A–C) An experiment representative of three independent repeats is presented. (D) Radiolabeled precursor of Ugo1 was imported into mitochondria isolated from *tom70Δtom71Δ*, *tom20Δ*, or their corresponding wild-type strains. After import, the mitochondria were analyzed by BN-PAGE. For comparison, wild-type mitochondria were analyzed by BN-PAGE and immunodecoration with an antibody

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To identify the involved receptors, we analyzed the import into mitochondria isolated from strains lacking either Tom20 or Tom70/71. In contrast to the dramatic effect on the import of porin, the absence of Tom20 did not cause any reduction in the membrane insertion of Ugo1 (Fig. 3 B). When we monitored the import into *tom70Δtom71Δ* mitochondria, we observed that the insertion of Ugo1 into these organelles was clearly compromised (although less than that of the prototype Tom70 substrate AAC; Fig. 3 C). To verify the importance of Tom70, we analyzed the in vitro import reaction by blue native (BN)–PAGE. Of note, endogenous and newly synthesized radiolabeled Ugo1 molecules were found in two oligomeric species with apparent molecular masses of ∼300 and 150 kD (Fig. 3 D, indicated as oligomer I and II, respectively). The bottom band represents a homodimer of Ugo1 (Hoppins et al., 2009), whereas the composition of the top one is unknown. In support of the aforementioned results, the formation of the Ugo1-containing oligomers was hampered in mitochondria lacking Tom70/71 but was unaffected by the absence of Tom20 (Fig. 3 D).

To further investigate the dependency on import receptors, we monitored the steady-state levels of Ugo1 in mitochondria lacking either Tom70/71 or Tom20. The endogenous levels of Ugo1 were indeed reduced in *tom70/71Δ* organelles but not in *tom20Δ* mitochondria (Fig. 3 E). Importantly, it appears that, in vivo, Tom70 plays a more crucial role in the biogenesis of Ugo1 than in that of AAC. We and others did not observe a reduction in the steady-state levels of AAC in *tom70/71Δ* mitochondria (Fig. 3 E; Bömer et al., 1996). Of note, the steady-state levels of Mim1, which is crucial for the membrane integration of Ugo1 (Fig. 4), are moderately reduced in mitochondria lacking Tom70 (Fig. 3 E). This observation raises the possibility that the hampered biogenesis of Ugo1 in *tom70Δ* cells is actually a result of reduced levels of Mim1. However, we regard this scenario as unlikely because removal of the exposed receptor domains by trypsin dramatically reduced the insertion of Ugo1 (Fig. 3 A), whereas deletion of the exposed N terminus of Mim1 does not result in a clear phenotype (Popov-Celeketić et al., 2008; Lueder and Lithgow, 2009) or alteration of the steady-state levels of Ugo1 (Fig. S1).

To substantiate a direct involvement of Tom70, we incubated newly synthesized Ugo1 molecules with either the recombinant cytosolic domain of Tom70 fused to GST or with GST alone and observed specific interactions only with the former construct (Fig. 3 F). Hence, the binding assay demonstrates the ability of Tom70 to directly recognize a precursor form of Ugo1. Collectively, the results of four different assays suggest that Tom70 plays an important role in the import of Ugo1. The receptor is not absolutely essential for the import of Ugo1 but rather accelerates and enhances this process as with other substrates of Tom70 (Steger et al., 1990; Ramage et al., 1993). In its absence, these tasks are probably partially performed by the other import receptor Tom20. Accordingly, Tom20 is actually found in higher amounts when Tom70 is deleted (Fig. 3 E).

We suggest that cytosolic chaperones help in delivering the multispan precursor proteins in an import-competent form to Tom70 that in turn provides the first recognition on the organelle surface. Supporting this notion is the involvement of Tom70 in the insertion of multispan MOM proteins in mammalian cells (Otera et al., 2007) and its function as a docking element for chaperone-associated precursor proteins of the inner membrane carrier proteins (Young et al., 2003).

Membrane integration of Ugo1 does not require the TOM complex pore, elements residing in the intermembrane space (IMS), and the topogenesis of MOM β-barrel protein (TOB) complex

Because β-barrel proteins are translocated through the import pore of the TOM complex before their insertion into the MOM (Pfanner et al., 2004; Ryan, 2004; Paschen et al., 2005), we asked whether multispan proteins follow a similar pathway. To this end, an excess of recombinant matrix-destined precursor (pSu9(1–69)-DHFR), which can compete with import of other TOM-dependent precursors, was added to the import reaction of Ugo1. This treatment did not affect the membrane integration of Ugo1 but, as expected, caused a strong reduction in the import of porin (Fig. S2 A). Thus, it appears that the TOM import pore is not required for the biogenesis of Ugo1.

Upon their translocation across the MOM, β-barrel proteins are engaged by the small Tim chaperones residing in the IMS (Hoppins and Nargang, 2004; Wiedemann et al., 2004; Habib et al., 2005). Interestingly, Otera et al. (2007) reported that mammalian multispan proteins also require elements in the IMS for their biogenesis. Hence, we asked whether swelling of mitochondria, which results in ruptured MOM and loss of most of IMS proteins, can affect the biogenesis of Ugo1. This treatment did not influence the membrane integration of Ugo1 under short incubation periods and had only a slight effect after incubation for 90 min. In sharp contrast, rupturing the MOM resulted in severely compromised assembly of the β-barrel protein Tom40 (Fig. S2 B). Of note, immunodecoration with antibodies against Tim10 and Tim13 showed that negligible amounts of these proteins are still associated with the swollen mitochondria. To exclude the possibility that these residual amounts are sufficient to support an efficient biogenesis of Ugo1, we analyzed the biogenesis of Ugo1 in a strain deleted for *TIM8/TIM13*. The double deletion did not interfere with the in vitro import of Ugo1 nor did it cause any reduction in the steady-state levels of the protein (Fig. S2, C and D). Similarly,

against Ugo1. Ugo1-containing complexes are indicated on the right (I and II). (E) Mitochondria were isolated from *tom70Δtom71Δ*, *tom20Δ*, or their corresponding wild-type strains. Mitochondrial proteins (10 and 30 µg) were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins. The intensity of the bands in three independent experiments was quantified, and the amount of proteins in mutant mitochondria is expressed as the mean (±SD) percentage of their level in the wild-type organelle. (F) The cytosolic domain of Tom70 can recognize newly synthesized Ugo1 molecules. Chemical amounts of Ugo1-HA (input) were mixed with either GST or GST fused to the cytosolic domain of Tom70 (GST-Tom70) bound to glutathione beads. The beads were washed, after which bound material was eluted with sample buffer. Aliquots of the input (10%), wash (W; 2%), and bound material (B; 100%) were analyzed by SDS-PAGE and either Ponceau staining (GST and GST-Tom70) or immunodecoration against HA tag.

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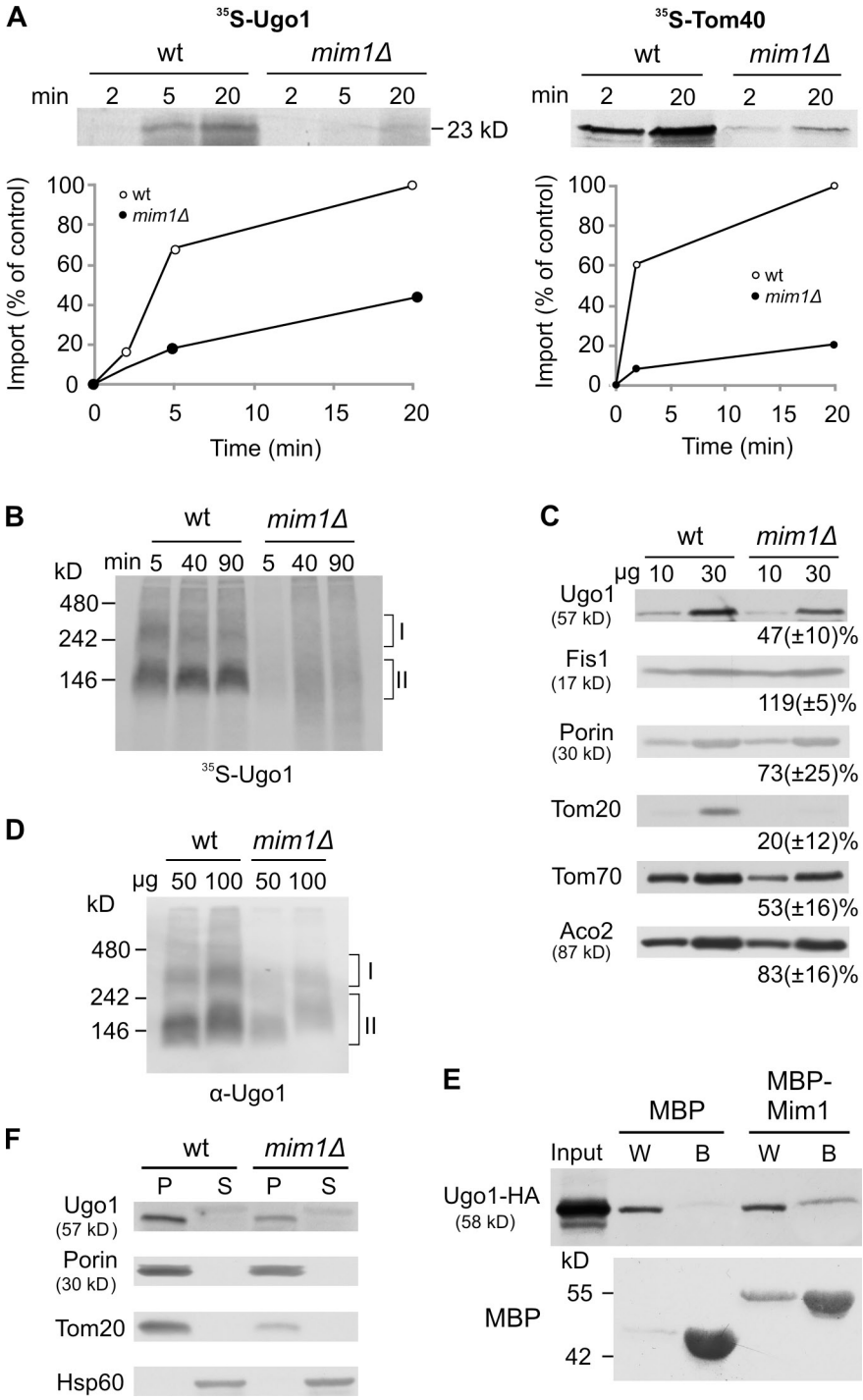

Figure 4. **Mim1 is playing an important role in the membrane integration of Ugo1.** (A) Radiolabeled Ugo1 and Tom40 were imported into mitochondria isolated from either wild-type (wt) or *mim1Δ* strains. The insertion of Ugo1 was analyzed as described in Fig. 3 A, whereas for Tom40, the PK-protected molecules were quantified. An experiment representative of three independent repeats is presented. (B) Radiolabeled precursor of Ugo1 was imported for the indicated time periods into mitochondria isolated from either wild-type or *mim1Δ* strains. After import, mitochondria were analyzed by BN-PAGE and autoradiography. Ugo1-containing complexes are indicated (I and II). (C) Mitochondria isolated from either wild-type or *mim1Δ* strains were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins. The intensities of the bands were quantified as described in Fig. 3 E. (D) The indicated amounts of mitochondria isolated from either wild-type or *mim1Δ* strains were analyzed by BN-PAGE and immunodecoration with antibody against Ugo1. Ugo1-containing complexes are indicated. (E) Chemical amounts of Ugo1-HA (input) were mixed with either MBP or MBP-Mim1 bound to maltose beads. The beads were washed, and then bound material was eluted with sample buffer. Aliquots of the input (10%), wash (W; 2%), and bound material (B; 80%) were analyzed by SDS-PAGE and immunodecoration with antibodies against HA tag and MBP. (F) Mitochondria isolated from either wild-type or *mim1Δ* strains were subjected to carbonate extraction. The pellet (P) and the supernatant (S) fractions were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins.

mitochondria isolated from a strain harboring a temperature-sensitive allele of *TIM10* were not compromised in their capacity to import in vitro newly synthesized Ugo1 molecules. In contrast, they were severely affected in their ability to import the known Tim10 substrate AAC (Fig. S2 E). Collectively, elements of the IMS do not appear to be essential for the biogenesis of Ugo1.

The TOB/SAM (sorting and assembly machinery) complex was initially reported to be a dedicated machinery for the membrane insertion of β-barrel proteins (Gentle et al., 2004; Pfanner et al., 2004; Paschen et al., 2005). However, later studies proposed its involvement also in the biogenesis of small helical Tom components (Stojanovski et al., 2007; Thornton et al., 2010). Hence, we investigated the biogenesis of Ugo1 in mitochondria lacking Mas37, a subunit that mediates the release of substrate proteins from the TOB complex (Wiedemann et al., 2003; Chan and Lithgow, 2008; Dukanovic et al., 2009). Deletion of *MAS37* caused, as expected, a clear reduction in the in vitro import of the β-barrel protein Tom40 and a minor one in steady-state levels of Tom40 and porin (Fig. S3, A and B). This deletion neither resulted in reduced in vitro membrane integration of Ugo1 nor compromised its steady-state levels (Fig. S3, A and B). Collectively, we conclude that the multispan protein Ugo1 does not require the TOB complex.

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The MOM protein Mim1 is crucial for the biogenesis of Ugo1

We next asked which membrane-embedded protein can mediate membrane integration of Ugo1. Based on its known functions, Mim1 is a good candidate to accomplish this role. The protein is important for the biogenesis of the TOM complex and is required for membrane integration of Tom helical components (Ishikawa et al., 2004; Waizenegger et al., 2005; Becker et al., 2008; Hulett et al., 2008; Popov-Celeketić et al., 2008; Lueder and Lithgow, 2009; Stefan Dimmer and Rapaport, 2010; Thornton et al., 2010).

When mitochondria lacking Mim1 were used, a strong reduction in the import of newly synthesized Ugo1 (and of Tom40 as a control) was observed by both the proteolytic assay and BN-PAGE (Fig. 4, A and B). Accordingly, the steady-state levels of Ugo1 and the amounts of endogenous Ugo1-containing oligomers were greatly reduced in *mim1Δ* mitochondria (Fig. 4, C and D). As the steady-state levels of Tom20, Tom70, and Tom40 are also reduced in *mim1Δ* mitochondria (Fig. 4 C; Ishikawa et al., 2004; Waizenegger et al., 2005), a theoretical scenario could be that the compromised biogenesis of Ugo1 is actually a result of reduced levels of these Tom components. However, this possibility is unlikely because deletion of Tom20 itself or blocking the Tom40 import pore does not have any influence on the biogenesis of Ugo1, and deletion of Tom70/71 causes a less severe phenotype. Next, we tested whether Mim1 can bind Ugo1 precursor molecules and observed that maltose-binding protein (MBP)–Mim1 but not MBP alone could interact with Ugo1 (Fig. 4 E). These results support a direct role of Mim1 in the membrane integration of Ugo1. The findings of a parallel study (see Becker et al. in this issue) underscore the importance of Mim1 in the biogenesis of multispanning outer membrane proteins and its capacity to directly bind these proteins during their membrane integration process. Of note, the residual amount of Ugo1 molecules in MOM lacking Mim1 behaved as membrane-embedded proteins that cannot be extracted by alkaline solution (Fig. 4 F). Thus, it might well be that an additional, yet to be identified, element also contributes to the membrane integration of Ugo1.

The newly discovered function of Mim1 in the integration of multispan proteins raises the question of how Mim1 performs this role. Based on our previous observation that Mim1 can form homooligomers (Popov-Celeketić et al., 2008), we speculate that multiple molecules of Mim1 form a distinct site in the MOM that can provide an entry platform for the transmembrane helices of single-span and multispan proteins.

Conclusions

We propose that the integration of Ugo1 into the MOM occurs via a novel pathway. This pathway involves initial docking of chaperone-associated Ugo1 to the import receptor Tom70. Ugo1 precursor is then inserted into the membrane in a process that is facilitated by the membrane-embedded protein Mim1.

Materials and methods

Yeast strains and growth media

Standard genetic techniques were used for growth and manipulation of yeast strains. Unless stated otherwise, the wild-type strains YPH499 and W303 were used. For construction of *ugo1Δ* and *tom20Δ* mutant strains in W303 background, the *UGO1* and *TOM20* genes were deleted by PCR-mediated gene replacement with *kan*MX4 and *HIS3*-MX6 cassette, respectively. The *mas37Δ* (Habib et al., 2005) and *tim8Δ/tim13Δ* (Paschen et al., 2000) strains were previously described. *mim1Δ*, *mim1Δ*+MIM1ΔN, and *mim1Δ*+MIM1−FL strains were constructed as reported by Popov-Celeketić et al. (2008). The *tom70Δ/tom71Δ* double deletion (Kondo-Okamoto et al., 2008) and *TIM10-1* (Koehler et al., 1998) strains were gifts from K. Okamoto (Osaka University, Osaka, Japan) and C. Koehler (University of California, Los Angeles, Los Angeles, CA), respectively. Transformation of yeast was performed according to the lithium-acetate method. Yeast cells were grown under aerobic conditions in yeast peptone dextrose, YPGal (1% yeast extract, 2% bactopeptone, and 2% galactose), Lac, synthetic dextrose–Trp, or synthetic dextrose–Ura media.

Recombinant DNA techniques

To obtain a C-terminally HA-tagged Ugo1, a sequence encoding 2× HA was PCR amplified from the pFA6a-3HA-KanMX4 vector and inserted into the target vector pYX113 using SalI and XhoI restriction sites. *UGO1* without its stop codon was amplified via PCR from genomic DNA isolated from the YPH499 strain and introduced into the modified vector using EcoRI and SalI restriction sites. For cell-free experiments, this construct (pYX113 UGO1-2HA) was used as a template for PCR amplification of UGO1-2HA. PCR product obtained in this way was inserted into pGEM4 vector by use of the SmaI and XbaI restriction sites. The cytosolic domain of Tom70 (Δ amino acid residues 1–34) was amplified by PCR and introduced into the pGEX4T vector using the BamHI and HindIII restriction sites.

Biochemical procedures

Mitochondria were isolated from yeast cells by differential centrifugation as previously described (Daum et al., 1982). For swelling experiments, isolated mitochondria were incubated with a hypotonic buffer (20 mM Hepes, pH 7.2) for 30 min on ice. Then, it was supplemented with urea to a final concentration of 1 M and incubated on ice for a further 5 min. Swollen mitochondria were reisolated by centrifugation and resuspended in import buffer. Chemical amounts of Ugo1-HA were produced in wheat germ lysate according to the manufacturer´s instructions (RTS 100 Wheat Germ CECF kit; 5Prime). The recombinant proteins GST, GST-Tom70 (cytosolic domain), MBP, and MBP-Mim1 were expressed in *Escherichia coli* BL21 cells as soluble proteins. Purification of recombinant proteins was performed by affinity chromatography according to the manufacturer´s instructions using either glutathione beads (Macherey-Nagel) or maltose-coupled beads (New England Biolabs, Inc.).

Protein samples were analyzed by SDS-PAGE and blotting to nitrocellulose membranes followed by visualization through autoradiography. Alternatively, incubation with antibodies was performed according to standard procedures, and visualization was performed via the ECL method. The antibody against Ugo1 was a gift from S. Hoppins and J. Nunnari (University of California, Davis, Davis, CA). Intensity of the observed bands was quantified with automatic imaging data analysis software (raytest GmbH). Unless stated otherwise, each presented experiment represents at least three repetitions.

In vitro protein import

Import experiments with radiolabeled precursor proteins and isolated mitochondria were performed in an import buffer containing 250 mM sucrose, 0.25 mg/ml BSA, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS-KOH, 2 mM NADH, and 2 mM ATP, pH 7.2. Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. In some cases, the import reaction was treated with 0.3 U/µl apyrase. Trypsin treatment of mitochondria was performed by adding 50 µg/ml trypsin for 25 min on ice. Trypsin was then inhibited by adding 1.5 mg/ml soybean trypsin inhibitor for 10 min on ice. For blocking the TOM complex import pore, the recombinant precursor protein pSu9-DHFR was added to 30 µg of isolated mitochondria immediately before the import reaction. In the carbonate extraction reaction, mitochondria were dissolved in 0.1 M Na_2CO_3. After 30 min on ice, the sample was centrifuged (75,000 *g* for 30 min at 2°C), and pellet and supernatant were analyzed.

BN-PAGE

Mitochondria were lysed in 40 µl digitonin-containing buffer (1–1.5% digitonin, 20 mM Tris-HCl, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, and 1 mM PMSF, pH 7.4). After incubation for 15 min at 4°C and a clarifying spin (30,000 *g* for 15 min at 2°C), 5 µl of sample buffer (5% [weight/volume] Coomassie brilliant blue G-250, 100 mM Bis-Tris, and 500 mM 6-aminocaproic acid, pH 7.0) was added, and the mixture was analyzed by electrophoresis in a 6–13% gradient BN gel

(Schägger et al., 1994). Gels were blotted to polyvinylidene fluoride membranes, and proteins were further analyzed by autoradiography or immunodecoration.

Online supplemental material

Fig. S1 presents data supporting the proposal that the N-terminal domain of Mim1 is not required for optimal biogenesis of Ugo1. Fig. S2 shows that the biogenesis of Ugo1 does not require the TOM import pore or elements in the IMS. Fig. S3 includes results of experiments demonstrating that the insertion of Ugo1 is independent of the TOB complex. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201102041/DC1.

We thank K. Rehn and E. Kracker for technical support, H. Kato and E. Merklinger for helpful discussions, C. Koehler, K. Tokatlidis, and K. Okamoto for yeast strains, and S. Hoppins and J. Nunnari for constructs and antibodies.

This work was supported by a Deutsche Forschungsgemeinschaft grant (RA 1048/4-1 to D. Rapaport) and a postdoctoral fellowship from the Carl Zeiss Stiftung (to K.S. Dimmer).

Submitted: 8 February 2011
Accepted: 11 July 2011

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Published August 8, 2011

Papić et al., http://www.jcb.org/cgi/content/full/jcb.201102041/DC1

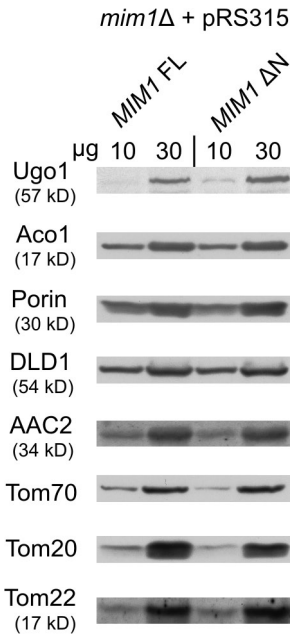

Figure S1. **The N-terminal domain of Mim1 is not required for the biogenesis of Ugo1.** The indicated amounts of mitochondria isolated from the *mim1Δ* strain transformed with a plasmid encoding either full-length Mim1 (*MIM1* FL) or an N-terminal truncated variant of Mim1 (*MIM1* ΔN) were analyzed by SDS-PAGE and immunodecoration with antibodies against mitochondrial proteins.

Published August 8, 2011

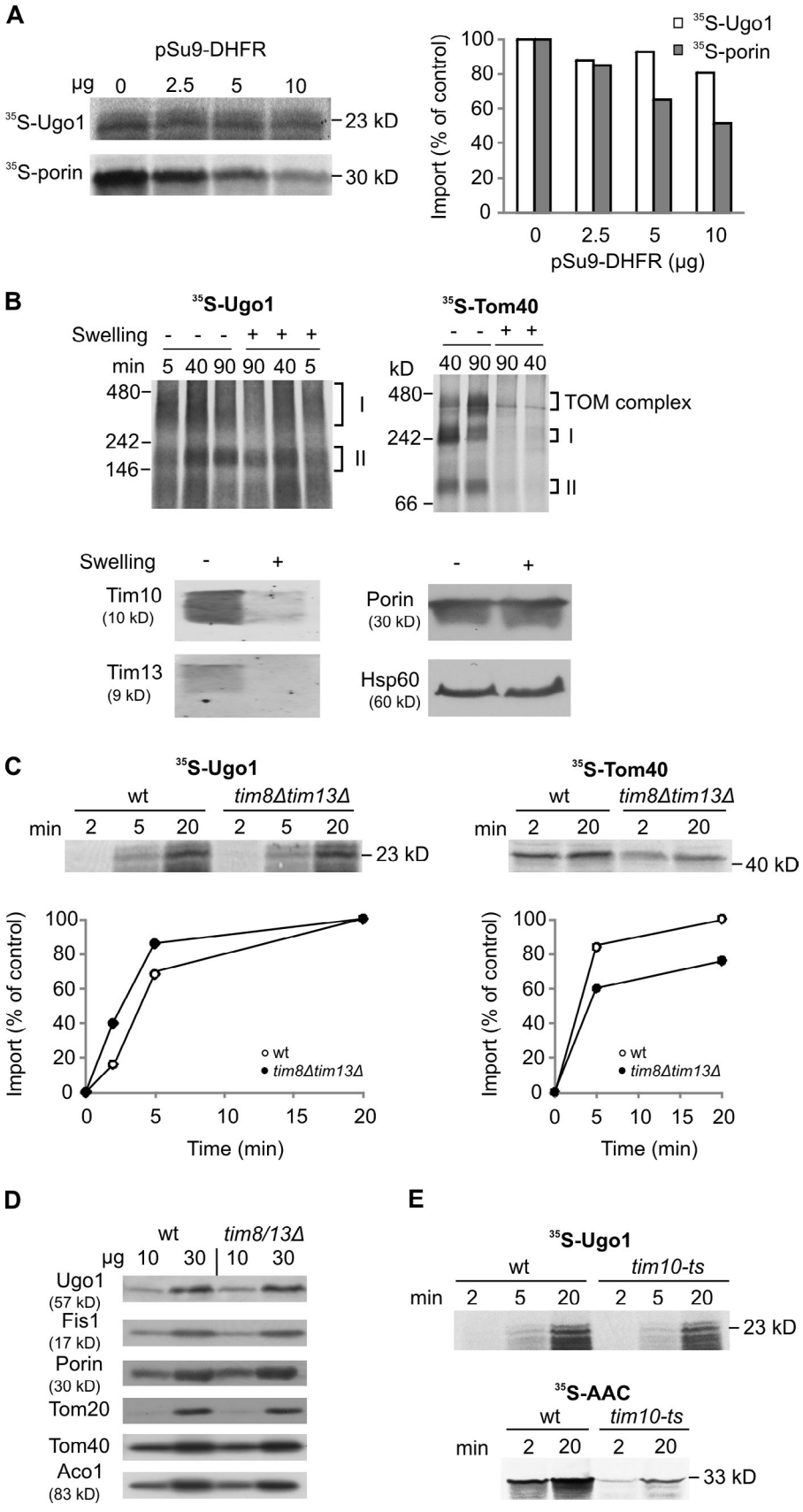

Figure S2. **The biogenesis of Ugo1 does not require the TOM import pore or elements in the IMS.** (A) Radiolabeled precursors of Ugo1 and porin were imported into mitochondria in the absence or presence of the indicated amounts of recombinant pSu9-DHFR. (left) Imported proteins were analyzed as described in Figs. 2 A and 3 A. (right) Bands representing the imported proteins were quantified, and the amount of precursor proteins imported into untreated mitochondria was set to 100%. (B) Rupturing of the outer membrane does not compromise the assembly of Ugo1. Radiolabeled precursors of Ugo1 and Tom40 were incubated for the indicated time periods with isolated intact mitochondria or with mitochondria that had been subjected to osmotic swelling. After import, mitochondria were pelleted, solubilized in buffer containing 1.5% digitonin, and analyzed by BN gel electrophoresis. (top) Ugo1-containing complexes, as well as intermediates and the assembled forms of Tom40, are indicated on the right side of the respective panels (I and II). (bottom) The swelling efficiency was monitored by immunodecoration with antibodies against proteins residing in the IMS (Tim10 and Tim13), the outer membrane (porin), and the matrix (Hsp60). (C) Insertion and assembly of Ugo1 are not affected in mitochondria lacking the Tim8–Tim13 complex. Radiolabeled precursors of Ugo1 and Tom40 were imported into mitochondria isolated from either wild-type (wt) or *tim8Δtim13Δ* strains. Imported proteins were analyzed as described in Figs. 3 A and 4 A. (A–C) An experiment representative of three independent repeats is presented. (D) Mitochondria were isolated from wild-type and *tim8Δ/tim13Δ* strains, and mitochondrial proteins were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. (E) Insertion and assembly of Ugo1 are not dependent on functional Tim10. Radiolabeled precursors of Ugo1 and AAC were imported into mitochondria isolated from either a strain harboring a temperature-sensitive allele of TIM10 (*tim10-ts*; Koehler et al., 1998) or its corresponding parental strain. The cells were grown at 24°C, and the isolated mitochondria were shifted to 37°C for 15 min before the import reactions. Imported proteins were analyzed as described in Fig. 3 (A and C).

Published August 8, 2011

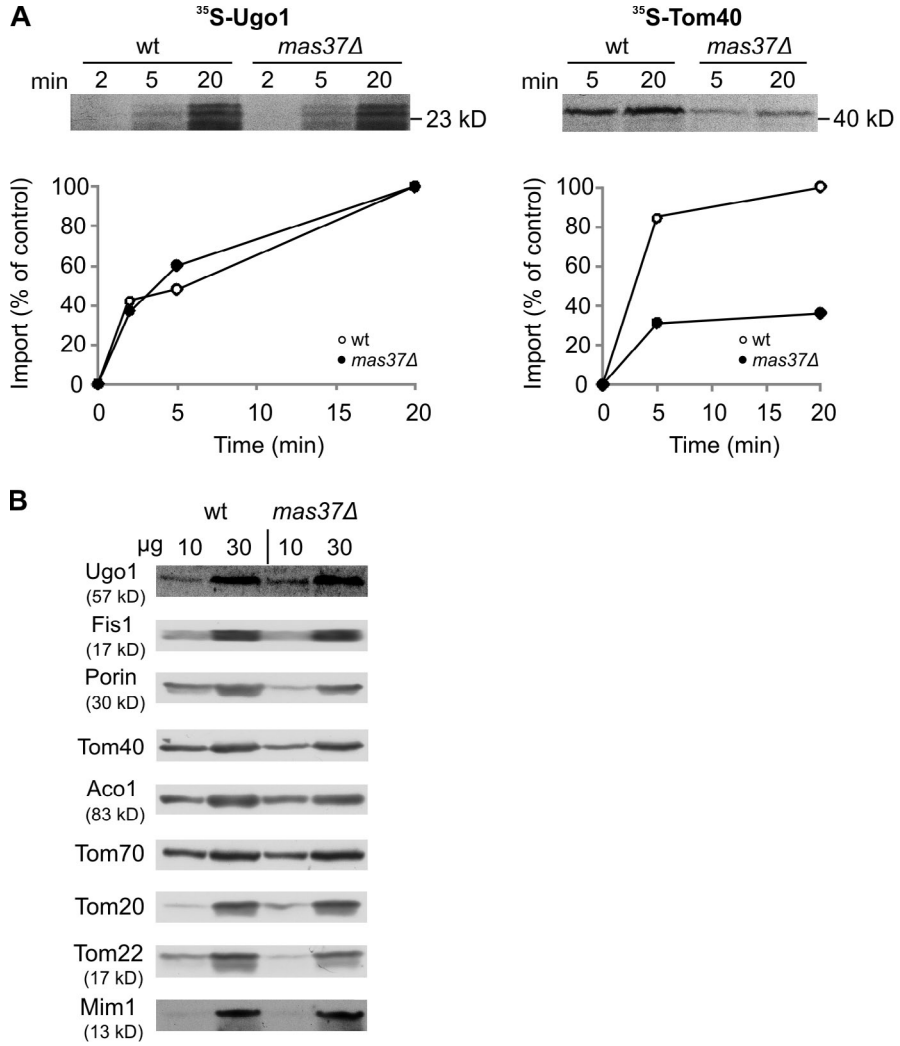

Figure S3. **Insertion of Ugo1 is independent of the TOB complex.** (A) Radiolabeled precursors of Ugo1 and Tom40 were imported into mitochondria isolated from either wild-type (wt) or the *mas37Δ* strain. Imported proteins were analyzed by SDS-PAGE and radiography. Imported proteins were quantified as described in Figs. 2 A and 4 A. The amount of precursor proteins imported into wild-type mitochondria for 20 min was set to 100%. An experiment representative of three independent repeats is presented. (B) The indicated amounts of mitochondria isolated from either wild-type or *mas37Δ* cells were analyzed by SDS-PAGE and immunodecoration with antibodies against mitochondrial proteins.

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A crucial role for Mim2 in the biogenesis of mitochondrial outer membrane proteins

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Accepted 12 March 2012
Journal of Cell Science 000, 1–10

doi: 10.1242/jcs.103804

Summary

Most of the mitochondrial outer membrane (MOM) proteins contain helical transmembrane domains. Some of the single-span proteins and all known multiple-span proteins are inserted into the membrane in a pathway that depends on the MOM protein Mitochondrial Import 1 (Mim1). So far it has been unknown whether additional proteins are required for this process. Here, we describe the identification and characterization of Mim2, a novel protein of the MOM that has a crucial role in the biogenesis of MOM helical proteins. Mim2 physically and genetically interacts with Mim1, and both proteins form the MIM complex. Cells lacking Mim2 exhibit a severely reduced growth rate and lower steady-state levels of helical MOM proteins. In addition, absence of Mim2 leads to compromised assembly of the translocase of the outer mitochondrial membrane (TOM complex), hampered mitochondrial protein import, and defects in mitochondrial morphology. In summary, the current study demonstrates that Mim2 is a novel central player in the biogenesis of MOM proteins.

Key words: Mim2, MIM complex, Mitochondria, Outer membrane, Protein import

Introduction

The mitochondrial outer membrane (MOM) harbors a diverse set of proteins with functions ranging from biosynthetic pathways, morphogenesis and inheritance of the organelle to protein import into mitochondria (Burri et al., 2006; Schmitt et al., 2006; Zahedi et al., 2006). As all MOM proteins are encoded in the nucleus and translated on ribosomes in the cytosol, they have to be targeted to the organelle and inserted into the membrane (Neupert and Herrmann, 2007; Chacinska et al., 2009; Endo and Yamano, 2009; Walther and Rapaport, 2009). Despite recent progress, the various insertion mechanisms by which MOM proteins are incorporated into the membrane are still poorly understood.

MOM proteins can be divided according to their topologies into different families (Dukanovic and Rapaport, 2011). The β-barrel proteins form one family and are unique to the outer membranes of chloroplasts, mitochondria and Gram-negative bacteria. ~~Their mitochondrial import route via TOB/SAM complex is best studied among the MOM proteins.~~ Three additional protein families contain a single helical transmembrane domain (TMD). The so called tail-anchored (TA) and signal-anchored (SA) proteins bear this domain at their very C- or N-terminus, respectively (Wattenberg and Lithgow, 2001; Waizenegger et al., 2003; Ahting et al., 2005). An additional group is comprised of proteins that contain a central TMD, thus exposing domains to both the cytosol and the intermembrane space (IMS). Finally, a unique group is composed of MOM proteins that transverse the membrane via multiple helical TMDs.

The import pathways of helical MOM proteins are ill defined. Some evidence exists that tail- and signal-anchored proteins insert into the MOM without participation of a dedicated insertion machinery (Setoguchi et al., 2006; Kemper et al., 2008; Meineke et al., 2008). Other reports suggest a partial overlap in insertion pathways of polytopic and TA proteins (Rojo et al., 2002; Otera et al., 2007).

Two recent reports shed new light on the insertion mechanism of multispan proteins. They demonstrate that the outer membrane protein Mitochondrial Import 1 (Mim1) plays a crucial role in the insertion of multispan MOM proteins (Becker et al., 2011; Papic et al., 2011). The results suggest that precursor proteins are first recognized by Tom70 and then handed over to a Mim1-containing complex. Mim1 was originally identified in a systematic screen ~~for mutants that accumulate~~ mitochondrial precursor proteins. It is a small integral protein of the MOM with a molecular mass of roughly 13 kDa (Mnaimneh et al., 2004; Dimmer and Rapaport, 2010). Later studies reported that Mim1 is a component of a higher molecular weight complex and that the protein is necessary for biogenesis of Tom20 and Tom70 and therefore also for the assembly of the TOM complex (Ishikawa et al., 2004; Waizenegger et al., 2005; Becker et al., 2008; Hulett et al., 2008; Popov-Celeketić et al., 2008; Lueder and Lithgow, 2009; Becker et al., 2010; Thornton et al., 2010).

Whereas the involvement of Mim1 in the biogenesis of ~~OM~~ helical proteins is well documented, it has been unclear so far whether additional proteins are required for this process. Furthermore, the actual composition of the Mim1-containing complex and its mode of function are still unknown. Here, we report on the identification and characterization of a novel outer membrane protein, Mim2 that is crucial for proper growth of yeast cells. Mim2 and Mim1 are components of the same functional complex that is playing a central role in the biogenesis of MOM proteins.

Results

Identification of Mim2

Mim1 was reported to be a subunit of a higher molecular weight complex of unknown composition. To search for additional components of the Mim1-containing complex, we performed immunoprecipitation in combination with stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al., 2002). This method has been widely used to identify protein-protein interactions (Selbach and Mann, 2006; Hubner et al., 2010; Vermeulen et al., 2010; Walther and Mann, 2010). Mitochondria were isolated from a *mim1Δ* strain transformed with a vector encoding either Mim1 or GFP–Mim1, organelles were lysed with digitonin, and the lysate was incubated with beads specifically binding GFP. Bound material was digested with the protease LysC and resulting peptides were analyzed by high-resolution mass spectrometry followed by data processing with the MaxQuant software environment (Cox and Mann, 2008). Among the identified proteins particularly the putative open reading frame (ORF) *YLR099W-A* displayed an enrichment very similar to that of the bait protein Mim1 (supplementary material Fig. S1). Due to its identification as an interaction partner of Mim1, we named this ORF *MIM2*. According to the Saccharomyces Genome Database (SGD, www.yeastgenome.org), this small ORF is an essential gene and encodes a protein of 87 amino acids. Mim2 has no homologues in higher eukaryotes but is conserved in fungi like *Schizosaccharomyces pombe* and *Neurospora crassa* (Fig. 1A). Although several hydrophobic amino acids are clustered in the middle of the primary sequence (Fig. 1A), no transmembrane domain was predicted by commonly used programs.

Mim2 is an integral protein of the MOM

To investigate the subcellular localization of Mim2, yeast cells deleted for the chromosomal copy of *MIM2* were transformed with a vector encoding either native Mim2 or Mim2 with a C-terminal HA-tag. Both Mim2 and Mim2–HA expressed in this way were functional as they rescued the growth defect of *mim2Δ* cells (supplementary material Fig. S2 and text below). Subcellular fractionation demonstrated that Mim2–HA is present in the mitochondrial fraction (Fig. 1B). Next, we subjected mitochondria harboring Mim2–HA to an alkaline extraction treatment in which soluble and peripheral membrane proteins can be separated from integral membrane proteins by centrifugation. As shown in Fig. 1C, Mim2–HA, like the integral membrane protein Tom40, was enriched in the pellet fraction, suggesting that Mim2 is a mitochondrial membrane protein.

Since mitochondria have two distinct membranes, we wanted to investigate in which membrane Mim2 is located and study its membrane topology. Mitochondria containing Mim2–HA were either left intact or their OM was ruptured under hypo-osmolar conditions. Thereafter samples were treated with proteinase K (PK). In intact mitochondria, Mim2–HA is cleaved and a smaller fragment of about 11 kDa was detected (Fig. 1D, second lane). This fragment was not observed when the MOM was ruptured or when mitochondria were solubilized with detergent (Fig. 1D). The IMS localized protein Dld1 and the matrix protein Mge1 served to control the integrity of the outer and inner membranes, respectively. These results demonstrate that Mim2 is anchored in the MOM with its C-terminus facing the IMS.

An unusual feature of Mim2 is the distribution of charged amino acid residues along its sequence. Negatively charged

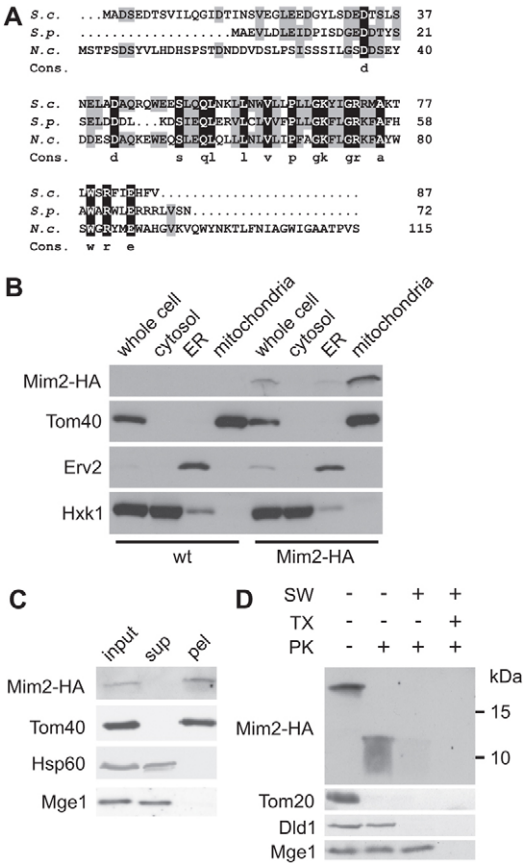


Fig. 1. Mim2 is an integral protein of the MOM with its C-terminus facing the intermembrane space. (**A**) Mim2 is conserved among fungi. Amino acid sequences of Mim2 from *Saccharomyces cerevisiae* (*S.c.*), *Schizosaccharomyces pombe* (*S.p.*) and *Neurospora crassa* (*N.c.*) are shown. Identical residues are depicted in white on black background, similar residues are highlighted in grey. (**B**) Mim2 is a mitochondrial protein. Whole-cell lysate (whole cell) and fractions corresponding to cytosol, light microsomal fraction (ER) and mitochondria of either wild-type cells or cells expressing Mim2–HA were analyzed by SDS-PAGE and immunodecoration with antibodies against the HA-tag, the mitochondrial protein Tom40, the ER protein Erv2 and a marker protein for the cytosol (hexokinase, Hxk1). (**C**) Mim2 is a membrane-embedded protein. Mitochondria isolated from cells expressing Mim2–HA were subjected to carbonate extraction. The supernatant (sup) and pellet (pel) fractions were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins. Tom40, an integral OM protein; Hsp60 and Mge1, soluble matrix proteins. (**D**) The C-terminus of Mim2 is protected from protease digestion by the MOM. Mitochondria isolated from cells expressing Mim2–HA were treated with proteinase K (PK) under different conditions. Mitochondria were kept intact, the outer membrane was ruptured by hypo-osmolar swelling (SW) or mitochondria were lysed completely by the addition of the detergent Triton X-100 (TX). Samples were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and immunodecoration with antibodies against the HA-tag, or the indicated mitochondrial proteins. Tom20, an OM protein exposed to the cytosol; Dld1, an IMS protein; Mge1, a matrix protein.

residues cluster at the N-terminal region, whereas the C-terminal part is positively charged. While low concentrations of the rather unspecific protease PK were sufficient to cleave Mim2–HA, treating intact mitochondria with high concentrations of trypsin, a protease cutting C-terminally to positively charged amino acids, did not result in a cleavage of Mim2–HA (supplementary

material Fig. S3). These results further support our proposal that the positively charged C-terminal region of Mim2 is protected by the MOM. Taken together our findings suggest that Mim2 is an integral membrane protein of the MOM with its N-terminus located in the cytosol and the C-terminus residing in the IMS.

Mim1 and Mim2 physically interact and are components of the same complex

Although we identified Mim2 as a protein that associates with GFP–Mim1 we wanted to substantiate the interaction between the two proteins by additional pull-down experiments. Mitochondria

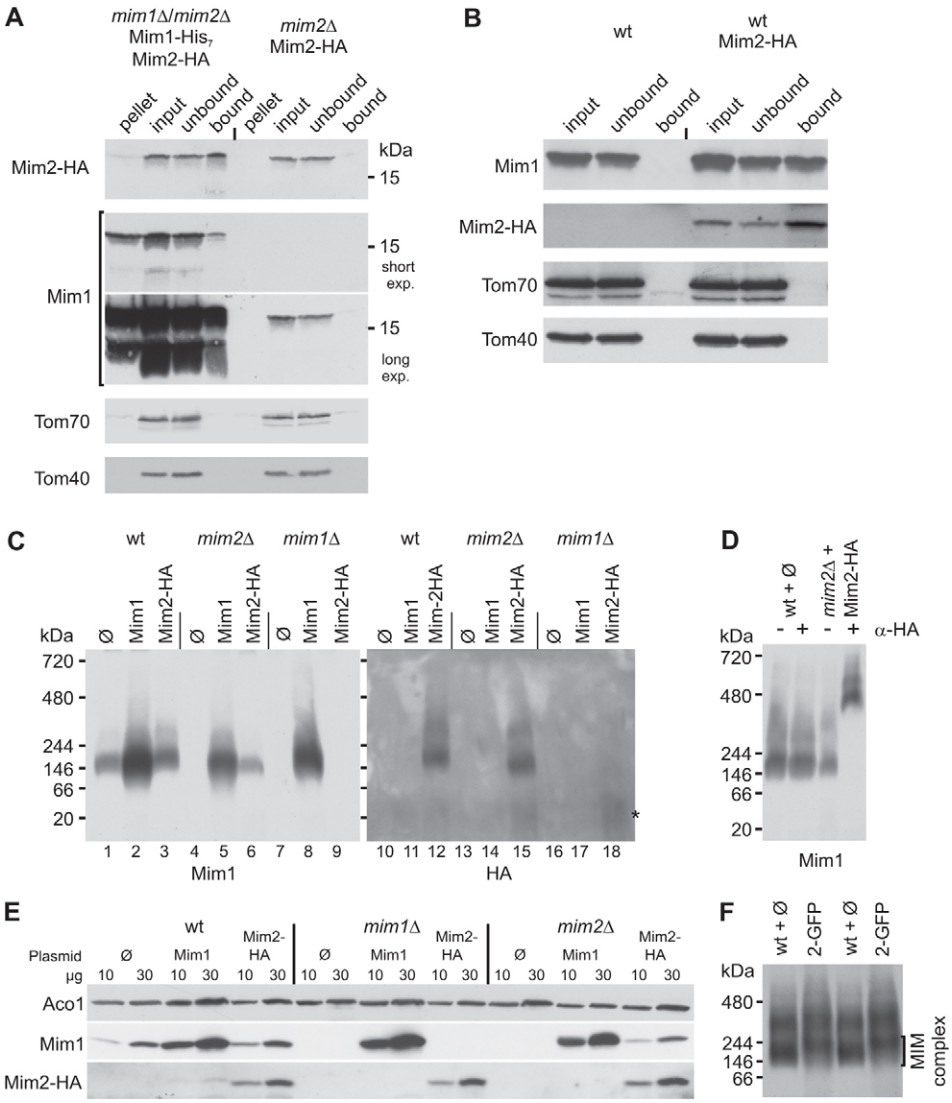


Fig. 2. Mim1 and Mim2 physically interact and are components of the same protein complex. (**A**) Mitochondria isolated from either the double-deletion strain *mim1*Δ/*mim2*Δ overexpressing Mim1-His$_7$ and Mim2–HA or a single deletion strain *mim2*Δ overexpressing Mim2–HA were employed. Organelles were lysed in digitonin-containing buffer and cleared supernatants were incubated with Ni–NTA beads. Non-solubilized matter (pellet), cleared supernatant (input, 20% of total), supernatant after binding to the beads (unbound, 20% of total) and material bound to the beads (bound, 100% of total) were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. (**B**) Mitochondria isolated from a strain overexpressing Mim2–HA (Mim2-HA) and the corresponding wild-type strain were lysed in digitonin-containing buffer. Cleared supernatants were incubated with ProteinG Sepharose beads preincubated with an antibody against the HA-tag. Supernatants before (input, 10% of total) and after (unbound, 10% of total) binding to the beads as well as bound material (bound, 100% of total) were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. (**C**) Mitochondria isolated from wild-type, *mim1*Δ or *mim2*Δ strains harboring an empty plasmid (Ø) or overexpressing either Mim1 or Mim2–HA were lysed in digitonin and analyzed by BN-PAGE. For analysis of Mim1 and Mim2–HA containing complexes, ~~membranes were~~ immunodecorated with antibodies against Mim1 and the HA-tag, respectively. An unassembled species of Mim2–HA is indicated with an asterisk. (**D**) Mitochondria isolated from a wild-type or *mim2*Δ strains containing either empty plasmid (Ø) or overexpressing Mim2–HA were solubilized in digitonin, the lysate was cleared by centrifugation and then incubated with or without an antibody against the HA–tag (α-HA). Samples were analyzed by BN-PAGE and immunodecoration with an antibody against Mim1. (**E**) Two different amounts (10 μg and 30 μg) of the mitochondria described above in the legend to panel (C) were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. The matrix protein aconitase (Aco1) served as a control. (**F**) Mitochondria isolated from a wild-type strain containing empty plasmid (Ø) and a *mim2*Δ strain overexpressing GFP–Mim2 (2-GFP) were solubilized in digitonin, and samples were analyzed by BN-PAGE and immunodecoration with an antibody against Mim1. For easier observation of the small size difference, the same samples were loaded twice in alternating lanes. The ~~NIN~~ complex is indicated.

were isolated from a *mim1Δ/mim2Δ* double-deletion strain overexpressing Mim1-His$_7$ (Popov-Celeketić et al., 2008) and Mim2–HA. A *mim2*Δ strain overexpressing Mim2–HA that contains non-tagged endogenous Mim1 served as a control (Fig. 2A). The isolated organelles were lysed and proteins were incubated with Ni–NTA beads to pull down Mim1-His$_7$. Subsequent SDS-PAGE and immunodecoration showed that Mim2–HA specifically bound to the affinity beads together with Mim1-His$_7$. No unspecific binding of Mim2–HA to the beads was observed with the control sample. Of note, the enrichment of Mim2–HA in the bound material was even higher than that of Mim1-His$_7$, suggesting a tight association of both proteins. A further potential explanation for this enrichment of Mim2 is that the binding of Mim2 to Mim1-His$_7$ causes a conformational change in the latter protein that in turn results in an increased accessibility of the His-tag for binding to the affinity beads.

To further verify this interaction, we performed the reciprocal co-immunoprecipitation experiment. Mitochondria isolated from a strain expressing Mim2–HA were solubilized with the mild detergent digitonin and then incubated with beads loaded with antibody specific for the HA-tag. A significant amount of the endogenous Mim1 was co-precipitated together with Mim2–HA (Fig. 2B). No unspecific binding of Mim1 to the beads was observed when the corresponding wild-type mitochondria were used as a control (Fig. 2B).

Mim1 was reported to be a component of a high molecular weight complex (Ishikawa et al., 2004; Waizenegger et al., 2005; Becker et al., 2008; Popov-Celeketić et al., 2008). Our results show that Mim1 and Mim2 tightly interact and indicate that Mim2 is a novel component of this Mim1-containing complex that we named the MIM complex. To confirm this hypothesis, mitochondria from *mim1*Δ or *mim2*Δ strains overexpressing either Mim1 or Mim2–HA were analyzed by blue native gel electrophoresis (BN-PAGE). Both Mim1 and Mim2-HA migrated as a complex of approximately 200 kDa (Fig. 2C, compare lanes 3 and 12 and supplementary material Fig. S4) confirming that the two proteins are indeed components of the same oligomeric structure. Expression of Mim2–HA in the strain lacking endogenous Mim2 only partially restored the levels of the MIM complex as assessed by BN-PAGE (Fig. 2C, compare lane 6 to lane 1) although the steady-state levels of Mim1 as monitored by SDS-PAGE were almost normal (Fig. 2E). These observations suggest that even though Mim2–HA complements the *mim2*Δ growth phenotype, the HA-tag might interfere with the optimal interaction of Mim2 with Mim1.

Next, we investigated the importance of Mim2 and Mim1 for the formation of the MIM complex. Of note, no Mim1-containing oligomeric species could be detected in the absence of Mim2 and the protein could not be detected in SDS-PAGE and immunodecoration (Fig. 2C, lane 4; Fig. 2E). Hence, Mim2 is a crucial player in the biogenesis of Mim1 and the MIM complex. The absence of Mim1 has different effects as it leads to a loss of a detectable Mim2–HA-containing complex but unassembled species of the protein is present (Fig. 2C, lane 18; supplementary material Fig. S5) and expression levels of the protein are unaffected (Fig. 2E).

To further substantiate the participation of both proteins in the same complex we used mitochondria isolated from wild-type and *mim2*Δ cells transformed with either Mim2–HA encoding vector or an empty plasmid as control. Next we lysed the organelles with detergent and performed an antibody-shift assay where antibodies against the HA-tag were added to the lysed organelles before their analysis by BN-PAGE. The antibodies caused a shift in the migration of the Mim1 signal (Fig. 2D), suggesting that both Mim1 and Mim2 are subunits of the same MIM complex.

Of note, overexpression of Mim1 in the absence of Mim2 resulted in a complex with apparent similar migration behaviour to the native complex (Fig. 2C, compare lane 1 to 5). Hence, it seems that Mim2 is not absolutely required for the formation of Mim1-containing complex. This observation

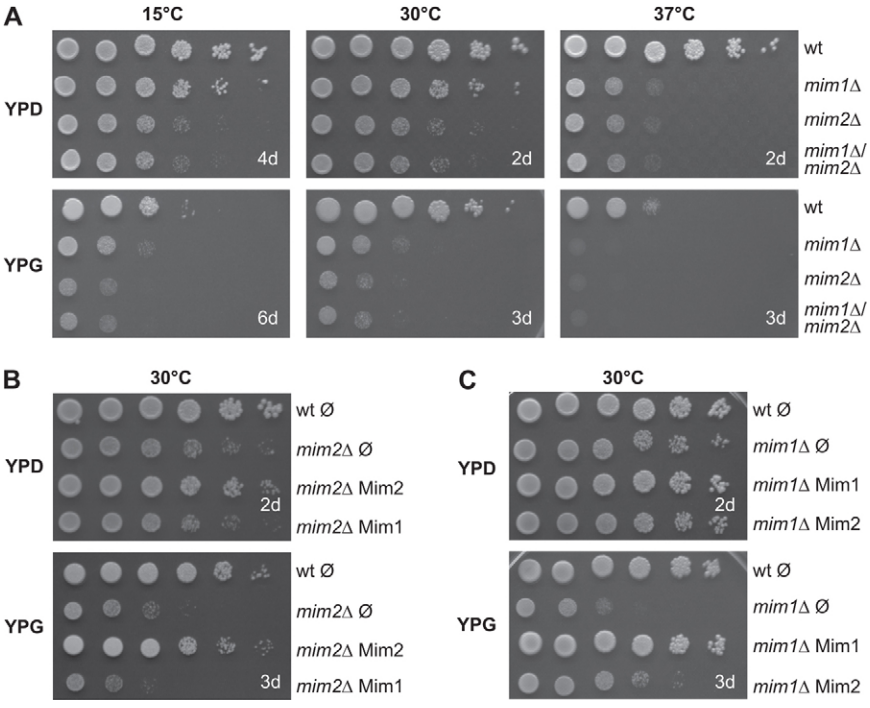


Fig. 3. Deletion of *MIM2* results in severe growth phenotypes. (**A**) Cells that lack Mim1, Mim2 or both proteins show reduced growth at all conditions. The indicated strains were tested at three different temperatures by drop-dilution assay for growth on rich medium containing the fermentable carbon source glucose (YPD) or the non-fermentable carbon source glycerol (YPG). Pictures were taken after the indicated number of days. (**B**) Overexpression of Mim1 does not rescue the growth defect of a *mim2*Δ strain. Wild type cells transformed with an empty plasmid and *mim2*Δ cells transformed with an empty plasmid, Mim2 encoding plasmid or Mim1 encoding plasmid were analyzed by drop-dilution assay on YPD or YPG medium. (**C**) Overexpression of plasmid-borne Mim2 partially rescues the growth defect of the *mim1*Δ strain. Wild type cells transformed with an empty plasmid and *mim1*Δ cells transformed with an empty plasmid, Mim1 encoding plasmid, or Mim2 encoding plasmid were analyzed by drop-dilution assay on YPD or YPG medium.

further suggests that the native MIM complex probably contains only one or two copies of Mim2. Therefore the absence of Mim2 causes only a minor difference of 10–20 kDa in the mass of the MIM complex and such a difference in turn is hard to resolve by BN-PAGE. In order to obtain further support for our assumption that the two proteins are components of the same native complex, we analyzed the MIM complex in organelles harbouring GFP-tagged Mim2. If Mim1 is a component in the same complex as Mim2, the additional mass of the GFP moiety should shift also the band of the Mim1-containing complex as analyzed by BN-PAGE. Indeed, clear slower migration behaviour of the Mim1-complex was observed in the organelles harbouring the GFP-tagged Mim2 (Fig. 2F). Taken together, our results suggest that Mim1 and Mim2 are components of the same protein complex.

Deletion of MIM2 causes severe growth phenotype

The ORF *YLR099W-A/MIM2* was reported in a systematic deletion attempt to be an essential gene (Kastenmayer et al., 2006). We wanted to confirm the reported lethality by deleting the complete ORF of *MIM2* in the diploid yeast strain W303a/α and then performing tetrad analysis. After sporulation and tetrad dissection, haploid *mim2*Δ strains were retrieved as confirmed by PCR (data not shown). In contrast to the reported lethality, this deletion strain was viable although it showed a severe growth reduction on fermentable and non-fermentable carbon sources at all tested temperatures (Fig. 3A). The growth behavior of the *mim2*Δ strain is even worse than that of the strain lacking Mim1 and the double-deletion strain grows like the *mim2Δ* strain (Fig. 3A). To exclude the possibility that the observed phenotypes were caused by unrelated changes, e. g. changes in the promoter region of the essential *ERG27* gene –which is in close proximity on the chromosome to the *MIM2* gene – we aimed to complement these phenotypes by plasmid-encoded Mim2. Overexpression of native or the C-terminally tagged version of Mim2 could rescue the growth phenotype of the deletion mutant confirming that the observed phenotypes are related to the absence of the Mim2 protein (supplementary material Fig. S2).

MIM2 and MIM1 genetically interact

Our results suggest that Mim1 and Mim2 physically interact and are components of the same protein complex. Hence we asked whether the two ORFs also genetically interact. We could not observe a synthetic growth phenotype by deletion of both genes (Fig. 3A). Of note, overexpression of Mim1 in yeast cells lacking Mim2 slightly hampered the growth of the *mim2*Δ strain (Fig. 3B). Accordingly, the steady-state levels of the MIM substrate Ugo1 are somewhat reduced in these cells (supplementary material Fig. S6). On the other hand, overexpression of Mim2 in a *mim1*Δ strain led to partial rescue of the growth phenotype (Fig. 3C). This partial rescue was observed in six independent transformants and was paralleled by elevated levels of Ugo1. Furthermore, the overexpression of Mim2–HA in the *mim1*Δ strain caused higher levels of Tom40 and less unassembled Tom40 molecules as compared to *mim1*Δ cells (supplementary material Fig. S7). These results suggest that higher levels of Mim2 can reduce for some processes the dependency on Mim1. Collectively, in addition to their physical association, *MIM1* and *MIM2* genetically interact.

Deletion of MIM2 leads to abnormal mitochondrial morphology

It was previously reported that downregulation of Mim1 leads to altered mitochondrial morphology (Altmann and Westermann, 2005; Dimmer and Rapaport, 2010). It is assumed that this phenotype results from the impaired assembly of the TOM complex and the subsequent insufficient import of morphology relevant proteins. We verified this phenotype by deletion of *MIM1* in the wild-type background W303 (Fig. 4A). Typically for this deletion strain, mitochondria were fragmented and aggregated in approximately 90% of the cells (Fig. 4B). Very similar morphological phenotype was observed upon deletion of

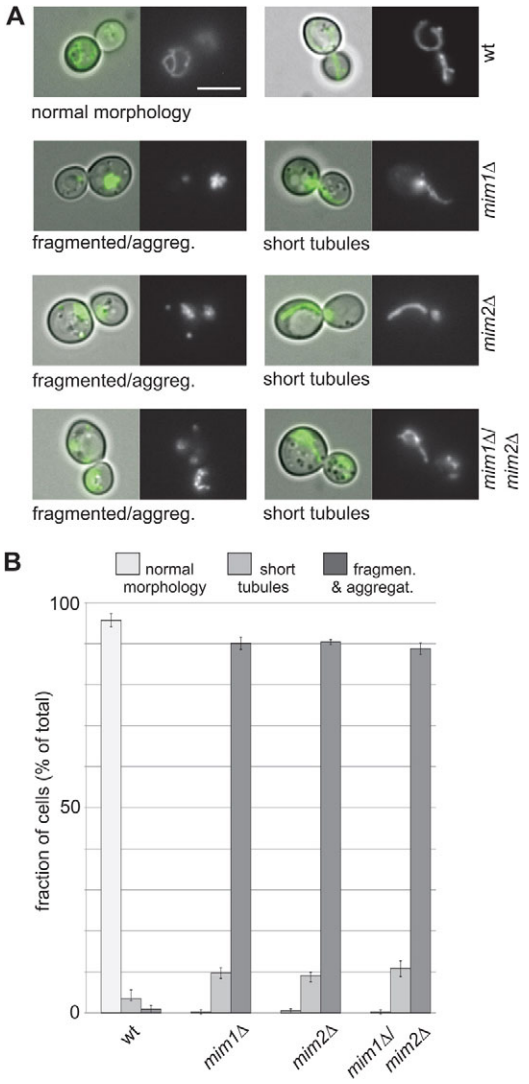


Fig. 4. Cells that lack Mim1, Mim2 or both proteins show altered mitochondrial morphology. (**A**) Cells of the indicated strains transformed with mitochondrially targeted GFP (pSu9-GFP) were analyzed by fluorescence microscopy. Examples of predominant phenotypes (fragmented/aggregated and short tubular) for each strain are shown (scale bar: 5 µm). (**B**) Analyzed cells of the various strains were grouped into three different morphology phenotypes (normal, short tubules, and fragmented and aggregated). Statistical analysis of four different experiments in which ⩾ 100 cells per experiment were analyzed was performed and the various occurrences of the phenotypes are presented.

MIM2 alone or in the *mim1*Δ/*mim2*Δ double-deletion strain (Fig. 4A, B). These results provide further evidence that Mim1 and Mim2 function in the same molecular pathway.

Loss of Mim2 leads to reduced biogenesis of mitochondrial proteins

To gain further insight into the function of Mim2, we analyzed the steady-state levels of proteins in mitochondria isolated from *mim2*Δ cells. Of note, Mim1 was hardly detectable in these organelles and a severe reduction was observed in the levels of the MOM proteins Tom20, Fzo1 and Ugo1 – known substrates of Mim1 (Fig. 2E; Fig. 5A, left panel) (Waizenegger et al., 2005; Becker et al., 2011; Papic et al., 2011). In contrast to Tom20, the levels of all other TOM components tested – Tom40, Tom22 and Tom70 – did not show a significant reduction in mitochondria lacking Mim2. Similarly, the steady-state levels of other mitochondrial proteins like the MOM β-barrel protein Por1, the tail-anchored protein Fis1, the inner membrane proteins Oxa1 and Dld1, as well as the matrix proteins Hsp60 and aconitase were unaltered in comparison to those in wild-type organelles (Fig. 5A, right panel).

We next compared the assembly status of the TOM complex in mitochondria isolated from strains lacking Mim2, Mim1 or both. The amount of assembled TOM complex as assessed by immunodecoration with antibodies against Tom40 and Tom22 was drastically reduced when *MIM1*, *MIM2* or both were deleted (Fig. 5B). Concomitantly, an unassembled species of Tom40 was observed in the mutated cells. The observations regarding the reduced stability of the TOM complex in *mim1Δ* cells are in line with previous reports (Ishikawa et al., 2004; Waizenegger et al., 2005). The assembly of the TOB complex as monitored by BN-PAGE was unchanged in these deletion strains (Fig. 5B). Collectively, the absence of Mim2 resulted in reduced steady-state levels of Tom20 and multispan MOM proteins as well as reduced stability of the TOM complex.

Mitochondria lacking Mim2 show compromised import of multispan MOM proteins

Since the steady-state levels of certain mitochondrial proteins were reduced in mitochondria lacking Mim2, we investigated its role in mitochondrial protein import. To this end we first analyzed whole-cell extracts for accumulation of mitochondrial precursor proteins, a phenotype that was observed in cells lacking Mim1 (Ishikawa et al., 2004; Mnaimneh et al., 2004; Waizenegger et al., 2005). We observed a clear accumulation of unprocessed precursor form of the matrix protein Hep1 in extracts from cells lacking Mim1 or Mim2 or both proteins (Fig. 6A). This indicates a global import defect of mitochondria lacking Mim2.

Next we investigated the in vitro import for model substrates located in the different mitochondrial compartments. Isolated mitochondria were incubated with radioactive precursor for different time points and import was assessed by SDS-PAGE and autoradiography. The import efficiencies for the matrix destined preprotein pSu9-DHFR, the inner membrane protein AAC, as well as the β-barrel precursor porin were reduced (Fig. 6B). Of note, the most pronounced reduction was in the case of the MOM multispan proteins Ugo1 and Fzo1 (Fig. 6B). In contrast, the import of the tail-anchored MOM protein Fis1, which is inserted independently of any known import factors (Kemper et al., 2008), was unaffected by the absence of Mim2 (data not shown). The global defect in mitochondrial import results most probably from a reduced number of functional TOM complexes. In accordance

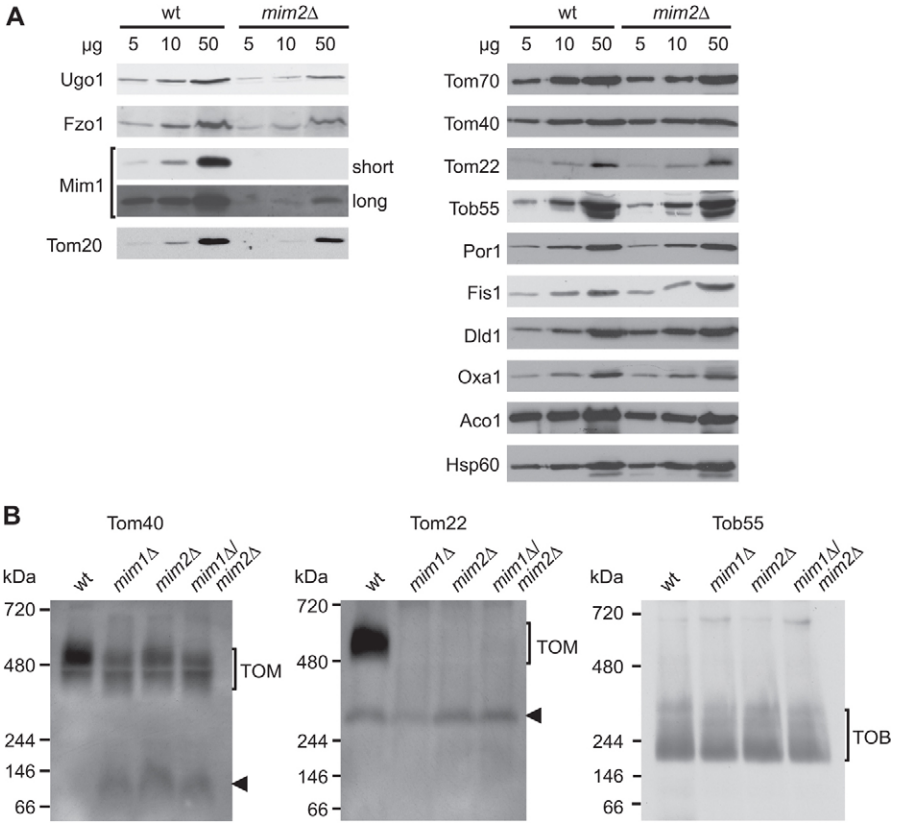
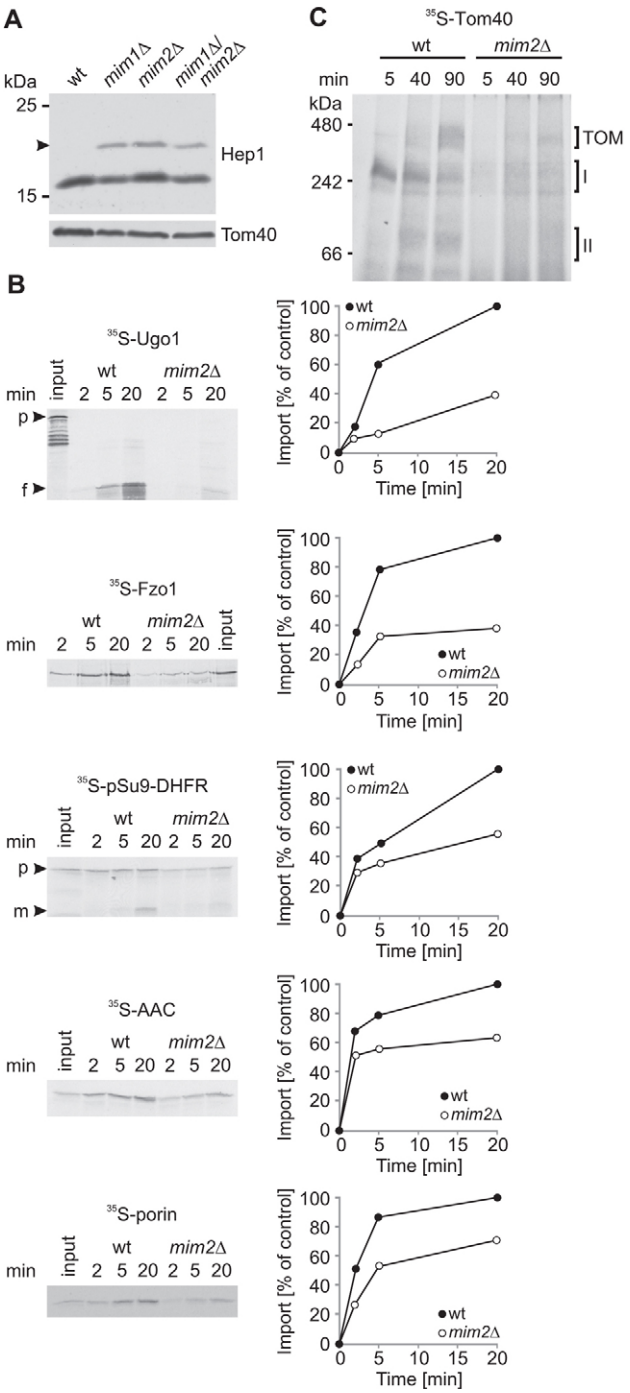


Fig. 5. Absence of Mim2 leads to reduced steady-state levels of helical MOM proteins and a compromised assembly of the TOM complex. (**A**) Various ~~concentrations~~ of mitochondria (5, 10 and 50 μg) isolated from wild-type and *mim2*Δ cells were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. A representative experiment of three different independent repeats is presented. (**B**) Mitochondria of the indicated strains were first lysed in 1% digitonin (for TOM analysis) or in 0.5% Triton X-100 (for TOB analysis) and then subjected to BN-PAGE and immunoblotting with the indicated antibodies. Arrowheads indicate the Tom40-containing low molecular mass species and the Tom22 assembly intermediate. The assembled TOM and TOB complexes are indicated. A representative experiment of three different independent repeats is presented.

with the reduced steady-state levels of assembled TOM complex in mitochondria lacking Mim2 (Fig. 5B), the assembly of newly synthesized Tom40 molecules into the TOM complex is severely hampered in *mim2*Δ cells (Fig. 6C).

We aimed to analyze the direct import defects due to lower levels of Mim2 avoiding the global outcome resulting from compromised biogenesis of Tom components and hampered assembly of the TOM complex. To that end a yeast strain in which the expression of *MIM2–HA* was under the control of the *GAL1* promoter was constructed. In the presence of galactose the cells grew like wild type cells whereas growth on glucose was strongly compromised. We first tested the levels of various mitochondrial proteins in total cell lysates from the *GAL1–MIM2–HA* cells grown at various time periods after the shift from galactose- to glucose-containing medium (data not shown). On the basis of this analysis we isolated mitochondria from cells grown for 15 h on glucose and analyzed their proteins by immunodecoration. Of note, Mim2 and its partner protein Mim1 were hardly detectable in these organelles whereas the Tom components were still in normal levels (supplementary material Fig. S8A). Furthermore, the TOM complex as analyzed by BN-PAGE was also detected in normal levels (supplementary material Fig. S8B). Next, in vitro import assays were performed with mitochondria depleted for Mim2. Importantly, whereas the insertion of the MIM substrate Ugo1 into these organelles was compromised, no import defects were observed for the TOM substrates pSu9-DHFR and porin (supplementary material Fig. S8C). Taken together the results suggest that the absence of Mim2 causes two effects: a specific reduction in membrane integration of some outer membrane helical proteins and subsequently a global import defect due to altered stability of the TOM complex.



Mim2 is directly involved in the import of Ugo1

Finally, we asked whether Mim2 actually participates in interactions with substrate proteins. To that end, we analyzed import reactions of newly synthesized [^{35}S]Ugo1 by BN-PAGE in combination with an antibody-shift assay. Mitochondria were isolated from wild-type and *mim2*Δ cells transformed with an empty plasmid and a plasmid encoding Mim2–HA, respectively. After import of Ugo1, mitochondria were lysed in digitonin, halved and an antibody specific for the HA-tag was added to one portion. Strikingly, addition of the antibody resulted in a shift of the radioactive signal of [^{35}S]Ugo1 to higher molecular weights only if mitochondria harboring Mim2–HA were used (Fig. 7, left panel, arrowhead). Thus, Mim2 interacts with substrate proteins. A similar shift was observed for the Mim1 signal (Fig. 7, right

Fig. 6. Deletion of *MIM2* leads to various import defects and impaired assembly of the TOM complex. (**A**) Whole-cell lysates of wild type cells and those lacking Mim1, Mim2 or both proteins were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. The precursor of the mitochondrial matrix protein Hep1 is indicated by an arrowhead. (**B**) Mitochondria isolated from a wild-type or *mim2*Δ strain were incubated with the indicated radiolabelled precursor proteins for the indicated time periods. At the end of the import reactions samples were treated as described below, analyzed by SDS-PAGE and autoradiography, and bands corresponding to imported material were quantified. Samples containing radiolabeled Ugo1 were trypsinated in order to generate a specific 23 kDa fragment (f) (see Papic et al., 2011). After import of Fzo1, carbonate extraction was performed and the membranous fraction was analysed; when pSu9-DHFR was imported, the mature protein (m) was quantified. After import of porin and AAC mitochondria were treated with PK and the protected molecules were quantified. The intensity of bands representing imported material into wild-type mitochondria for the longest time period was set as 100%. (p) precursor form of pSu9-DHFR ~~or~~ Ugo1. A representative experiment of three independent repeats is presented. (**C**) Radiolabelled precursor of Tom40 was imported into mitochondria that had been isolated from *mim2*Δ or the corresponding wild-type strain. After import, the mitochondria were solubilized with digitonin and analyzed by BN-PAGE and autoradiography. The two assembly intermediates of Tom40 (I, II) and the assembled TOM core complex (TOM) are indicated.

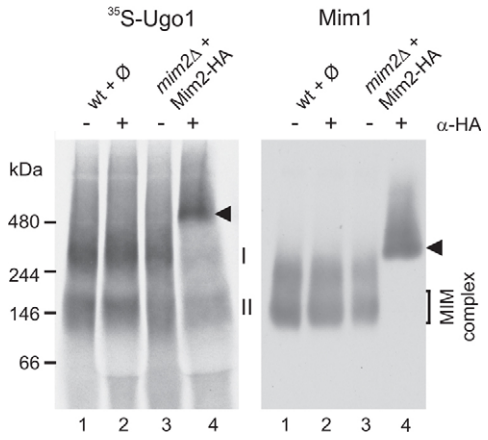


Fig. 7. Mim2 is directly involved in Ugo1 import. Mitochondria isolated from a wild-type strain containing the empty plasmid (Ø) and from a *mim2*Δ strain overexpressing Mim2–HA were incubated with radiolabelled precursor of Ugo1. After the import reactions mitochondria were solubilized in digitonin; the lysate was cleared by centrifugation and then incubated with or without an antibody against the HA-tag (α-HA). Samples were analyzed by BN-PAGE, autoradiography (left panel) and then immunodecoration with an antibody against Mim1 (right panel). Mim2-containing complexes that were shifted by the antibody are indicated by an arrowhead.

panel) suggesting that both Mim1 and Mim2 are subunits of the functional substrate-binding MIM complex.

Taken together, this study reveals that the integral MOM protein Mim2 is a novel component of the MIM complex that mediates the import of integral MOM helical proteins.

Discussion

In this work we report on the identification of Mim2 as a novel protein with a crucial function in the biogenesis of mitochondria. Mim2 is located in the MOM, exposing its N-terminus to the cytosol and the C-terminus to the IMS. The protein shares this topology with its binding partner Mim1 and it also shows functional similarity to the latter protein. Altered mitochondrial morphology, reduced growth, lower steady-state levels of several mitochondrial components as well as compromised assembly of TOM complex are consequences of both *MIM2* and *MIM1* deletion. Mim1, Tom20 and the multispan proteins of the MOM seem to be the main substrates that are affected by the absence of Mim2. We propose that hampered biogenesis in the absence of Mim2 results in reduced steady-state amounts and assembly of different proteins in *mim2*Δ cells that in turn cause the other observed phenotypes in these cells. For example, the altered morphology of mitochondria in *mim1Δ* and *mim2Δ* cells can be explained by the lower levels of their substrate proteins Fzo1 and Ugo1. The latter two proteins mediate mitochondrial fusion and thus their reduced levels interfere with the balance between fusion and fission of the organelles.

Strikingly, the steady-state level of Mim1 is severely reduced in mitochondria lacking Mim2. This finding might suggest that the observed effects in *mim2*Δ cells are solely due to the loss of Mim1. Yet several observations are in contrast to this scenario. First, the growth phenotype of *mim1*Δ cells can be partially rescued by the overexpression of Mim2. Therefore additional copies of Mim2 can, to a certain extent, reduce the requirement for Mim1. Second, overexpression of Mim1 in a *mim2*Δ strain does not improve the growth retardation but rather has even somewhat negative effect on growth. These observations suggest a unique function of Mim2 and might indicate that in the absence of Mim2 some unassembled Mim1 molecules exert a dominant-negative effect by competing with the function of the MIM complex. Third, our pull-down experiments and native electrophoresis assays demonstrate that both proteins are present in a stable functional complex that interacts with substrate proteins.

Our results shed new light on the stoichiometry of the MIM complex as they show that reduced levels of Mim1 or its overexpression have a minor effect on complex size. Thus it seems that the actual complex of around 200 kDa contains a rather fixed number of copies of Mim1 and Mim2. In the absence of Mim1 we could not observe a Mim2-containing sub-complex suggesting that Mim1 is a crucial component for the formation of the MIM complex. In contrast, a Mim1-containing complex was formed even in the absence of Mim2 suggesting that the latter protein is not absolutely essential for complex formation. Naturally, we cannot exclude the possibility that additional, yet to be identified, proteins are further components of the MIM complex. Future efforts to functionally reconstitute the complex from isolated subunits can provide an answer to this open question. Taken together, the identification and characterization of Mim2 get us one step ahead in solving the riddle of import of outer membrane proteins, yet the elucidation of the precise composition of the MIM complex and its molecular mode of action has to be the next venture.

Materials and Methods

SILAC-based immunoprecipitation

*Mim1*Δ (YPH499 background) cells expressing plasmid encoding either native Mim1 or GFP-Mim1 were grown in synthetic media containing either $^{15}N_2$-$^{13}C_6$ lysine (heavy) or (light) lysine (Ong et al., 2002). Cells were harvested in mid-exponential phase and mitochondria were isolated after enzymatic spheroblastation, using an abridged protocol. EDTA was omitted from all buffers. Mitochondria were lysed in 1% digitonin, 10% (v/v) glycerol, 150 mM NaCl, 50 mM MOPS/KOH, pH 7.4, containing complete protease inhibitor cocktail (Roche). Samples were clarified by centrifugation and incubated with GFP-binder beads (gta100, Chromotek). Beads were isolated, washed and pooled according to forward (Mim1 light and GFP–Mim1 heavy) and reverse (Mim1 heavy and GFP–Mim1 light) experiments. Proteins were eluted with 4% SDS, 100 mM Tris–HCl pH 8.0, 0.1 M DTT and subjected to filter aided sample preparation method and digestion with endoproteinase (Wiśniewski et al., 2009).

Mass spectrometry and data analysis

Peptides were separated by nLC at 4 h gradient length without prior fractionation, electrosprayed online and analyzed with LTQ-Orbitrap-XL or Orbitrap-Velos mass spectrometers using collision-induced dissociation or higher-energy collisional dissociation fragmentation, respectively (Olsen et al., 2005; Olsen et al., 2009). Data analysis was performed using the MaxQuant software environment (Cox and Mann, 2008) version 1.0.13.9. Searches of generated peak lists were carried out with Mascot (Perkins et al., 1999) against the translation of all 6809 gene models from the Saccharomyces Genome Database (release date 12 December 2007) and 175 frequently observed contaminants. Identifications were accepted at a false discovery rate of 1% both at the peptide and protein level using a decoy database strategy with reversed protein sequences (Elias and Gygi, 2007).

Yeast strains and growth conditions

Standard genetic techniques were used for growth and manipulation of yeast strains. Unless otherwise stated, the wild-type strain W303 was used. The *mim1*Δ/*mim2*Δ double-deletion strain was obtained by mating of the single deletion strains followed by tetrad dissection. Transformation of yeast was carried out according to the lithium-acetate method. For drop-dilution assays, yeast cells were grown in synthetic medium to an OD_{600} of 1.0, diluted in fivefold increment, and then 5 µl of each dilution were spotted onto solid media and growth was monitored for few days.

Recombinant DNA techniques

To express Mim1 or Mim2 in yeast cells with or without a C-terminal HA-tag, the ORF of *MIM1* or *MIM2* (systematic name *YHR099W-A*) was amplified by PCR

with or without its stop codon using yeast genomic DNA as template. Primers used contained *Eco*RI and *Hind*III restriction sites which were used to introduce the amplified fragment into the expression vector pYX142 which contains the HA-tag sequence. Constructs were verified by sequencing. For expression of Mim1 with a C-terminal His_7-tag, the plasmid pRS426-TPIpro-Mim1-His_7 was used (Popov-Celeketić et al., 2008).

Yeast genes were deleted by a PCR-based approach using the HIS3 marker amplified from pFA6a-His3MX6 (Wach et al., 1997) or the kanamycin resistance cassette amplified from pFA6a-kanMX4 (Wach et al., 1994). For the deletion of *MIM1* the primers KSD311 (5′-AGAAACATCACCCCCTTCTTACGAAACT-GCCACAAGACAGAAATCGTACGCTGCAGGTCGAC-3′) and KSD 312 (5′-GTGTGTGTATTTATTTATGTAGGTTGCTAATGCTTTGGTGATCGTATCGA-TGAATTCGAGCTCG-3′) were used, for MIM2 KSD099f (5′-CCCAGCACC-ACAGCACATCACTGCACGAGCAACAATAACTAGAACCGTACGCTGCAGG-TCGAC-3′) and KSD099r (5′-TTATCTGTTATAACTGCTATATGCGGATACA-TAAACAACAAACACATCGATGAATTCGAGCTCG-3′). Deletion of genes was confirmed by screening-PCR. Haploid deletions strains were obtained by tetrad dissection.

A yeast strain harbouring Mim2 under the control of an inducible promoter was obtained by transforming the pYX113-GAL1pro-*MIM2*-HA vector into *mim2*Δ strain. To construct this plasmid the *MIM2* ORF without the stop codon was subcloned from pYX142-*MIM2*-HA. For expression of GFP–Mim2 the ORF of *MIM2* was cloned into pYX132-Nterm-GFP using *BamH*I and *Hind*III sites. The pYX132-Nterm-GFP plasmid contains the coding sequence for GFP without a stop codon cloned between *Eco*RI and *BamH*I sites.

Biochemical procedures

Mitochondria were isolated from yeast cells by differential centrifugation as previously described (Daum et al., 1982). Subcellular fractionation was performed according to published procedures (Walther et al., 2009). Import experiments with radiolabeled precursor proteins and isolated mitochondria were performed in an import buffer containing 250 mM sucrose, 0.25 mg/ml BSA, 80 mM KC1, 5 mM $MgCl_2$, 10 mM MOPS–KOH, 2 mM NADH, 2 mM ATP, pH 7.2. Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Import assays for the mitochondrial precursor proteins pSu9-DHFR, AAC, Porin, and Ugo1 were performed as described before (Papic et al., 2011). For swelling experiments, isolated mitochondria were incubated with a hypotonic buffer (20 mM HEPES, pH 7.2) for 30 min on ice. In the carbonate extraction reaction mitochondria were dissolved in 0.1 M Na_2CO_3. After 30 min on ice, samples were centrifuged (75000 ***g***, 60 min, 2℃) and pellet and supernatant were analyzed.

For pull-down experiments, mitochondria from the *mim1*Δ/*mim2*Δ yeast strain expressing Mim2–HA and Mim1-His_7 or the *mim2*Δ strain expressing Mim2–HA were used. After lysis in digitonin buffer (0.5% digitonin, 20 mM Tris-HCl, 50 mM NaCl, 10% glycerol, 1 mM PMSF, pH 7.4) and clarifying spin (20000 ***g***, 20 min, 2℃) supernatants were incubated for 1 h at 2℃ with Ni-NTA agarose beads (NEB) that were pre-equilibrated in digitonin-buffer. After washing twice, bound material was analyzed by SDS-PAGE and immunodecoration.

Co-immunoprecipitation experiments were performed using isolated wild-type mitochondria and mitochondria isolated from a strain expressing Mim2–HA. After binding of the HA-antibody to Protein G Sepharose beads these were incubated with cleared lysate of the mitochondria in digitonin buffer (1% digitonin, 20 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 1 mM PMSF, pH 7.4). After several washes, bound proteins were analyzed by SDS-PAGE and immunodecoration.

Protein samples were analyzed by SDS-PAGE and blotting to nitrocellulose membranes followed by visualization through autoradiography. Alternatively, incubation with antibodies was carried out according to standard procedures and visualization was performed via the ECL method. Intensity of the observed bands was quantified with the AIDA software (Raytest). Unless stated otherwise, each presented experiment represents at least three independent repetitions.

Blue Native PAGE

Mitochondria were lysed in 40 µl TX-100 or digitonin buffer (0.5% TX-100 or 1-1.5% digitonin, 20 mM Tris-HCl, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM PMSF, pH 7.4). After incubation for 15 min at 4℃ and a clarifying spin (30000 ***g***, 15 min, 2℃), 5 µl sample buffer (5% [w/v] Coomassie Brilliant Blue G-250, 100 mM Bis-Tris, 500 mM 6-aminocaproic acid, pH 7.0) were added, and the mixture was analyzed by electrophoresis in a 6 to 13% gradient blue native gel (Schägger et al., 1994). Gels were blotted to polyvinylidene fluoride membranes and proteins were further analyzed by autoradiography or immunodecoration. For antibody shift, the antibody was added to the cleared mitochondrial lysate and the samples were incubated 30 min on ice prior to the addition of the sample buffer.

Fluorescence microscopy

For visualization of mitochondria, cells were transformed with a yeast expression vector harboring the mitochondrial presequence of subunit 9 of the F_o-ATPase of *N. crassa* fused to GFP, pVT100U-mtGFP (Westermann and Neupert, 2000). Microscopy images were acquired with an Axioskop20 fluorescence microscope equipped with an Axiocam MRm camera using the 43 Cy3 filter set and the AxioVision software (Zeiss).

Acknowledgements

We thank K. Rehn and E. Kracker for technical support, F. Essmann for helpful discussions and critically reading the manuscript, and J. Müller (institute, town, country) and A. Barna (institute, town, country) for providing plasmids.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft [grant number RA 1048/4-1 to D.R.], and a postdoctoral fellowship from the Carl Zeiss Stiftung (K.S.D.).

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.103804/-/DC1

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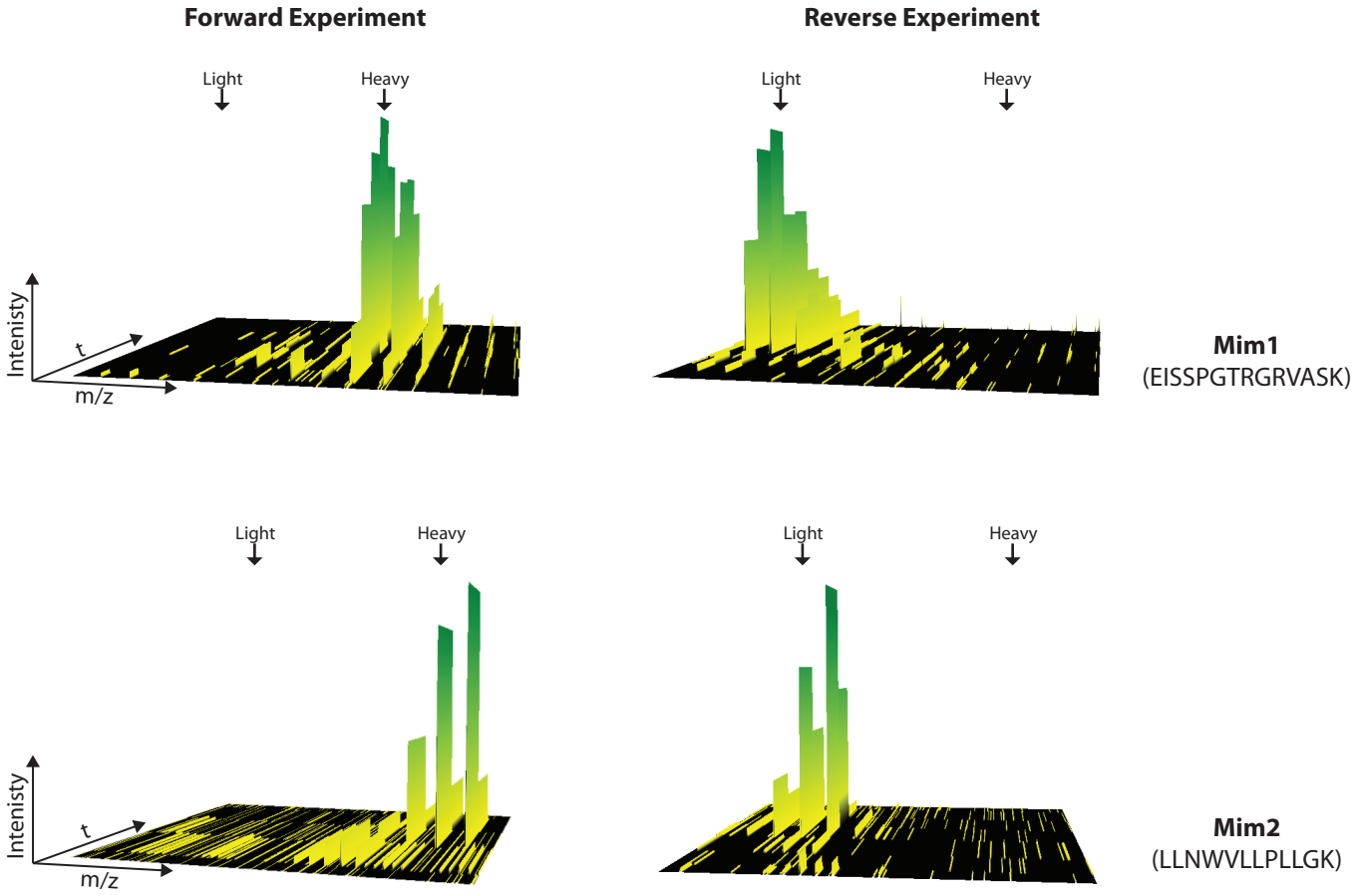
A



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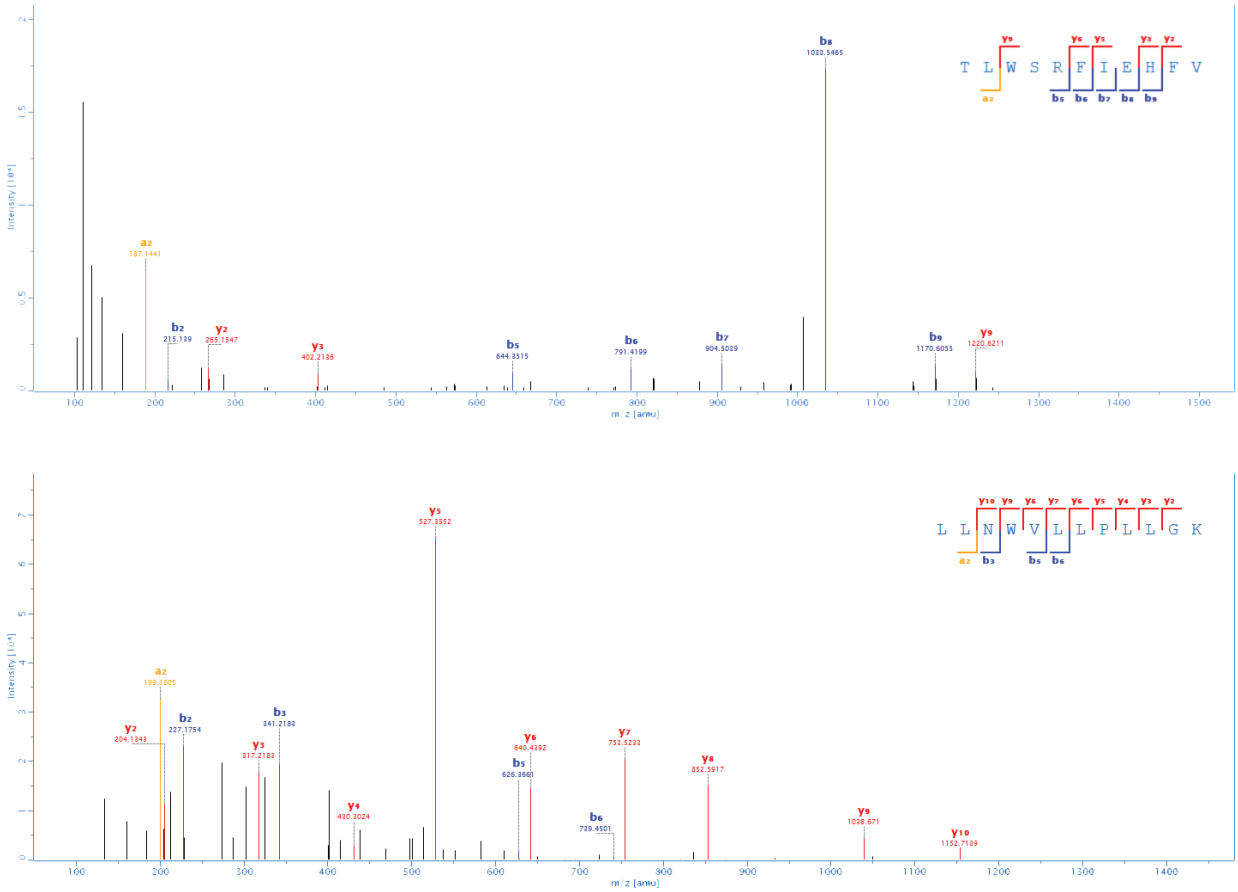
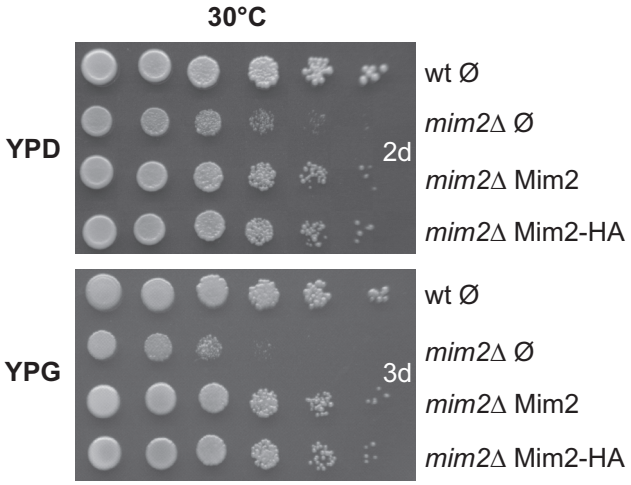
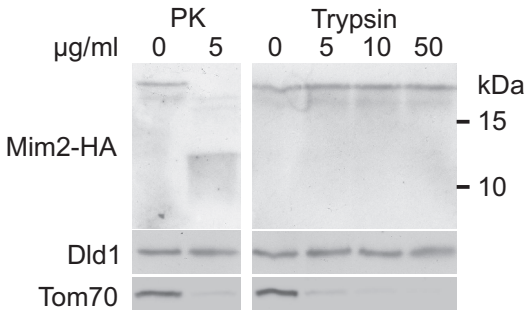


Dimmer et al. Supp. Fig. 1

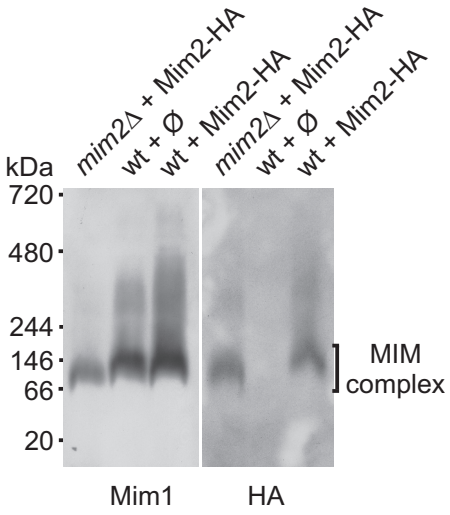
Supplementary Figure 1: Identification of Mim2 as an interactor of Mim1 by quantitative mass spectrometry-based proteomics. **(A)** Three dimensional representations of MS signals from the SILAC-based immunoprecipitation experiment obtained with an LTQ-OrbitrapXL mass spectrometer. In the forward experiment *mim1Δ* cells expressing GFP-Mim1 were grown on media containing heavy lysine whereas *mim1Δ* cells expressing native Mim1 were grown in light media. In the reverse experiment, the media used were swapped between strains. After performing immunoprecipitation, samples from each experiment were pooled, digested with endoproteinase LysC and analyzed by mass spectrometry. A similar enrichment was observed for peptides derived from Mim1 or Mim2 as exemplified by the profiles corresponding to the two indicated peptide sequences. **(B)** Annotated fragmentation spectra of two identified peptides of Mim2 (Ylr099w-a) with unique amino acid sequences acquired with an Orbitrap-Velos mass spectrometer. Precursor ions were isolated in the linear ion trap, fragmented by higher-energy collisional dissociation (HCD) and analyzed in the orbitrap mass analyzer at high resolution.



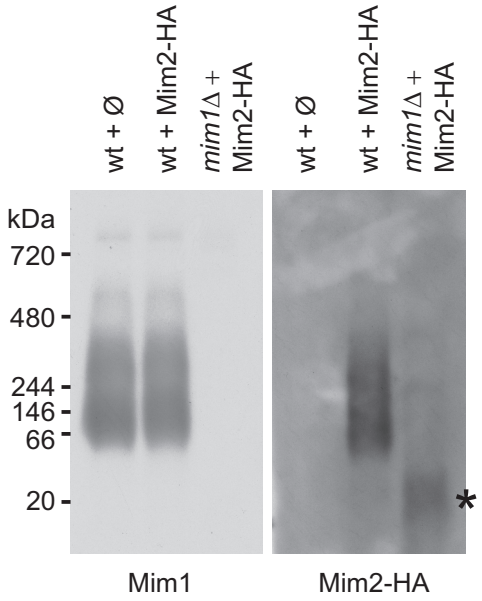
Supplementary Figure 2: Overexpression of plasmid-borne Mim2 or Mim2-HA rescues the growth defect of a *mim2*Δ strain. Wild type cells transformed with an empty plasmid and *mim2*Δ cells transformed with an empty plasmid, Mim2 or Mim2-HA encoding plasmid were analyzed by drop-dilution assay on YPD or YPG medium (dilutions were in fivefold increments).



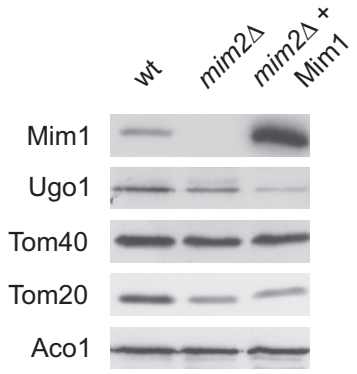
Supplementary Figure 3: Mim2 is insensitive to digestion by trypsin. Mitochondria containing Mim2-HA were treated with the indicated concentrations of PK or trypsin. After reisolation, samples were analyzed by SDS-PAGE and immunodecoration with antibodies against the HA-tag, the IMS protein Dld1 and the MOM protein Tom70.



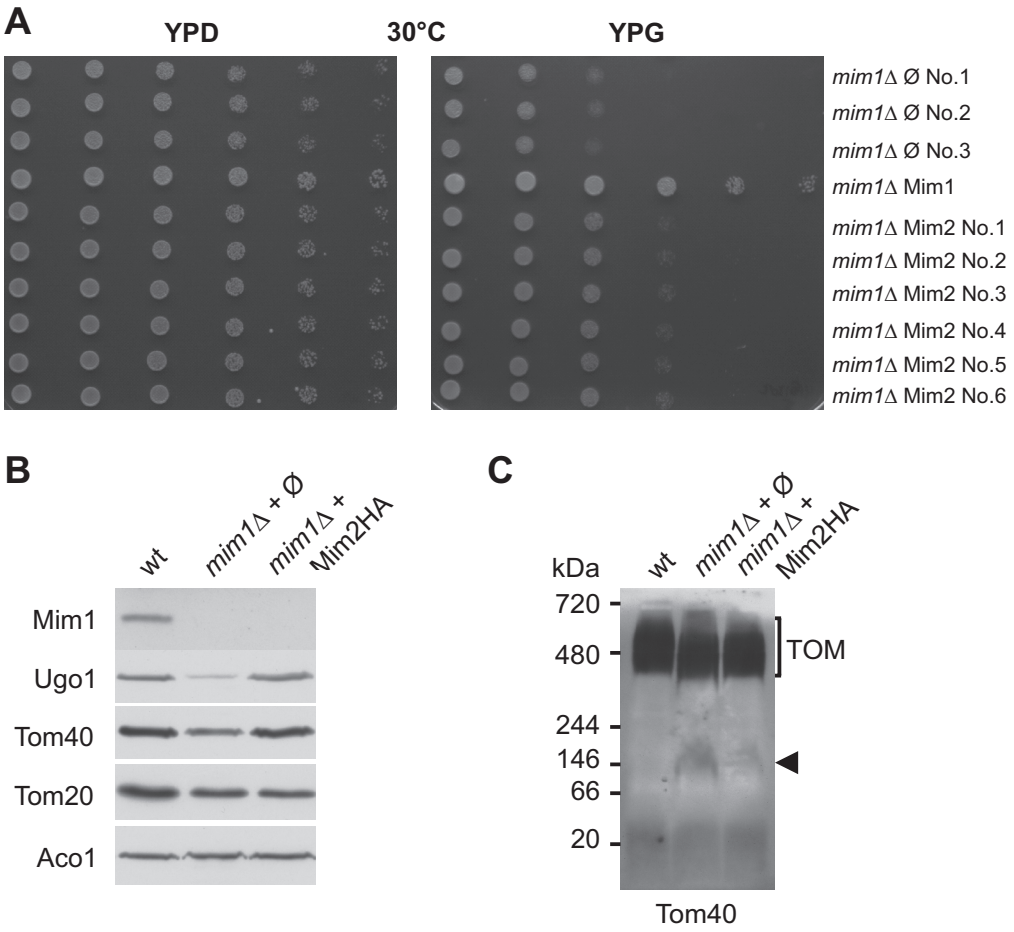
Supplementary Figure 4: Mim2-HA is assembled into the MIM complex. Mitochondria isolated from either wild-type or *mim2*Δ strains overexpressing Mim2-HA (Mim2-HA↑) or harboring the empty plasmid (Ø) were lysed in digitonin and analyzed by BN-PAGE. For analysis of Mim1- and Mim2-containing complexes membranes were immunodecorated with antibodies against Mim1 and the HA-tag, respectively.



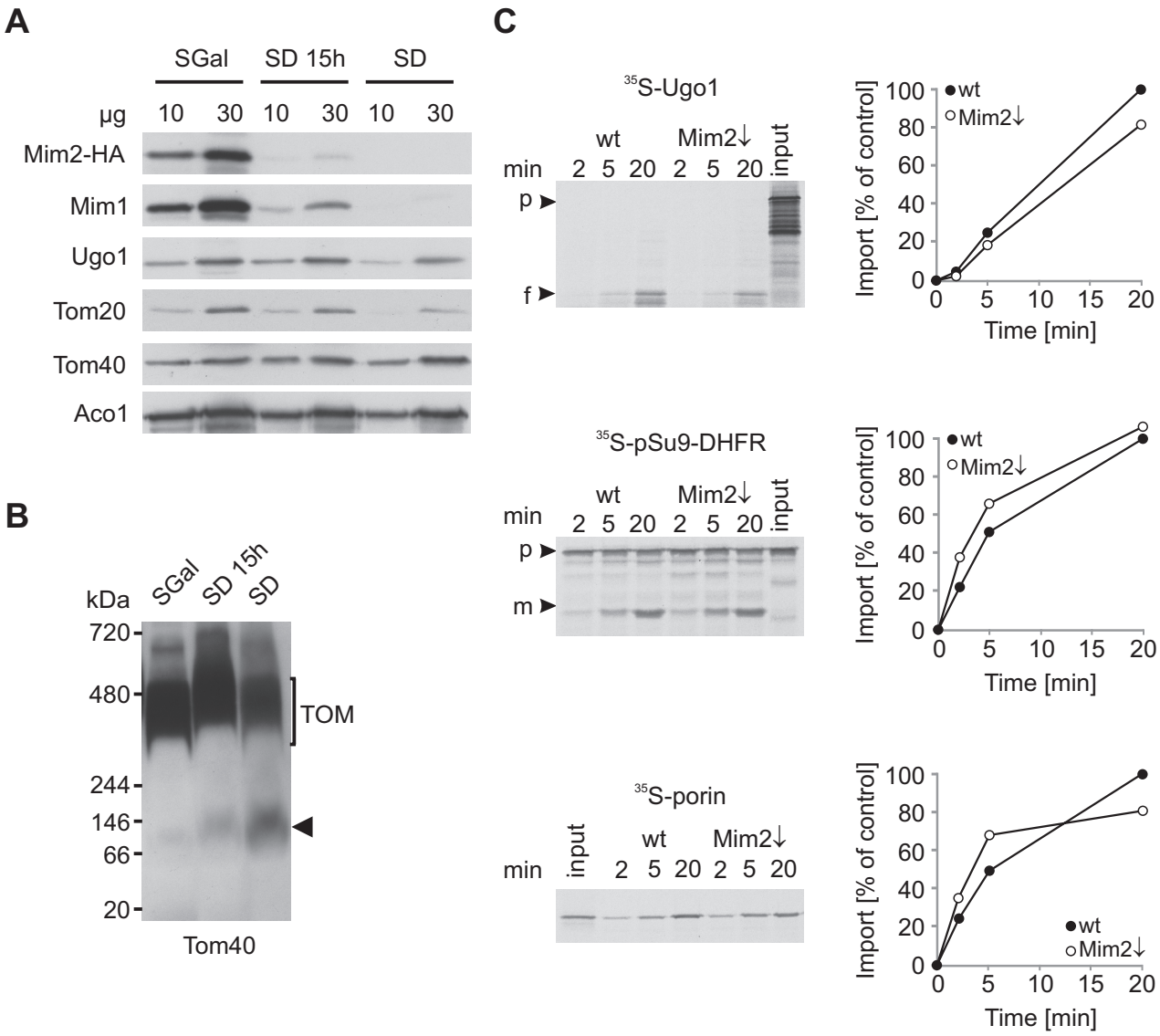
Supplementary Figure 5: Overexpression of Mim2-HA in *mim1*Δ cells results in an unassembled Mim2 species. Mitochondria isolated from wild-type or *mim1*Δ cells overexpressing Mim2-HA (Mim2-HA↑), or harboring the empty plasmid (Ø) were lysed in digitonin and analyzed by BN-PAGE. For analysis of Mim1 and Mim2-HA containing complexes, membranes were immunodecorated with antibodies against Mim1 and the HA-tag, respectively. The unassembled species of Mim2-HA is indicated with an asterisk.



Supplementary Figure 6: Overexpression of Mim1 in *mim2*Δ cells leads to a further reduction of Ugo1 steady state levels. Proteins of mitochondria isolated from the indicated cells were separated by SDS-PAGE, transferred on nitrocellulose and analyzed by immunodecoration with the indicated antibodies.



Supplementary Figure 7: Overexpression of plasmid-borne Mim2 or Mim2-HA partially rescues the growth defect of a *mim1*Δ strain. (A) Growth phenotype of different transformants of *mim1*Δ cells transformed with either empty plasmid or a plasmid encoding Mim2. Overexpression of Mim1 serves as a control. (B) Steady state levels of mitochondrial proteins in wt cells or *mim1*Δ cells transformed with either control plasmid or a plasmid containing Mim2-HA. Isolated mitochondria (20 µg) were analyzed by SDS-PAGE gel followed by immunodecoration with the indicated antibodies. (C) Mitochondria of the indicated strains were lysed in 1% digitonin and subjected to BN-PAGE and immunoblotting with an antibody against Tom40. An arrowhead indicates unassembled Tom40 species. The assembled TOM complex is indicated.



Dimmer *et al*. Supplementary Figure 8

Supplementary Figure 8: Mitochondria depleted for Mim2 show a reduced import capacity for Ugo1. **(A)** Cells lacking Mim2 were transformed with a plasmid encoding Mim2-HA under the control of the GAL1 promoter. These cells were grown on galactose (SGal), glucose (SD), or were grown initially on galactose for two days and then shifted for 15 h on glucose (SD 15h). The indicated amounts of mitochondria isolated from these cells were subjected to SDS-PAGE followed by immunodecoration with the indicated antibodies. **(B)** Mitochondria as in (A) were lysed in 1% digitonin and subjected to BN-PAGE and immunoblotting with an antibody against Tom40. An arrowhead indicates the Tom40-containing low-molecular-mass species. The assembled TOM complex is indicated. **(C)** Mitochondria as in (A) were incubated with the indicated radiolabelled precursor proteins for the indicated time periods. At the end of the import reactions samples were treated as described below, analyzed by SDS-PAGE and autoradiography, and bands corresponding to imported material were quantified. The intensity of bands representing imported material into wild-type mitochondria for the longest time period was set as 100%. Samples containing radiolabeled Ugo1 were treated with trypsin for the generation of a specific 23 kDa fragment (f, see Papic et al., 2011). In the case of import of pSu9-DHFR, the mature protein (m) was quantified. After import of porin, mitochondria were treated with PK and the protected molecules were quantified. (p) precursor form of pSu9-DHFR or Ugo1. A representative experiment of three independent repeats is presented.

Signals in bacterial β-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria

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Edited by William T. Wickner, Dartmouth Medical School, Hanover, NH, and approved December 15, 2008 (received for review August 11, 2008)

The outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts harbor β-barrel proteins. The signals that allow precursors of such proteins to be targeted to mitochondria were not characterized so far. To better understand the mechanism by which β-barrel precursor proteins are recognized and sorted within eukaryotic cells, we expressed the bacterial β-barrel proteins PhoE, OmpA, Omp85, and OmpC in *Saccharomyces cerevisiae* and demonstrated that they were imported into mitochondria. A detailed investigation of the import pathway of PhoE revealed that it is shared with mitochondrial β-barrel proteins. PhoE interacts initially with surface import receptors, and its further sorting depends on components of the TOB/SAM complex. The bacterial Omp85 and PhoE integrated into the mitochondrial outer membrane as native-like oligomers. For the latter protein this assembly depended on the C-terminal Phe residue, which is important also for the correct assembly of PhoE into the bacterial outer membrane. Collectively, it appears that mitochondrial β-barrel proteins have not evolved eukaryotic-specific signals to ensure their import into mitochondria. Furthermore, the signal for assembly of β-barrel proteins into the bacterial outer membrane is functional in mitochondria.

outer membrane | PhoE | protein import | TOB complex | TOM complex

Mitochondria and chloroplasts contain β-barrel proteins in their outer membranes (1–3). The only other biological membrane known to harbor β-barrel proteins is the outer membrane of Gram-negative bacteria (4). This situation reflects the evolutionary origin of mitochondria and chloroplasts from endosymbionts that belong to the class of Gram-negative bacteria.

Precursors of mitochondrial β-barrel proteins are synthesized on cytosolic ribosomes and recognized initially by the import receptors of the translocase of the mitochondrial outer membrane (TOM complex). They are then translocated across the outer membrane via the general-import pore of the TOM complex (5–8). From the TOM complex, β-barrel precursors are relayed to a specialized hetero-oligomeric protein complex termed either topogenesis of outer-membrane β-barrel proteins (TOB complex) (9) or sorting and assembly machinery (SAM complex) (10).

The major component of the TOB complex is Tob55/Sam50 (9, 11, 12). Its sequence is similar to that of the highly conserved bacterial protein Omp85/YaeT, which mediates the insertion of β-barrel proteins into the bacterial outer membrane (13, 14). Hence, this function has apparently been conserved during evolution of mitochondria from bacteria. Although the mitochondrial machinery for insertion of β-barrel proteins was derived from the bacterial system, some modifications were necessary during evolution to meet the requirements of the organelle. It is believed that the evolvement of 2 further TOB subunits, Mas37/Sam37/Tom37 and Tob38/Sam35/Tom38, is part of such an adaptation process. These latter 2 components are associated with Tob55 at the cytosolic side of the outer membrane (10, 15–17). The role of Mas37 in the biogenesis of mitochondrial β-barrel membrane proteins has not yet been identified. Recent studies pointed to a possible function in the release of β-barrel precursors from the TOB complex (18, 19). Tob38 is tightly bound to Tob55, and both proteins form a functional TOB core complex even in the absence of Mas37. Tob38 is probably required for the stability and assembly of the TOB complex. Furthermore, a recent study suggests that Tob38 recognizes a sorting signal within β-barrel proteins (20).

Like all other mitochondrial outer-membrane proteins, β-barrel precursors do not contain a cleavable N-terminal presequence for their targeting to mitochondria but rather a noncleavable internal signal. The nature of such a signal element has not been characterized so far. Similarly unclear is whether the interaction of the mitochondrial import machinery is specific for β-barrel proteins of the eukaryotic cell or whether this machinery can recognize equivalent structural motifs independent of the origin of the protein. A study addressing this point revealed that PorB of pathogenic *Neisseria* species can target mitochondria when expressed in eukaryotic cells whereas porins of closely related nonpathogenic bacteria were unable to do so (21). Thus, it is still an open question whether PorB contains unique virulence-related features that make it an exceptional case. Furthermore, very recently, a specific signal that is conserved in eukaryotic β-barrel proteins was suggested to promote the intramitochondrial sorting of these proteins (20). However, because this signal is not conserved in prokaryotic β-barrel proteins, it is particularly interesting to test the fate of the latter proteins when expressed in eukaryotic cells.

To better understand the mechanism by which the eukaryotic cell recognizes β-barrel precursor proteins, we expressed bacterial β-barrel proteins in yeast cells. The bacterial proteins were imported into mitochondria via a pathway shared with mitochondrial β-barrel proteins. Furthermore, these proteins could be assembled in a native-like conformation into the mitochondrial outer membrane. Our results imply that although the machinery that sorts these proteins had to be modified during evolution of mitochondria, such an adaptation of substrate proteins was not required for allowing the machinery to recognize them.

Results

Bacterial β-Barrel Proteins Expressed in Yeast Cells Are Targeted to Mitochondria. In the present study, we wanted to test whether mitochondrial β-barrel proteins contain specific targeting and

Author contributions: D.M.W., D.P., M.P.B., J.T., and D.R. designed research; D.M.W., D.P., and M.P.B. performed research; D.M.W. and M.P.B. contributed new reagents/analytic tools; D.M.W., D.P., M.P.B., J.T., and D.R. analyzed data; and D.M.W., J.T., and D.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0807830106/DCSupplemental.

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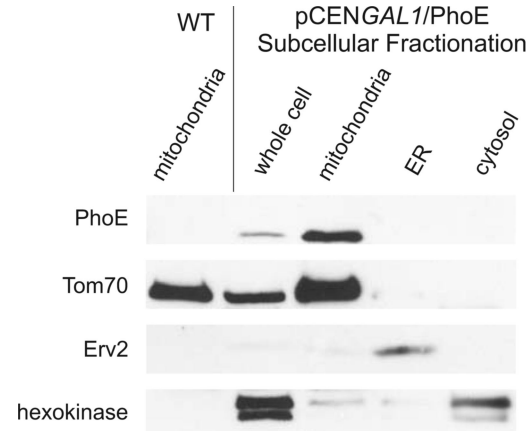

Fig. 1. Bacterial β-barrel proteins are targeted to mitochondria in yeast cells. Whole-cell lysate of cells expressing PhoE and fractions corresponding to mitochondria, endoplasmic reticulum (ER), and cytosol were analyzed by SDS/PAGE and immunodecoration with antibodies against the bacterial protein, the mitochondrial protein Tom70, the ER protein Erv2, and a control marker protein for the cytosol (hexokinase). Mitochondria isolated from wild-type, untransformed cells were coanalyzed as a control.

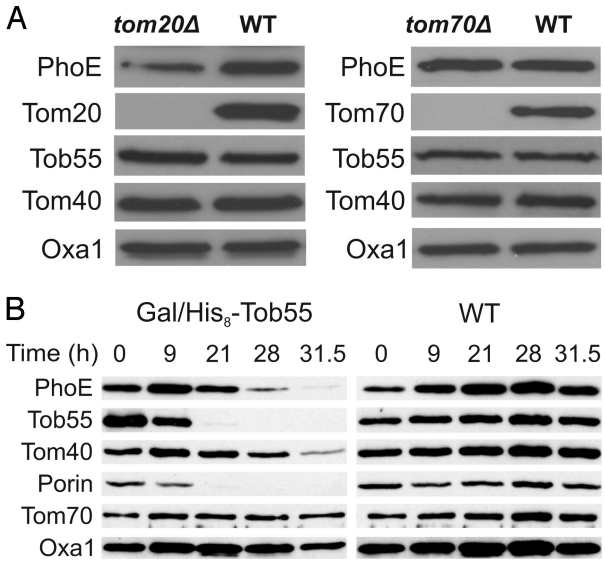

Fig. 2. PhoE is sorted to mitochondria in a TOM- and TOB complex-dependent manner. (*A*) Mitochondrial import of PhoE depends on the import receptor Tom20. Mitochondria isolated from *tom20*Δ, *tom70*Δ, and their corresponding wild-type cells transformed with p2μ*TPI*/PhoE were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE and the indicated mitochondrial proteins. (*B*) Cells from wild-type strain or from a strain expressing Tob55 under the control of the *GAL10* promoter (Gal/His$_8$-Tob55) were harvested at the indicated time points after a shift to glucose-containing medium (see Fig. S4*A*). Crude mitochondria were isolated and proteins were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE and the indicated mitochondrial proteins. Tob55, Tom40, and porin, β-barrel proteins; Tom70, a signal-anchored protein in the outer membrane; Oxa1, an inner membrane protein.

sorting signals that are absent in bacterial β-barrel proteins. To address this point, we used the outer-membrane porin PhoE of *Escherichia coli* that was used extensively as a model protein for studying the biogenesis of bacterial outer-membrane proteins. We constructed a PhoE variant without the signal sequence required for transport across the bacterial inner membrane and produced it in yeast cells by using the strong *GAL1* promoter on a low-copy plasmid. We chose high-expression conditions to enhance detection and to challenge the mitochondrial import and sorting systems. Subcellular fractionation of the transformed cells revealed that PhoE was located solely in the mitochondrial fraction (Fig. 1). As a control for the specificity of the antibody against PhoE, we verified that no signal was observed in mitochondria isolated from a nontransformed strain (Fig. 1). The mitochondrial localization of PhoE was confirmed by immunofluorescence microscopy where PhoE colocalized with a mitochondrial marker protein (Fig. S1).

We investigated whether other bacterial β-barrel proteins are targeted to mitochondria when expressed in yeast cells. To that end, we examined the outer-membrane proteins OmpA and OmpC of *E. coli* and Omp85 from *Neisseria meningitidis*. This group of proteins presents a wide array of β-barrel proteins ranging from the 8-stranded monomer OmpA to the 16-stranded trimers OmpC and PhoE. These 3 additional proteins were constructed without a signal sequence and expressed in yeast cells under the control of a strong promoter. Similarly to PhoE, these proteins were exclusively found in the mitochondrial fractions (Fig. S2). Thus, the property of being targeted to mitochondria when expressed in eukaryotic cells is shared by several bacterial proteins.

PorB from pathogenic *Neisseria gonorrhoeae* expressed in mammalian cells was reported to dissipate the membrane potential across the mitochondrial inner membrane and to induce apoptosis (21). Therefore, we investigated whether the bacterial β-barrel proteins under study interfere with the biogenesis of mitochondrial β-barrel proteins or with other crucial mitochondrial functions. The levels of outer-membrane β-barrel proteins, such as Tob55, porin, and other mitochondrial proteins, were not affected by the expression of PhoE, Omp85, or OmpC and were only slightly reduced in mitochondria containing OmpA (Fig. S3 *A* and *B*). The capacity of isolated mitochondria harboring PhoE to import in vitro the matrix-destined preprotein pSu9 (1–69)-DHFR was similar to that of organelles isolated from control cells (Fig. S3*C*). Next, the growth rate of cells expressing PhoE was monitored and found to be comparable to that of nontransformed cells under all tested conditions, including growth on a nonfermentable carbon source where yeast cells depend on mitochondrial respiration for energy production (Fig. S3*D*). Thus, it appears that high-level expression of bacterial β-barrel proteins in yeast does not obstruct major mitochondrial features.

PhoE Is Imported into Mitochondria in a TOM- and TOB-Complex-Dependent Manner. Precursors of the mitochondrial β-barrel proteins like Tom40, porin, and Tob55 are initially recognized at the surface of the organelle by the import receptor Tom20 (5, 6, 8, 18). Therefore, we asked whether the import receptors of the TOM complex play a role in the import of the bacterial β-barrel precursor proteins. To that end, we expressed PhoE in cells lacking either Tom20 or Tom70 and monitored its level in mitochondria. Mitochondria isolated from strains lacking Tom20 had significantly reduced amounts, whereas deletion of Tom70 hardly affected the mitochondrial level of PhoE (Fig. 2*A*). Hence, similar to their function in recognizing precursors of mitochondrial β-barrel proteins, the import receptor Tom20 plays an important role in the recognition of PhoE precursor, whereas Tom70 has only a minor function in this process.

Does the TOB complex mediate the membrane insertion of PhoE? To address this point, we transformed a plasmid encoding PhoE into cells where the essential component Tob55 is under the control of *GAL10* promoter (9). The growth of these transformed cells was slowed down ≈32 h after shifting them to glucose-containing medium (Fig. S4*A*). Mitochondria were isolated from these Tob55-depleted cells at various time points after the shift, and the levels of various mitochondrial proteins were monitored. Clearly, Tob55 was gradually depleted from cells grown on glucose-containing medium. The lower amounts of Tob55 caused a reduction in the detected amounts of the mitochondrial β-barrel proteins porin and Tom40 as well as in the amounts of PhoE (Fig. 2*B*). The

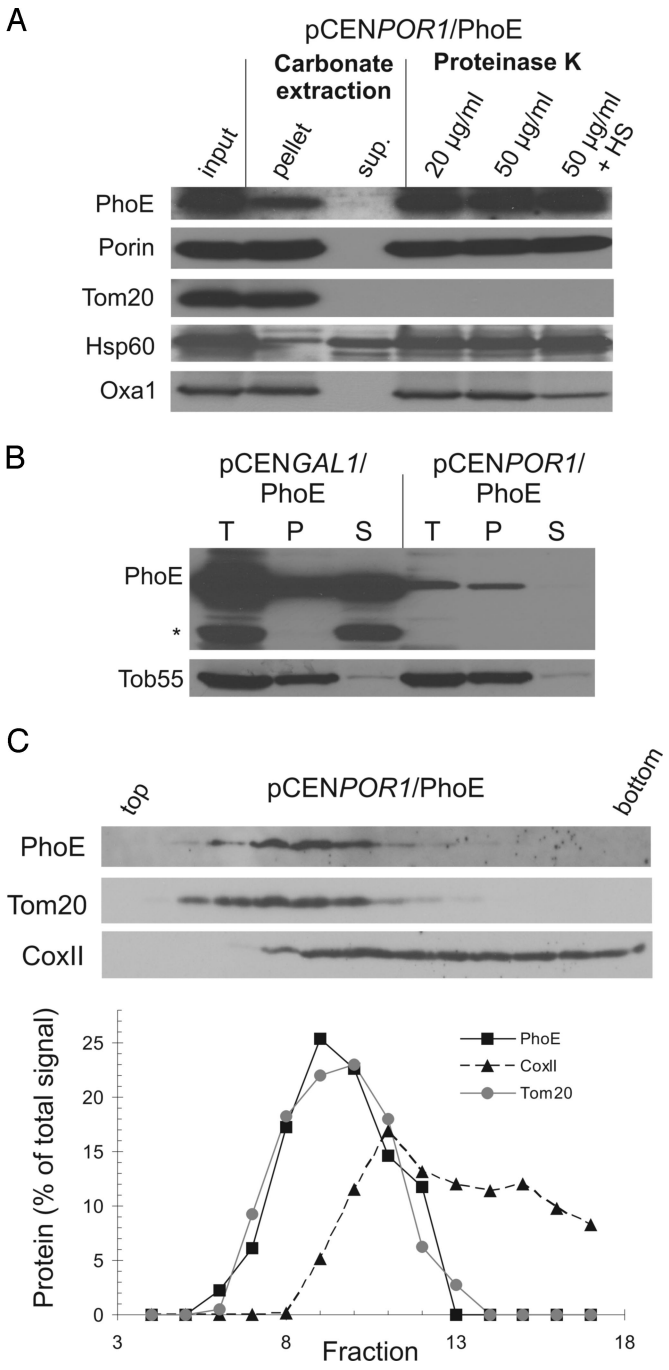

Fig. 3. PhoE expressed at low levels is properly integrated into the mitochondrial outer membrane. (*A*) Mitochondria isolated from cells expressing PhoE under the control of the *POR1* promoter were loaded directly on SDS/PAGE gel (input) or were subjected first to alkaline extraction and then centrifuged to discriminate between membrane proteins (pellet) and soluble proteins in the supernatant (sup.). Additional aliquots of mitochondria were left intact or were treated by hypoosmotic swelling (HS) before their incubation with the indicated amounts of proteinase K. Proteins were analyzed by SDS/PAGE and immunodecorated with antibodies against the indicated proteins. Of note, for unknown reason, the recovery of PhoE in the carbonate extraction experiment was only 75%. Porin, protein embedded in the outer membrane; Tom20, outer-membrane protein exposed to the cytosol; Hsp60, soluble matrix protein; OxaI, an inner-membrane protein exposed to IMS. (*B*) Mitochondria isolated from PhoE-expressing cells were loaded directly on SDS/PAGE gel (T, Total) or were treated with urea and then centrifuged to separate the nonextractable pellet (P) from the urea-soluble fraction (S). Proteins were analyzed by SDS/PAGE and immunodecoration with antibodies against the indicated proteins. A proteolytic fragment of PhoE is labeled with an asterisk. (*C Upper*) PhoE is sorted to the mitochondrial outer membrane when expressed at low levels. Mitochondria from cells expressing PhoE under the *POR1* promoter were analyzed by sucrose density gradient centrifugation. Proteins from fractions were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE, Tom20, and the inner-membrane protein CoxII. (*C Lower*) The bands corresponding to the detected proteins were quantified by densitometry. The relative amount in each fraction is presented as the portion from the total amount of this protein.

levels of non-β-barrel proteins were unaffected. Of note, the reduction in the amounts of PhoE preceded that of Tom40, suggesting that the depletion of PhoE is not a secondary effect to the reduction in the levels of Tom40. Next, PhoE was expressed in cells lacking Mas37, a peripheral subunit of the TOB complex. The absence of Mas37 resulted in a dramatic reduction in the amount of PhoE detectable in the corresponding mitochondria (Fig. S4*B*). Collectively, these results demonstrate that PhoE follows a TOM- and TOB-dependent insertion pathway.

Integration of PhoE in a Native-Like Structure into the Mitochondrial Outer Membrane. We asked whether PhoE is correctly assembled into the mitochondrial membranes. To that end, the association of PhoE with the mitochondrial membranes was analyzed by alkaline treatment of mitochondria isolated from PhoE-expressing cells. PhoE was recovered mainly in the pellet fraction just like the known membrane proteins porin, Tom20, and OxaI, although a considerable portion was also found in the supernatant (Fig. S5*A*). The membrane topology of PhoE was further studied by protease treatment of mitochondria because native PhoE is resistant against proteolysis from either side of the bacterial outer membrane (22). PhoE was indeed unaffected by such treatment in intact mitochondria consistent with its uptake into the organelles, but it was partially degraded upon rupturing the outer membrane by hypoosmotic swelling (Fig. S5*A*). Furthermore, when the membranes were treated with 6 M urea, a common method to analyze the membrane insertion of bacterial outer-membrane proteins (13, 23), a significant fraction of the overexpressed PhoE was extractable (Fig. 3*B*). Thus, a large portion of PhoE produced at high amounts is present in the mitochondria in a conformation and location where the protein is not embedded within a membrane, and at least some parts of the molecule are exposed to the intermembrane space (IMS).

Next, we wished to determine in which of the 2 mitochondrial membranes PhoE was located. To that end, the organelles were sonified, and membrane vesicles were separated by sucrose density gradient centrifugation where the 2 mitochondrial membranes can be enriched in distinct fractions. Interestingly, PhoE behaved neither like a typical outer-membrane protein nor as an inner-membrane protein (Fig. S5*B*). Significant amounts of the protein were found in fractions with high density suggesting that PhoE molecules in these fractions might represent precursors that aggregated in the IMS and/or mistargeted to the IMS surface of the inner membrane. Taken together, it appears that most of the overexpressed PhoE molecules are not properly inserted into the mitochondrial membranes.

Do the high amounts of PhoE cause overloading of the TOB machinery, and therefore, the protein is not efficiently inserted from the IMS into the outer membrane? To address this question, we investigated the membrane integration of PhoE expressed under the control of the promoter of the mitochondrial β-barrel protein *POR1*. Subcellular fractionation confirmed that mitochondrial targeting of PhoE and the other bacterial proteins, OmpA and Omp85, was retained under these low expression levels (Fig. S6), although detection of OmpC was not possible with the available antibody. Hence, the bacterial β-barrel proteins are targeted to mitochondria independently of their expression level.

The vast majority of the PhoE produced at a low level was in the

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membrane pellet of the alkaline extraction, and it was protected from proteinase K even when the outer membrane was ruptured (Fig. 3*A*). Next, we analyzed the membrane integration of PhoE by resistance to extraction with urea. When expressed in reduced amounts, practically all PhoE molecules were not extractable with urea, similar to the endogenous β-barrel protein, Tob55. The submitochondrial location of PhoE expressed at reduced levels was further studied by sucrose density gradient centrifugation and revealed that PhoE migrated like the outer-membrane marker, Tom20 (Fig. 3*C*). Similarly, when mitochondria harboring OmpA and Omp85 expressed at reduced amounts were analyzed by this method, both proteins were found to comigrate with the outer-membrane marker (Fig. S7). Collectively, these results suggest that low expression levels of bacterial β-barrel proteins allow the precursor molecules to fully integrate into the mitochondrial outer membrane.

The C-Terminal Phenylalanine Residue of PhoE Is Crucial for Its Assembly into Trimeric Structures. PhoE, like the majority of bacterial β-barrel proteins, contains a C-terminal signature sequence, of which Phe at the ultimate C-terminal position is the most important feature (24). To test whether this signature plays a similar role in the sorting of PhoE in eukaryotic cells, we constructed a PhoE variant where the C-terminal Phe residue was deleted (PhoEΔF). This variant was expressed in yeast cells under the strong *GAL1* promoter or the *POR1* promoter, and in both cases the mutant protein was targeted to mitochondria with a similar or even slightly higher efficiency as compared with full-length PhoE (Fig. S8 *A* and *B*). Is the ultimate Phe residue required for the correct membrane insertion of PhoE? In contrast to the full-length protein, both low- and high-level expression of PhoEΔF resulted in a situation where the vast majority of the protein was extractable by urea (Fig. 4*A*). Furthermore, at both expression levels, PhoEΔF was sensitive to protease treatment when the outer membrane was not intact (Fig. 4*B* and Fig. S8*C*). We next tested whether PhoEΔF is imported into mitochondria in a TOB-dependent manner. As observed for the full-length protein, the absence of Mas37 resulted in a significant reduction in the amount of PhoEΔF molecules in the corresponding mitochondria (Fig. S8*D*). Hence, PhoEΔF is imported into mitochondria via a pathway shared with the mitochondrial β-barrel proteins. However, its protease sensitivity and extractability by urea suggest that the protein is not assembled correctly into the outer membrane.

PhoE is known to form homotrimers in the bacterial outer membrane. We monitored whether PhoE expressed in yeast cells is able to form such oligomeric structures. Only a small fraction of the overexpressed mitochondrial PhoE was found in the trimeric structures when mitochondria were analyzed by seminative SDS/PAGE. Most of the PhoE molecules migrated as a monomeric form. In contrast, practically all PhoE molecules were found in the trimeric form when PhoE was expressed under the *POR1* promoter (Fig. 5*A*). The ability to form such assembled trimeric form requires C-terminal Phe residues, because the variant lacking this residue was unable to form such structures independently of its expression level (Fig. 5*B*).

The formation of the oligomeric structure was further studied after solubilizing the organelles with the mild detergent digitonin. Of note, most of the overproduced PhoE was recovered in the pellet fraction when a clarifying spin was performed with this lysate. In contrast, PhoE at lower levels was soluble under these conditions and recovered in the supernatant (Fig. S9*A*). These observations support the notion that a significant portion of the PhoE molecules is aggregated under high expression levels. The soluble fractions of the lysates were further analyzed by BN/PAGE, and a band corresponding to an oligomeric form of PhoE was detected (Fig. S9*B*). Of note, because solubilization of bacterial membranes with digitonin is very inefficient, a direct comparison of the migration behavior with that of PhoE derived from *E. coli* membranes was not possible in this case. Omp85 is also known to form oligomers, and such forms of the purified in vitro refolded *E. coli* Omp85/YaeT were observed by BN/PAGE (25). Thus, we used the same technique to analyze mitochondria isolated from cells expressing this protein. High molecular-weight oligomers were observed upon solubilization with digitonin (Fig. S9*C*). Collectively, these results demonstrate that the outer membrane of mitochondria can support the formation of oligomeric structures of bacterial β-barrel proteins.

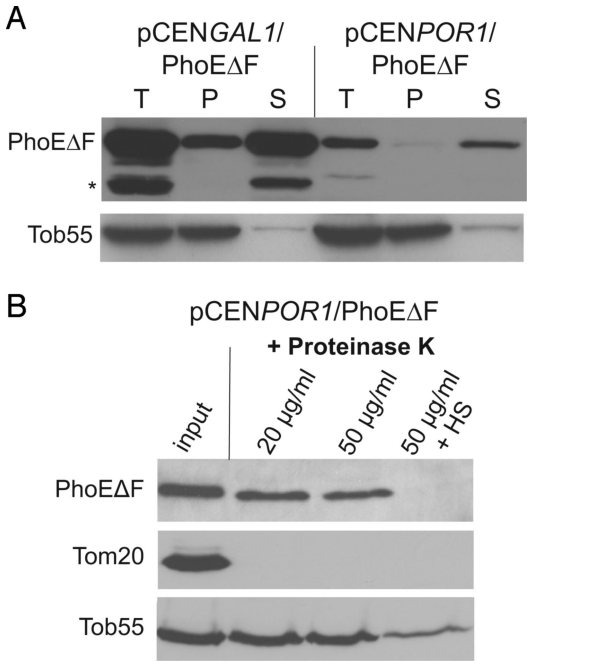

Fig. 4. The C-terminal phenylalanine residue of PhoE is dispensable for targeting to mitochondria but not for proper assembly. (*A*) Mitochondria isolated from PhoEΔF-expressing cells were loaded directly on SDS/PAGE gel (T, Total) or were treated with urea and then centrifuged to separate the nonextractable pellet (P) from the urea-soluble fraction (S). Proteins were analyzed by SDS/PAGE and immunodecoration with antibodies against the indicated proteins. A proteolytic fragment of PhoE is labeled with asterisk. (*B*) PhoEΔF is present in mitochondria in a protease-sensitive conformation. Mitochondria isolated from cells expressing low amounts of PhoEΔF were treated and analyzed as described in Fig. 3*A*.

The formation of correctly folded forms was further monitored by immunofluorescence microscopy employing mitochondria isolated from PhoE-expressing cells and a monoclonal antibody that recognizes specifically native PhoE (26). Specific staining at the surface of intact mitochondria was observed when full-length PhoE was expressed. In agreement with a stronger trimer band in the case of overexpression (Fig. 5*A*), the signal was stronger for mitochondria from cells overproducing PhoE (Fig. 5 *Ci* and *Cii*). Only background staining was observed when mitochondria isolated from cells producing high levels of PhoEΔF were inspected (Fig. 5*Ciii*). Taken together, PhoE expressed in eukaryotic cells is imported into mitochondria independent of its C-terminal signature sequence. In contrast, like in bacteria, the insertion into the mitochondrial outer membrane in a native-like structure requires this signature motif.

Discussion

Precursors of mitochondrial β-barrel proteins are recognized initially by the import receptors of the TOM complex and are then translocated via the import pore of the translocase through the outer membrane into the IMS. Next, β-barrel precursors are relayed to the TOB complex, which mediates their insertion into the

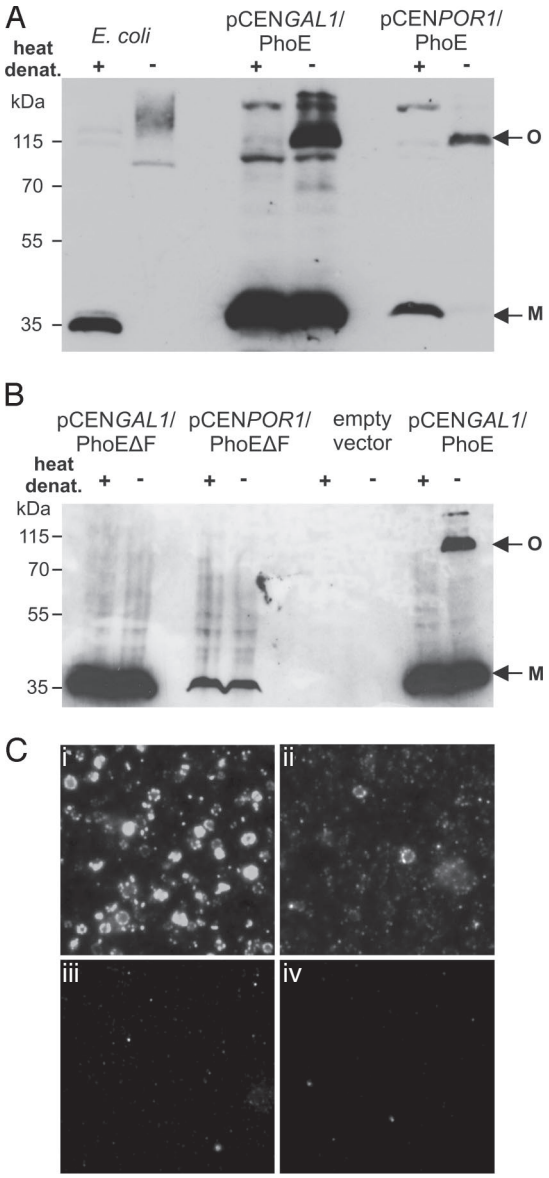

Fig. 5. PhoE can form native-like trimeric structures in mitochondria. (*A*) Full-length PhoE assembles into a trimeric structure in mitochondrial membranes. Cell envelopes from *E. coli* or mitochondria isolated from cells transformed with plasmid encoding PhoE under either the *GAL1* or *POR1* promoter were analyzed by seminative PAGE after heat denaturation or without further treatment. The positions of monomeric and oligomeric forms of PhoE are indicated with M and O, respectively. (*B*) Deletion of the C-terminal Phe residue abolishes PhoE trimer formation in mitochondrial membranes. Mitochondria isolated from cells transformed with the empty plasmid or expressing the PhoEΔF variant from the *GAL1* or *POR1* promoter were treated and analyzed as in Fig. 5*A*. Mitochondria harboring high levels of full-length PhoE are coanalyzed as control. (*C*) Mitochondria derived from cells expressing PhoE (*i*), PhoE under control of the *POR1* promoter (*ii*), PhoEΔF under the control of the *GAL1* promoter (*iii*), or no PhoE (empty vector control) (*iv*) were stained with a monoclonal antibody that specifically recognizes native PhoE trimers.

outer membrane (27, 28). Very recently, a sorting signal that facilitates the interaction of eukaryotic β-barrel precursors with the TOB complex was identified. This signal appears to be an intramitochondrial sorting signal and is not involved in targeting of the precursor proteins to the organelle (20). The signals that facilitate the specific targeting of β-barrel precursors to mitochondria are not characterized yet. Studies have failed to identify a linear, well-defined sequence that can function as a targeting sequence. Hence, it can be assumed that the mitochondrial protein-import machinery recognizes β-barrel-specific structural elements (2).

To test this hypothesis, we expressed bacterial β-barrel proteins in yeast cells. These bacterial proteins do not share significant sequence similarity with endogenous mitochondrial β-barrels and should be devoid of any eukaryotic targeting sequence that evolved when mitochondria developed from the endosymbiont. Nevertheless, the proteins were imported into mitochondria suggesting that the ability of a protein to adopt a membrane-embedded, β-barrel conformation could be sufficient to ensure its specific targeting to mitochondria. This proposal is in line with our observation that a partially folded conformation of β-barrel precursors is required for efficient import into mitochondria (8). These findings also imply that mitochondrial β-barrel proteins, which evolved most probably from bacterial β-barrel structures, were not forced to develop specific targeting signals to assure their correct subcellular targeting.

Of note, our results differ from those of Müller *et al.* (21) who reported that, with the exception of PorB from pathogenic *Neisseriae*, bacterial porins were unable to be targeted to mitochondria. Differences in the experimental setup of both studies might explain this apparent discrepancy. For example, Müller *et al.* studied bacterial proteins in mammalian cells, whereas we used proteins expressed in yeast.

Assembly of outer-membrane proteins in bacteria depends on a C-terminal signature motif, which corresponds to the last transmembrane β-strand and is reminiscent of, but not identical to, the recently identified β-sorting signal in mitochondrial proteins (20). Most bacterial outer-membrane proteins contain a Phe residue at their C terminus, which is essential for efficient integration into the outer membrane in vivo but not for the formation of the trimeric structure as appeared from in vitro folding studies (29, 30). The high-level expression of a mutant PhoE lacking the C-terminal Phe in *E. coli* led to the periplasmic accumulation of the mutant protein in a protease-sensitive monomeric conformation (24). Furthermore, we demonstrated recently that the bacterial C-terminal signature sequence interacts directly with Omp85 (25). To assess whether the recognition of substrates is conserved between the TOB and the Omp85/YaeT complexes, we investigated whether deletion of the C-terminal Phe residue of PhoE affects assembly of the protein into the mitochondrial outer membrane. The PhoEΔF variant could not form the native-like trimers in the outer membrane. Collectively, our results demonstrate that the ancestral bacterial signal is still recognized and correctly processed by the mitochondrial assembly machinery.

We report that the expression levels of the β-barrel protein influence the fraction of molecules that can be properly integrated into the outer membrane. At high expression levels, all of the protein was found in mitochondria, thus, it appears that the TOM complex can process such elevated amounts of β-barrel precursors. In contrast, the TOB complex is probably the limiting factor in the biogenesis pathway as overexpressed precursor proteins accumulated in the IMS. Interestingly, even under these conditions, the import into the organelle depended on the TOB complex. Based on these findings, the function of the TOB complex can be divided into 2 distinct steps. First, the complex is involved in the translocation of β-barrel precursors via the TOM complex across the outer membrane. It is not entirely clear yet whether a direct interaction between the precursor and the TOB complex is required at this stage. If there is a direct interaction, it does not involve the intramitochondrial sorting signal of the β-barrel precursor or its bacterial substitute because PhoEΔF and mutant mitochondrial proteins with a deficient signal (20) were still efficiently imported into the mitochondria. Perhaps, the TOB complex, like the TOM complex, recognizes a high β-sheet content at this stage. In the second step, the TOB complex mediates the membrane integration of the β-barrel precursors. At this stage, an interaction between the

intramitochondrial sorting signal of the precursor and the Tob38 component of the TOB complex is required. This sorting signal is present in the last predicted β-strand of the mitochondrial proteins and was not recognized in bacterial outer-membrane proteins (20). Nevertheless, we found that the assembly of bacterial β-barrel proteins into a native-like oligomeric structure at the mitochondrial outer membrane depended on their ancestral recognition motif.

Collectively, the current study demonstrates that no eukaryotic-specific signals that are essential for the intracellular mitochondrial targeting of these proteins have evolved within mitochondrial β-barrel proteins. Moreover, it shows that bacterial outer-membrane proteins can be properly assembled into the mitochondrial outer membrane. These findings support the notion that β-barrel protein assembly is conserved between bacteria and eukaryotic cell organelles of endosymbiont origin.

Materials and Methods

SI Text includes a description of immunofluorescence microscopy, in vitro import assay, and drop-dilution assay.

Yeast Strains and Growth Methods. The wild-type strain YPH499 was used. The *GAL10-TOB55* and *mas37*Δ strains were described in refs. 9 and 18, respectively. The *tom20* null strain YTJB64 and its corresponding parental strain YTJB4 were used (31). The *tom70* deletion strain was obtained from Research Genetics.

Biochemical Procedures. Mitochondria were isolated from yeast cells by differential centrifugation, as described in ref. 32. Seminative gel electrophoresis and urea extraction were performed basically as described in refs. 13 and 33. The separation of mitochondrial membranes was based on published procedures (34). Mitochondria were subjected to hypoosmotic shock in swelling buffer, adjusted to a final sucrose concentration of 0.45 M, and disrupted by treatment with a Branson 250 sonifier. The homogenate was clarified by centrifugation (35,000 × *g* for 30 min at 2 °C). Vesicles were sedimented by ultracentrifugation (200,000 × *g* for 90 min at 2 °C) onto a 2 M sucrose cushion and overlaid with a sucrose step gradient ranging from 1.4 to 1 M. Gradients were centrifuged in a swing-out rotor (230,000 × *g* for 16 h at 4 °C), and fractions were analyzed by SDS/PAGE.

Recombinant DNA Techniques. The sequences encoding PhoE, OmpA, OmpC of *E. coli*, and Omp85 from *N. meningitidis* lacking their signal sequences were cloned by PCR amplification from corresponding plasmids encoding full-length proteins. PhoEΔF was generated in a similar way; however, the codon for Phe-330 was omitted from the 3′ primer of the PCR. The PCR products were inserted into the yeast expression vectors pRS426-TPIp-URA3 or pYX113-GAL1p-URA3. To construct low-expression plasmids, the *GAL1* promoter in pYX113 was replaced by the *S. cerevisiae POR1* promoter.

Fluorescence Microscopy. Mitochondria were fixed with 2% formaldehyde in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM Mops/KOH, pH 7.2) and immobilized on poly-L-lysine-coated glass cover slips. After removing the fixative by 3 washes with PBS, the cover slips were blocked with 3% BSA in PBS. The mitochondria were labeled with monoclonal antibody PP4–1, which specifically recognizes PhoE trimers (26), followed by labeling with Alexa 488-conjugated goat anti-mouse antibodies. Control samples were treated similarly but without the first antibody. Cover slips were mounted in PBS-buffered glycerol and viewed in a Zeiss Axioskop 2 plus microscope.

ACKNOWLEDGMENTS. We thank W. Neupert and M. Ott for helpful discussions; K. Rehn and J. Arenas for technical support; E. Kracker for help in performing some experiments; and K.S. Dimmer and G. Dodt for help with the fluorescence microscopy. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 446-A30, and the Netherlands Research Council for Earth and Life Sciences.

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Supporting Information

Walther *et al.* 10.1073/pnas.0807830106

SI Text

Immunofluorescence Microscopy of Yeast Cells. Cells were prepared for immunofluorescence according to ref. 1 except that only Zymolase 20T was used for spheroplasting. For immunostaining, a mouse monoclonal antibody against ADP-ATP carrier and a rabbit polyclonal antiserum against PhoE were used as primary antibodies. Pictures were taken by using a Zeiss Axiovert 200 M microscope.

In Vitro Import Assay. Radio-labeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine after in vitro transcription from pGEM4 vector encoding the chimeric protein pSu9(1–69)-DHFR. Import experiments were performed according to published procedures (2).

Drop-Dilution Assay. For dilution assays, yeast cells were grown to an OD_{600} of 1.0 in synthetic medium, diluted in 10-fold increments, and then 5 μl of each dilution were spotted onto solid media with different carbon sources.

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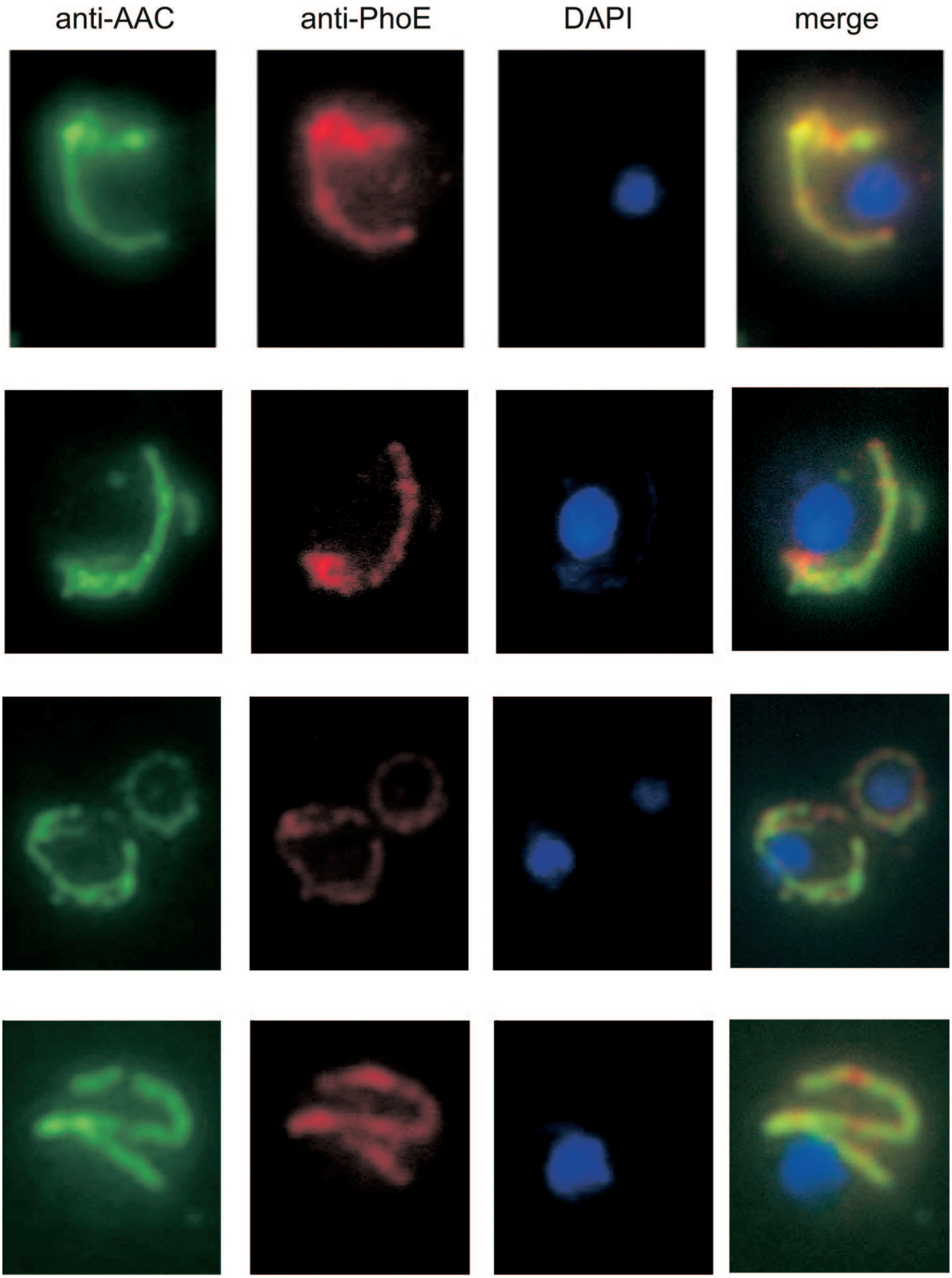

Fig. S1. PhoE colocalizes with mitochondria. Fluorescence images of yeast cells expressing PhoE at high levels were taken after fixation and staining with DAPI (DNA, blue), antibodies against PhoE (red), and ADP-ATP carrier (mitochondrial marker protein, green).

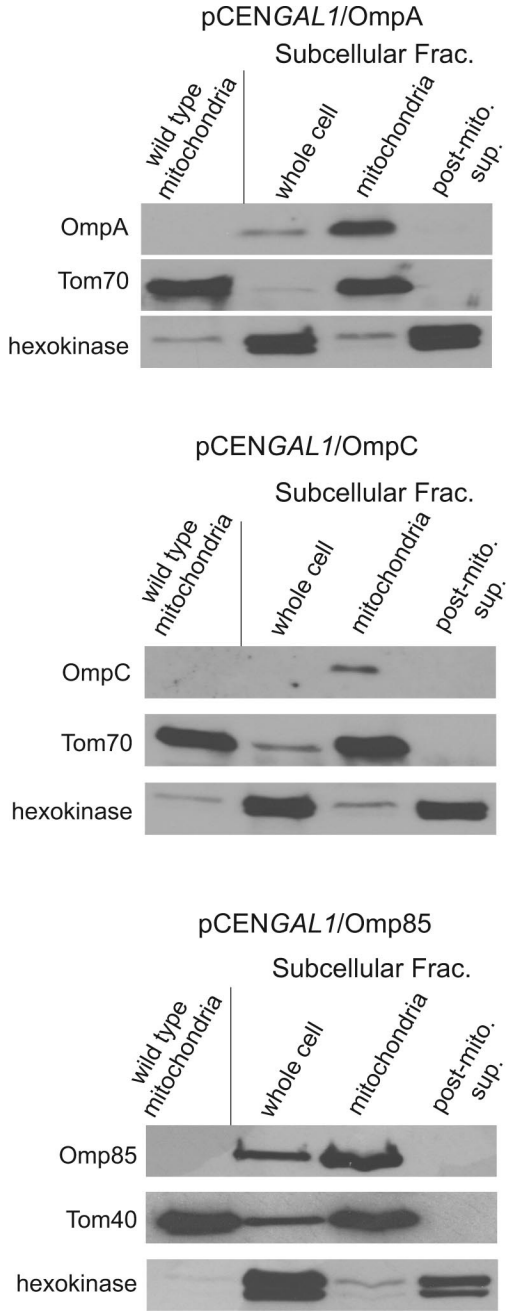

Fig. S2. Bacterial β-barrel proteins are targeted to mitochondria in yeast cells. Whole-cell lysate of cells expressing OmpA, OmpC, or Omp85 and fractions corresponding to mitochondria and postmitochondrial supernatant were analyzed by SDS/PAGE and immunodecoration with antibodies against the bacterial protein, the mitochondrial proteins Tom70 or Tom40, and a control marker protein for the cytosol (hexokinase). Mitochondria isolated from wild-type untransformed cells were coanalyzed as a control.

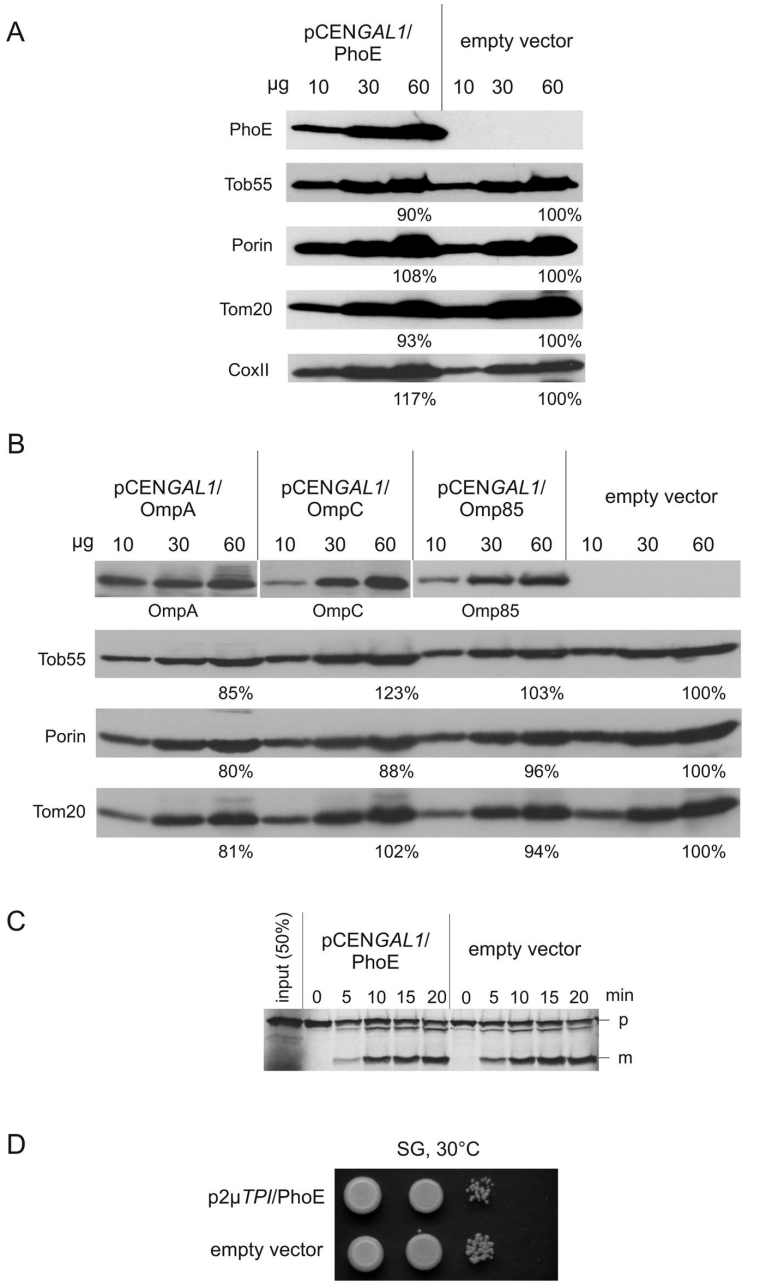

Fig. S3. Bacterial outer-membrane proteins do not interfere with mitochondrial functions. (*A*) Steady-state levels of endogenous mitochondrial β-barrel proteins are unaffected in cells expressing PhoE at high levels. The indicated amounts of mitochondria isolated from cells transformed with either a plasmid encoding PhoE or an empty plasmid were analyzed by SDS/PAGE and immunodetection with antibodies against PhoE, mitochondrial β-barrel proteins (Tob55 and porin), a signal-anchored protein of the outer membrane (Tom20), and a protein of the inner membrane (CoxII). The bands corresponding to 60 μg of mitochondria were quantified and the intensity of the band corresponding to mitochondria isolated from cells transformed with an empty plasmid was defined as 100%. (*B*) Steady-state levels of endogenous mitochondrial β-barrel proteins are unaffected in cells expressing OmpA, OmpC, or Omp85 at high levels. The indicated amounts of mitochondria isolated from cells transformed with either a plasmid encoding the indicated bacterial protein or an empty plasmid were analyzed by SDS/PAGE and immunodetection with antibodies against the bacterial proteins, mitochondrial β-barrel proteins (Tob55 and porin), and a signal-anchored protein of the outer membrane (Tom20). The bands corresponding to 60 μg of mitochondria were quantified and the intensity of the band corresponding to mitochondria isolated from cells transformed with an empty plasmid was defined as 100%. (*C*) Overexpression of PhoE does not reduce the capacity of isolated mitochondria to import precursor proteins in vitro. Mitochondria were isolated from cells harboring either plasmid-encoded PhoE or empty plasmid. Radiolabeled precursors of the matrix-destined protein pSu9 (1–69)-DHFR were incubated with the isolated mitochondria for the indicated time periods. The precursor and mature forms of pSu9 (1–69)-DHFR are indicated as p and m, respectively. (*D*) Overexpression of PhoE does not interfere with growth on a nonfermentable carbon source. Cells harboring either a multicopy plasmid encoding PhoE under the control of the *TPI* promoter or empty plasmid as control were tested by drop dilution assay for their ability to grow on synthetic glycerol-containing medium (SG) at 30 °C. Of note, to examine growth on various carbon sources, the *GAL* promoter in the plasmids expressing the bacterial proteins was replaced by the constitutive *TPI* promoter.

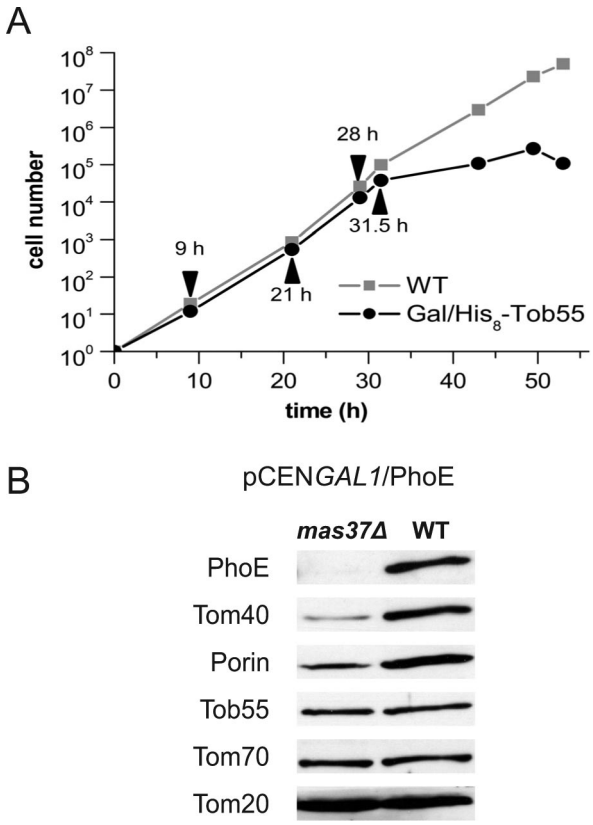

Fig. S4. Steady-state levels of PhoE are highly reduced when the function of the TOB complex is impaired. (*A*) Down-regulation of Tob55 affects cell growth. Cells from wild-type strain or from a strain expressing Tob55 under the control of the *GAL10* promoter (Gal/His_8-Tob55) were shifted from galactose- to glucose-containing medium at time 0. Relative cell number of the cultures was evaluated upon measuring the optical density at the indicated time points. (*B*) Mitochondria isolated from wild-type and *mas37*Δ cells transformed with a plasmid-encoding PhoE were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE and the indicated mitochondrial proteins.

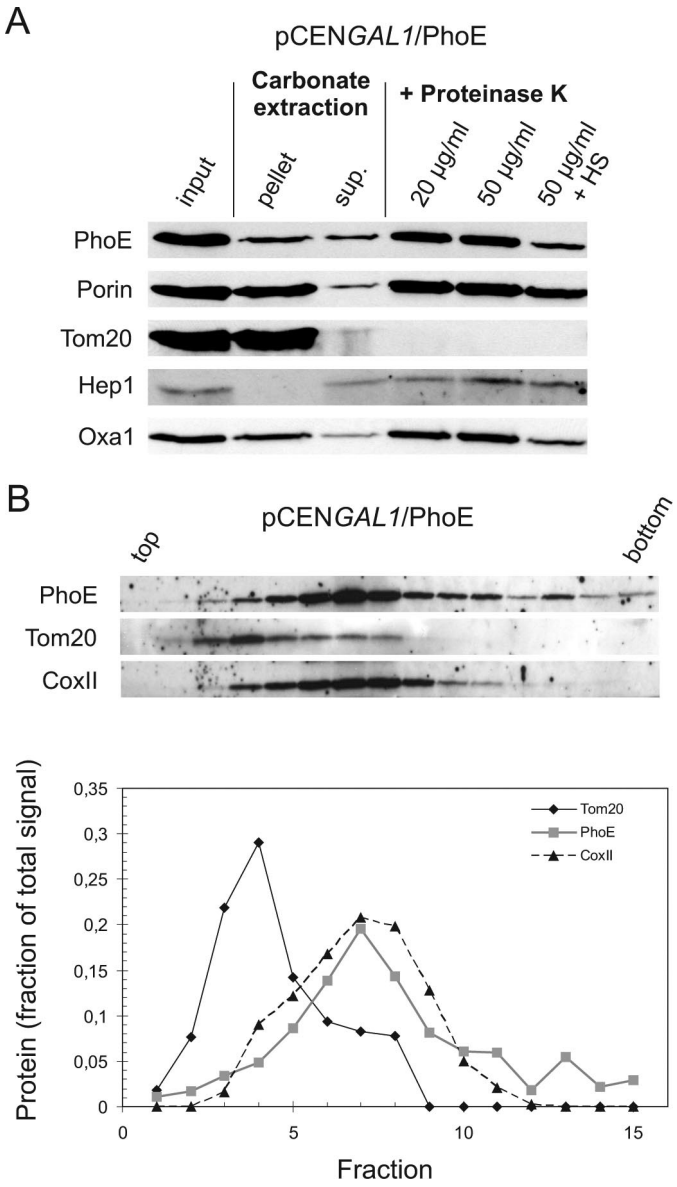

Fig. S5. Overexpressed PhoE is not properly inserted into mitochondrial membranes. (*A*) Assessment of membrane integration and assembly of PhoE. Mitochondria isolated from PhoE-overexpressing cells were loaded directly on SDS/PAGE gel (input) or were subjected first to alkaline extraction and then centrifuged to discriminate between membrane proteins (pellet) and soluble proteins in the supernatant (sup.). Additional aliquots of mitochondria were left intact or were treated by hypoosmotic swelling (HS) before their incubation with the indicated amounts of proteinase K. Proteins were analyzed by SDS/PAGE and immunodecorated with antibodies against the indicated proteins. Porin, protein embedded in the outer membrane; Tom20, outer membrane protein exposed to the cytosol; Hep1, soluble matrix protein; Oxa1, an inner membrane protein exposed to the intermembrane space (IMS). Of note, Hep1 was not affected by the protease treatment, demonstrating the intactness of the inner membrane under these conditions. (*B Upper*) At high expression levels, PhoE is not localized to a distinct membrane. Mitochondria from cells expressing PhoE under the *GAL1* promoter were submitted to hypoosmotic shock and subsequent disruption by sonication. Vesicles were then separated by a sucrose gradient centrifugation. Proteins from fractions were precipitated with trichloroacetic acid, analyzed by SDS/PAGE, and immunodecoration with antibodies against PhoE, the outer membrane protein Tom20, and the inner membrane protein CoxII. (*B Lower*) The bands corresponding to the detected proteins were quantified by densitometry. The relative amount in each fraction is presented as the portion from the total amount of this protein.

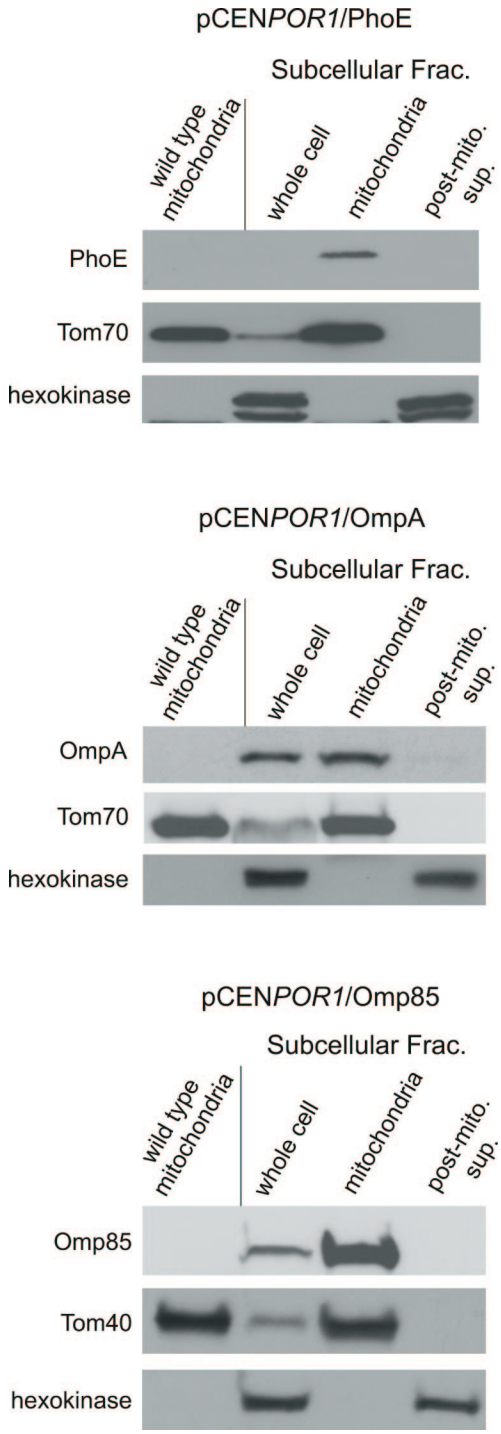

Fig. S6. Bacterial β-barrel proteins expressed at low levels are targeted to mitochondria in yeast cells. Whole-cell lysates of cells expressing the indicated bacterial protein under the control of the *POR1* promoter and fractions corresponding to mitochondria and postmitochondrial supernatant were analyzed by SDS/PAGE and immunodecoration with antibodies against the bacterial β-barrel protein, the mitochondrial proteins Tom70 or Tom40, and a control marker protein for the cytosol (hexokinase). Mitochondria isolated from wild-type, untransformed cells were coanalyzed as a control for the specificity of the antibodies.

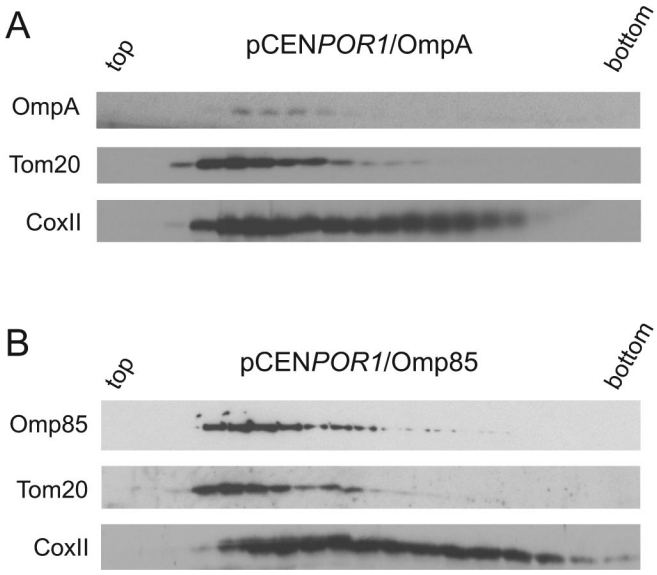

Fig. S7. OmpA and Omp85 are sorted to the mitochondrial outer membrane when expressed at low levels. Mitochondria from cells expressing OmpA (*A*) or Omp85 (*B*) under the *POR1* promoter were submitted to hypoosmotic shock and subsequent disruption by sonication. Inner- and outer-membrane vesicles were separated by sucrose density gradient centrifugation. Proteins from the collected fractions were analyzed by SDS/PAGE and immunodecoration with antibodies against either OmpA or Omp85, the outer membrane protein Tom20, and the inner membrane protein CoxII.

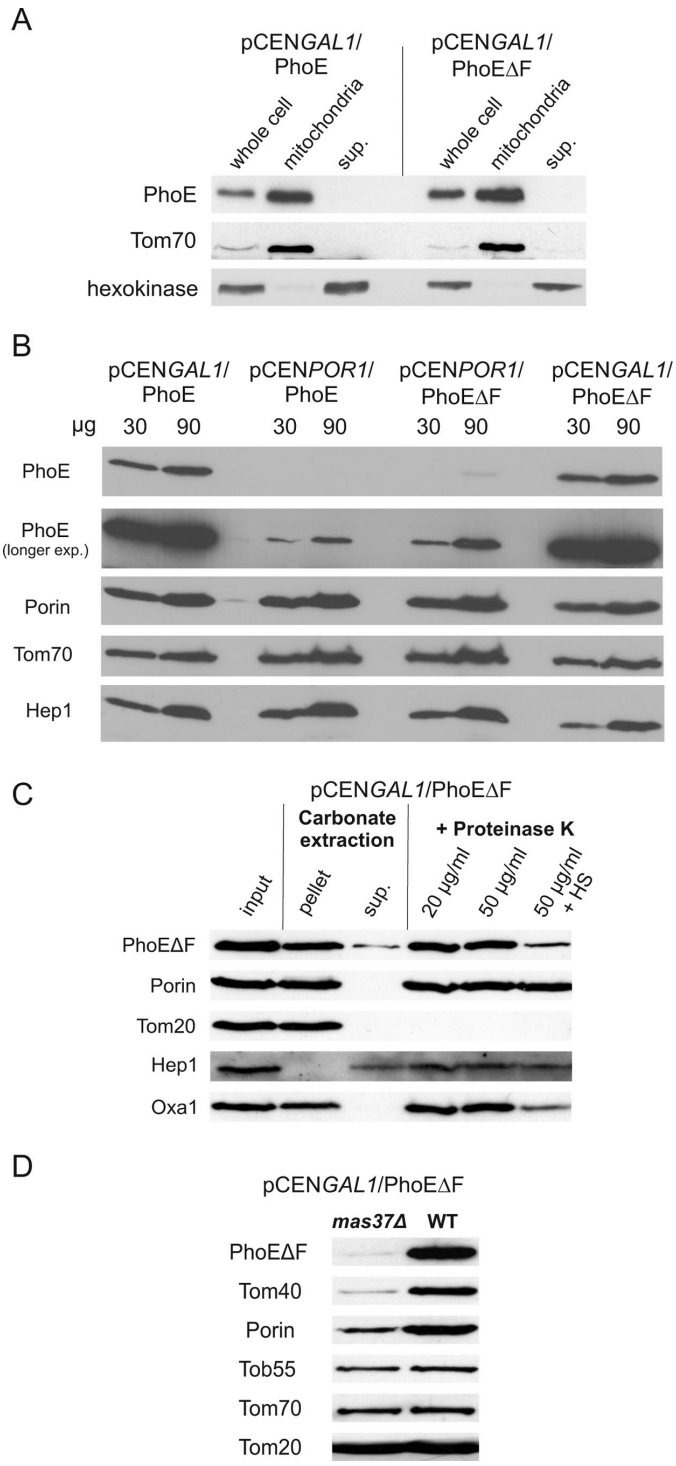

Fig. S8. PhoEΔF is imported into mitochondria but does not insert properly into the outer membrane. (*A*) PhoE variant lacking the C-terminal Phe residue (PhoEΔF) is targeted to mitochondria. Cells expressing full-length PhoE or PhoEΔF were treated and analyzed as described in Fig. S2. (*B*) The indicated amounts of mitochondria isolated from cells transformed with the specified plasmid were analyzed by SDS/PAGE and immunodetection with the indicated antibodies. A longer exposure of the immunodecoration with the antibody against PhoE is presented. (*C*) Mitochondria isolated from PhoEΔF-overexpressing cells were loaded directly on SDS/PAGE gel (input) or were subjected first to alkaline extraction and then centrifuged to discriminate between membrane proteins (pellet) and soluble proteins in the supernatant (sup.). Additional aliquots of mitochondria were left intact or were treated by hypoosmotic swelling (HS) before their incubation with the indicated amounts of proteinase K. Proteins were analyzed by SDS/PAGE and immunodecorated with antibodies against the indicated proteins. Porin, protein embedded in the outer membrane; Tom20, outer membrane protein exposed to the cytosol; Hep1, soluble matrix protein; OxaI, an inner membrane protein exposed to IMS. (*D*) Mas37 is required for import of PhoEΔF into mitochondria. Mitochondria were isolated from either wild-type or *mas37*Δ cells transformed with the plasmid expressing PhoEΔF under the *GAL1* promoter. Mitochondria were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE and the indicated mitochondrial proteins.

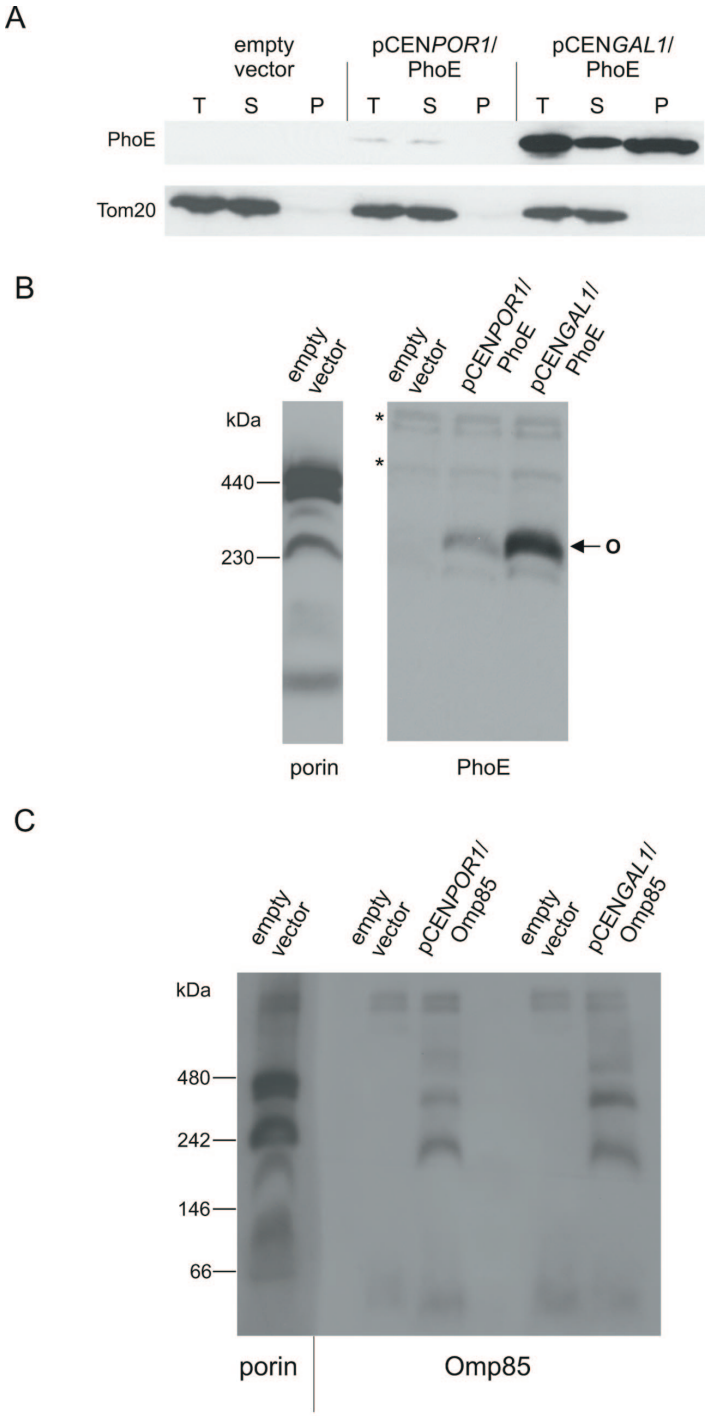

Fig. S9. PhoE and Omp85 form oligomers when expressed in yeast cells. (*A*) Mitochondria were isolated from cells transformed with empty plasmid and from cells expressing PhoE under the control of either *POR1* or *GAL1* promoters. The organelles were solubilized in a buffer containing 1% digitonin (T, Total) and the lysate was separated by centrifugation (36,700 × *g* for 10 min at 4 °C) to soluble fraction in the supernatant (S) and insoluble material in the pellet (P). Samples were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE and Tom20. (*B*) The supernatant fractions from *A* were analyzed by a 6–13% gradient BN/PAGE and immunodecoration with antibodies against PhoE or porin. The oligomeric form of PhoE and bands with which the antibody against PhoE cross-reacts are indicated with O and an asterisk, respectively. The migration of mitochondrial porin in this system is presented for comparison. Of note, the PhoE oligomer migrates similarly to one of the oligomeric forms of mitochondrial porin. This migration behavior excludes the possibility that the detected band represents folded PhoE monomer. (*C*) Mitochondria were isolated from cells transformed with empty plasmid and from cells expressing Omp85 under the control of either the *GAL1* or the *POR1* promoters. The organelles were solubilized in a buffer containing 1% digitonin. After a clarifying spin as in *B*, the supernatant fractions were analyzed by a 6–13% gradient BN/PAGE and immunodecoration with antibodies against Omp85. Porin oligomeric structures are immunodecorated for comparison.

Mitochondria can recognize and assemble fragments of a β-barrel structure

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ABSTRACT **β-barrel proteins are found in the outer membranes of eukaryotic organelles of endosymbiotic origin as well as in the outer membrane of Gram-negative bacteria. Precursors of mitochondrial β-barrel proteins are synthesized in the cytosol and have to be targeted to the organelle. Currently, the signal that assures their specific targeting to mitochondria is poorly defined. To characterize the structural features needed for specific mitochondrial targeting and to test whether a full β-barrel structure is required, we expressed in yeast cells the β-barrel domain of the trimeric autotransporter Yersinia adhesin A (YadA). Trimeric autotransporters are found only in prokaryotes, where they are anchored to the outer membrane by a single 12-stranded β-barrel structure to which each monomer is contributing four β-strands. Importantly, we found that YadA is solely localized to the mitochondrial outer membrane, where it exists in a native trimeric conformation. These findings demonstrate that, rather than a linear sequence or a complete β-barrel structure, four β-strands are sufficient for the mitochondria to recognize and assemble a β-barrel protein. Remarkably, the evolutionary origin of mitochondria from bacteria enables them to import and assemble even proteins belonging to a class that is absent in eukaryotes.**

Monitoring Editor
Benjamin Glick
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Received: Dec 6, 2010
Revised: Mar 5, 2011
Accepted: Mar 22, 2011

INTRODUCTION

Membrane-embedded β-barrel proteins transverse the membrane in the form of a cylindrically shaped structure built by interconnected β-strands (Wimley, 2003). These proteins are found in both prokaryotic and eukaryotic organisms. In prokaryotes, β-barrel proteins are found in the outer membrane of Gram-negative bacteria, whereas in eukaryotes they reside exclusively in the outer membrane of mitochondria and chloroplasts. Their presence in these organelles supports the endosymbiotic hypothesis, according to which mitochondria and chloroplasts evolved from prokaryotic ancestors. Indeed, the biogeneses of these proteins in the various systems bear significant similarities (Dolezal *et al.*, 2006; Walther *et al.*, 2009b).

Bacterial β-barrel proteins are synthesized in the cytoplasm with an N-terminal signal sequence for transport across the inner membrane into the periplasm via the SEC system (Bos *et al.*, 2007a). Their later integration into the outer membrane is facilitated by the β-barrel assembly machinery (BAM), the central component of which is the essential protein BamA (Omp85/YaeT) (Voulhoux *et al.*, 2003; Wu *et al.*, 2005). In mitochondria, precursors of β-barrel proteins are synthesized in the cytosol without a cleavable signal sequence. It is currently not clear why bacterial-like cleavable signal sequences were lost during the evolution of bacterial β-barrel proteins to their mitochondrial counterparts. Upon their synthesis, mitochondrial β-barrel precursors are translocated from the cytosol into the intermembrane space (IMS) via the translocase of the outer membrane (TOM) complex (Pfanner *et al.*, 2004; Paschen *et al.*, 2005). Their subsequent assembly into the outer membrane depends on a dedicated translocase, the topogenesis of mitochondrial outer membrane β-barrel proteins (TOB)

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10-12-0943) on April 1, 2011.

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Abbreviations used: BAM, β-barrel assembly machinery; DHFR, dihydrofolate reductase; ER, endoplasmic reticulum; IMS, intermembrane space; MA, membrane anchor; PK, proteinase K; SAM, sorting and assembly machinery; TOB, topogenesis of mitochondrial outer membrane β-barrel proteins; TOM, translocase of the outer membrane; YadA, Yersinia adhesin A.

(also known as sorting and assembly machinery [SAM]) complex. The central member of the latter complex is the essential protein Tob55/Sam50 that bears sequence and functional homology to BamA (Kozjak *et al.*, 2003; Paschen *et al.*, 2003; Gentle *et al.*, 2004). The other two subunits of the TOB complex, Mas37/Sam37 and Tob38/Sam35/Tom38, are peripheral membrane proteins exposed to the cytosol that share no obvious sequence similarity with the accessory lipoproteins of the bacterial Bam complex (Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004). Thus the biogenesis machineries in bacteria and mitochondria are similar in their central protein component and in an insertion into the outer membrane from the internal side of the membrane. In contrast, they vary with respect to the requirement of a signal sequence, the character of the accessory proteins, and the fact that precursors of mitochondrial β-barrel proteins initially have to cross the outer membrane.

To better understand the assembly process of β-barrel proteins in both bacteria and mitochondria, we expressed bacterial β-barrel proteins in the yeast *Saccharomyces cerevisiae* and demonstrated that they were imported into mitochondria and formed native-like oligomers. A detailed investigation of the import pathway revealed that it is shared with mitochondrial β-barrel proteins (Walther *et al.*, 2009a). The reciprocal expression approach was also successful, and we observed that expression of mitochondrial porin in *Escherichia coli* resulted in assembly of the protein into the bacterial outer membrane, where it formed conducting pores (Walther *et al.*, 2010). Taken together, it appears that despite the above-mentioned differences, the basic mechanism of β-barrel assembly in the outer membranes of bacteria and mitochondria is evolutionary conserved and that β-barrel proteins from one system can be dealt with and assembled by the other one.

Although some progress in our understanding of the biogenesis of β-barrel proteins has been made recently, the mitochondrial targeting signal in such proteins is still ill defined. A conserved linear sequence could not be identified, hence it was proposed that the signal is composed by β-barrel–specific structural elements (Walther *et al.*, 2009b). However, neither the character nor the size of such putative structural signal has been identified so far. A crucial question is whether precursor of a full β-barrel structure is required. To shed new light on this issue, we investigated whether mitochondria can recognize and assemble fragments of a β-barrel protein. Such fragments are found in nature in the single subunits of trimeric autotransporter β-barrel proteins. These proteins form a special subfamily of bacterial β-barrel proteins. They have a characteristic arrangement of functional domains, including an N-terminal signal peptide, an internal passenger domain (also called the effector domain), and a relatively short C-terminal β-domain. The passenger moiety mediates the various functions of the autotransporter, and the β-domain forms a β-barrel that anchors the protein to the outer membrane. This anchor is made by a single 12-stranded β-barrel structure to which each monomer contributes four β-strands (Hoiczyk *et al.*, 2000; Linke *et al.*, 2006).

We took advantage of this model system and expressed the β-barrel domain of the bacterial trimeric autotransporter Yersinia adhesin A (YadA) in yeast and analyzed its cellular localization and topology. Our findings demonstrate that four β-strands contain sufficient structural information to be recognized and processed by the mitochondrial assembly machinery. Surprisingly, the bacterial evolutionary origin of mitochondria enables them to assemble even proteins that are absent in modern eukaryotic organisms.

RESULTS

Bacterial signal sequence interferes with the assembly of PhoE in mitochondria

Bacterial β-barrel proteins are synthesized in the cytoplasm with a signal sequence that targets them to the SEC machinery (Bos *et al.*, 2007a). It is commonly believed that mitochondrial β-barrel proteins evolved from their bacterial counterparts. Nevertheless, in the process of developing from endosymbiont to modern time organelle, mitochondrial β-barrel proteins lost such an N-terminal extension. Hence, as part of our efforts to understand the sorting of these proteins in the eukaryotic cell we wanted to understand why during evolution mitochondrial β-barrels lost their bacterial signal sequence. To address this question, we used the capacity of the bacterial β-barrel protein PhoE to be assembled into the mitochondrial outer membrane of yeast cells (Walther *et al.*, 2009a). First, we compared the mitochondrial levels of mature PhoE that lacks the signal sequence to that of a protein containing the N-terminal signal sequence (Sig-PhoE). We observed that Sig-PhoE is present in mitochondria at significantly reduced levels as compared to the form without the signal sequence (Figure 1A). One potential explanation for this decline could be reduced mRNA levels encoding the Sig-PhoE protein. To address this point, we isolated mRNAs from both cell types and performed RT-PCR. The results suggested that the amounts of mRNAs encoding both PhoE forms are comparable (Figure 1B). The bacterial signal sequence has similar characteristics to the eukaryotic signal sequence that directs protein to the secretory pathway. Hence another theoretical possibility that could explain the reduced levels of Sig-PhoE in the mitochondria would be a secretion of a portion of the protein molecules from the yeast cells. However, when we analyzed the medium of the growing culture, we could not detect any PhoE signal (unpublished data).

We next explored whether an impaired biogenesis or an enhanced degradation of the signal sequence–containing protein can explain the observed reduction. To test the latter option, we added cycloheximide, which blocks protein synthesis, to the yeast culture and compared the levels of PhoE and Sig-PhoE in crude membrane preparations. Surprisingly, we detected in these samples a modified version of Sig-PhoE that had a relatively short half-life, and most of it was degraded after 2 h (Figure 1C). In contrast, the nonmodified form of Sig-PhoE and PhoE, as well as the control β-barrel protein porin, remained stable. Importantly, although nonmodified Sig-PhoE was present in lower levels than PhoE, the turnover rates of both proteins were indistinguishable. Tom70, which exposes a large domain to the cytosol, exhibited an enhanced turnover as compared to the membrane-embedded proteins (Figure 1C). Of note, this membrane fraction contains crude mitochondria and contaminations from other cellular compartments and proteins that are only loosely associated with the organelle. Thus we wondered whether the modified form of Sig-PhoE is membrane embedded. To answer that question, we performed alkaline extraction, after which membrane proteins remain in the pellet and soluble and membrane-peripheral proteins are found in the supernatant. Remarkably, the modified versions of Sig-PhoE were largely extracted under these conditions, whereas the nonmodified species behaved as membrane-embedded proteins (Figure 1D). Thus the modified forms are not integrated into cellular membranes.

We aimed to identify the nature of this modification. Our initial suspicion that the modified forms represent ubiquitination of Sig-PhoE was not confirmed, as an antibody against ubiquitin failed to recognize the modified species (unpublished data). Next we treated the membrane fraction with recombinant endoglycosidase H, which can remove oligosaccharides from N-linked glycoproteins.

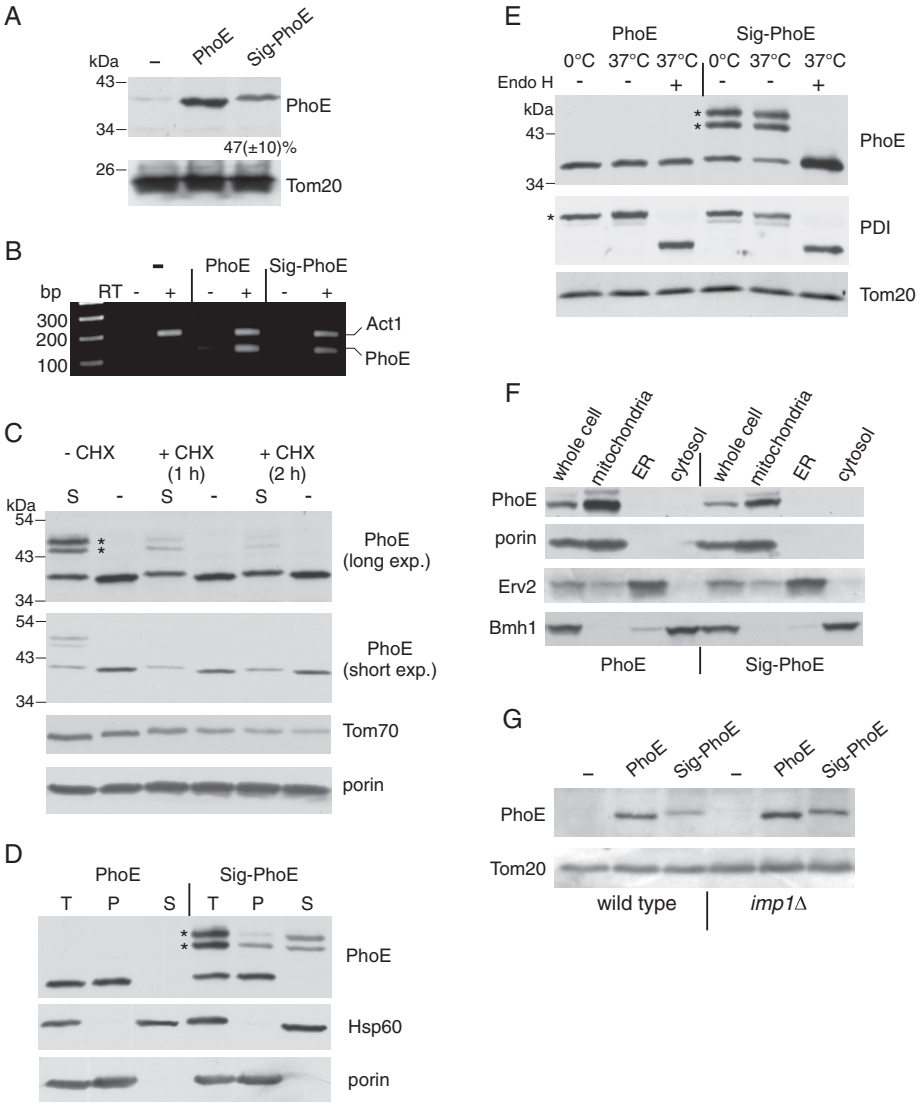

FIGURE 1: Bacterial signal sequence interferes with assembly of PhoE into mitochondria. (A) Mitochondria isolated from yeast cells transformed with an empty plasmid (–) or with a plasmid encoding either mature PhoE (PhoE) or PhoE with its signal sequence (Sig-PhoE) were analyzed by SDS–PAGE and immunodecoration with antibodies against PhoE and Tom20 as a loading control. The intensity of the PhoE and Sig-PhoE bands in three independent experiments was quantified, and the amount of Sig-PhoE is expressed as mean (±SD)% of the level of PhoE. (B) mRNAs were isolated from the cells just described, and reverse transcriptase (RT) was added to the indicated samples. Then, PCR using primers complementary to PhoE or actin (as a control) was performed using the obtained DNA as template. The PCR-amplified DNA fragments were analyzed on a 2% agarose gel by staining with ethidium bromide. (C) Cells expressing either PhoE (indicated as "–") or Sig-PhoE (indicated as "S") were grown in the presence or absence of cycloheximide (CHX). Membrane fraction isolated from these cells was analyzed by SDS–PAGE and immunodecorated with antibodies against PhoE, Tom70, and porin. To better view the different intensities of the various PhoE forms, short and long exposures are presented. Modified forms of Sig-PhoE are indicated with an asterisk. (D) Membrane fractions isolated from cells expressing either PhoE or Sig-PhoE (total, T) were subjected to alkaline extraction and then centrifuged to discriminate between membrane proteins in the pellet (P) and soluble proteins in the supernatant (S). Proteins were analyzed by SDS–PAGE and immunodecorated with antibodies against PhoE, Hsp60 (soluble matrix protein), and porin (embedded in the outer membrane). Modified forms of Sig-PhoE are indicated with an asterisk. (E) Membrane fractions isolated from cells expressing either PhoE or Sig-PhoE were solubilized with a buffer containing 0.5% SDS and 40 mM dithiothreitol in the presence of protease inhibitor cocktail. The samples were then incubated for 1 h at 0°C (as control) or at 37°C in the presence or absence of Endoglycosidase H_f (Endo H). Proteins were analyzed by SDS–PAGE and immunodecorated with antibodies against PhoE, protein disulphide isomerase (PDI, a glycosylated ER protein), and Tom20 (a nonglycosylated mitochondrial protein). Glycosylated forms of Sig-PhoE and PDI are indicated with an asterisk. (F) Lysate of cells expressing either PhoE or Sig-PhoE and fractions corresponding to mitochondria, light microsomal fraction (ER), and cytosol were analyzed by SDS–PAGE and immunodecoration with antibodies against PhoE, the mitochondrial protein porin, the ER protein Erv2, and a marker protein for the cytosol (Bmh1). (G) An empty vector (–) or vector encoding for either PhoE or Sig-PhoE was transformed into either wild type or cells lacking a functional inner membrane peptidase (*imp1*Δ). Mitochondria isolated from these cells were analyzed by SDS–PAGE and immunodecoration with antibodies against PhoE and Tom20 as a loading control.

Surprisingly, this treatment resulted in disappearance of the modified forms concomitantly with an enhancement of the signal of the unmodified Sig-PhoE (Figure 1E). Indeed, analysis of PhoE sequence with the glycosylation sites prediction program, NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) revealed several asparagine residues as candidates for N-glycosylation. As this modification occurs in the lumen of the endoplasmic reticulum (ER), it appears that the similarity between the signal sequence of PhoE to the eukaryotic signal sequence causes a subpopulation of Sig-PhoE to be mistargeted to this latter compartment. Collectively, these results demonstrate that both forms of PhoE are expressed to the same extent. Apparently, a large portion of Sig-PhoE molecules is guided by the signal sequence to the ER, gets glycosylated and then degraded.

Because a portion of Sig-PhoE molecules is getting glycosylated in the ER, we asked whether the membrane-embedded form of Sig-PhoE can also be found in the ER in addition to mitochondria. Upon performing subcellular fractionation, we could not detect Sig-PhoE in the light microsomal fraction (ER), and both PhoE forms were located exclusively in the mitochondria (Figure 1F). Of note, Sig-PhoE is migrating at an apparently higher molecular mass than PhoE is, suggesting that the signal sequence is not processed (Figure 1, A and C–F). Mitochondria contain in their IMS a peptidase (named Imp) belonging to type I signal peptidase family. This peptidase was suggested to share several key features with the bacterial leader peptidase that cleaves the bacterial signal sequence upon its translocation across the inner membrane (Schneider *et al.*, 1991). Thus we wanted to confirm by an additional approach that Imp does not cleave Sig-PhoE upon its import into mitochondria. To that end we used a strain deleted for one of the subunits of the Imp peptidase (*imp1*) and observed that the

migration behavior of Sig-PhoE was not altered (Figure 1G). Collectively, it appears that mitochondrial β-barrels lost their N-terminal extension due to the potential of this signal sequence to wrongly direct them to the ER and, by that, to reduce their assembly into the mitochondrial outer membrane.

The β-barrel domain of YadA is targeted to mitochondria

Next we wanted to narrow down the structural features required for specific targeting to mitochondria and thus asked whether the mitochondrial import machinery can deal with a fragment of a β-barrel structure. To address this point, we used the membrane anchor (MA) domain (amino acids 335 to 422) of YadA, which is located in the C-terminal region of the protein (Figure 2A). Based on our previous and current results, YadA-MA was constructed without the bacterial signal sequence. To allow detection, an HA-tag was introduced at the N terminus of YadA-MA (Figure 2A). The expression of this construct in yeast cells was under the control of the *GAL1* promoter. Subcellular fractionation of the transformed cells revealed that YadA-MA was located exclusively in the mitochondrial fraction (Figure 2B). As a control for the specificity of the antibody against the HA-tag, we confirmed the absence of the signal in mitochondria isolated from a nontransformed strain (Figure 2B, left lane).

YadA-MA migrated in SDS–PAGE as several bands with an apparent molecular weight of 42–50 kDa, a size expected for its trimeric structure (Figure 2C) (Wollmann *et al.*, 2006; Grosskinsky *et al.*, 2007). It is well documented that both full-length YadA and the MA domain build trimeric forms that are stable in SDS–PAGE (Wollmann *et al.*, 2006; Grosskinsky *et al.*, 2007; Ackermann *et al.*, 2008). The 42- to 50-kDa bands can represent various conformations of the native trimeric form. To support this notion, we also expressed HA-tagged YadA-MA in *E. coli* and heated both *E. coli* envelopes and mitochondria isolated from transformed yeast cells in a solution containing 1% SDS and 8 M urea. In both expression systems, a shift from the trimeric bands to a single monomeric band was observed (Figure 2C). The detection of a single monomeric band argues against the possibility that the multiple bands behavior reflects a situation in which various trimeric forms harbor different patterns of covalent modifications. Of note, YadA-MA expressed in bacteria also migrates as several bands, suggesting that this phenomenon is not an artifact due to expression in eukaryotic cells. The pattern of the bands differs slightly from bacteria to mitochondria probably due to different membrane composition in these two systems. Collectively, these results confirm the trimeric nature of the 42- to 50-kDa bands observed upon analysis of mitochondria.

We further investigated whether the expression of YadA-MA obstructs the biogenesis of other mitochondrial outer membrane proteins. The levels of outer membrane β-barrel proteins, such as Tob55 and porin, were not affected by the expression of YadA-MA (Figure 3A). Similarly, the growth rate of yeast cells expressing the bacterial protein was similar to that of nontransformed cells under all tested conditions, including growth on a nonfermentable carbon source where yeast cells require fully functional mitochondria (Figure 3B and unpublished data). Next we verified that expressing YadA-MA in yeast cells did not have any effect on the morphology of the organelle (unpublished data). Collectively, it seems that the expression of YadA-MA in yeast cells does not interfere with crucial mitochondrial processes.

Membrane topology of YadA-MA

To verify that YadA-MA was embedded within the membrane rather than associated on the surface of the organelle, mitochondria were subjected to alkaline extraction. The YadA-MA protein was found in

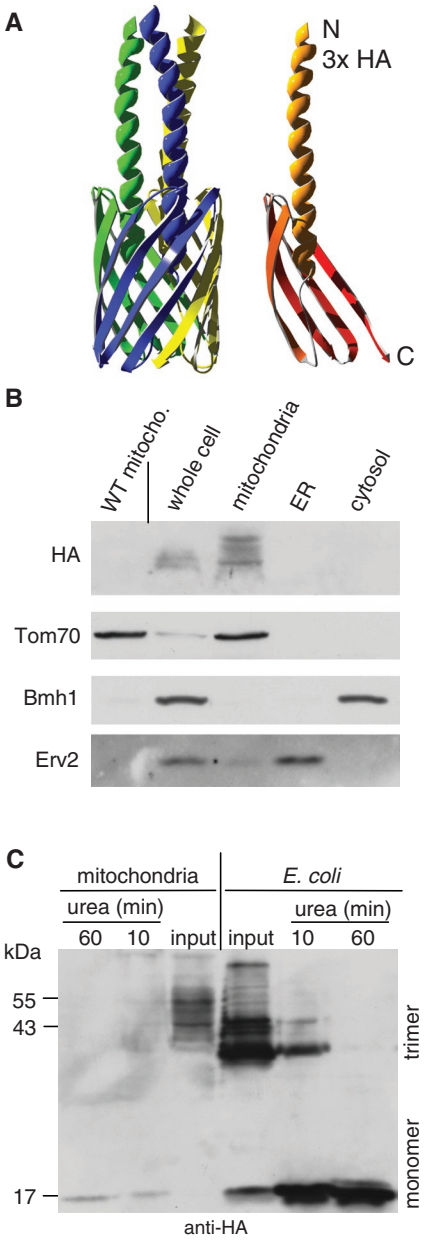

FIGURE 2: YadA-MA is assembled into mitochondria in a native trimeric conformation. (A) Atomic structure model of YadA-MA monomer with an HA-tag at its N-terminal (right) and trimeric form built from three monomers (left). Each YadA-MA monomer is composed of four β-strands that participate in the β-barrel structure and a linker domain (shown here as a helical structure). (B) YadA-MA is located in mitochondria. Lysate of cells expressing YadA-MA and fractions corresponding to mitochondria, ER, and cytosol were analyzed by SDS–PAGE and immunodecoration with antibodies against HA-tag, the mitochondrial protein Tom70, a marker protein for the cytosol (Bmh1), and the ER protein Erv2. Mitochondria isolated from untransformed wild-type cells were coanalyzed as a control. (C) Monomerization assay of YadA. Mitochondria isolated from yeast cells expressing YadA-MA and envelopes of *E. coli*–expressing YadA-MA were boiled for 5 min in Laemmli buffer without urea (input) or in Laemmli buffer containing 8 M urea for the indicated time periods. The samples were analyzed by SDS–PAGE and immunodecoration with HA-antibody. Molecular mass markers and the monomeric and trimeric forms of YadA are indicated (left and right, respectively).

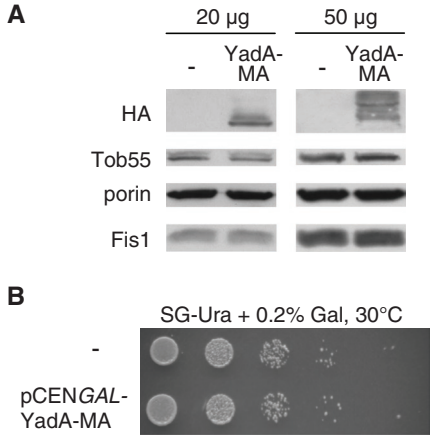

FIGURE 3: Expression of YadA-MA does not interfere with mitochondrial functions. (A) Mitochondria (20 or 50 µg) isolated from cells transformed with either an empty plasmid (–) or a plasmid encoding YadA-MA were analyzed by SDS–PAGE and immunodecoration with antibodies against HA, mitochondrial β-barrel proteins (Tob55 and porin), and a tail-anchored protein of the outer membrane (Fis1). (B) Expression of YadA-MA does not interfere with growth on a nonfermentable carbon source. Cells harboring either a plasmid encoding YadA-MA under the control of the *GAL1* promoter or an empty plasmid (–) as control were tested by drop dilution assay for their ability to grow on synthetic glycerol-containing (SG) medium at 30°C. Small amounts of galactose (0.2%) were added to assure activation of the promoter.

the pellet fraction together with other membrane-embedded mitochondrial proteins like Tom20 or porin (Figure 4A). In contrast, the soluble proteins aconitase and Tim10 were detected in the supernatant after this treatment (Figure 4A). Moreover, as further support for localization to the outer membrane, treatment of mitochondria isolated from YadA-MA–expressing cells with externally added proteinase K (PK) resulted in disappearance of the HA signal. Of note, the outer membrane was intact under these conditions as verified by the protease resistance of the small Tim10 chaperone residing in the IMS (Figure 4A). The results of the proteolytic assay can be explained by two alternative conformations of the protein. The first one is the native-like conformation with the HA-tags facing the cytosol, thereby being digested by PK (Figure 4B, left panel, I). The second conformation would be an upside-down conformation with the HA-tags facing the IMS (Figure 4B, left panel, II). In the latter case, the HA-tags themselves would be protected, but loops connecting the β-strands of YadA with the linker and the HA-tag might be accessible to the protease. According to an atomic model of the protein (Ackermann *et al.*, 2008) and the crystal structure of the homologous membrane-anchor domain of the autotransporter Hia (Meng *et al.*, 2006), cleavage at this loop would result in a HA-containing fragment with a size of approximately 6 kDa. To completely exclude the upside-down conformation, mitochondria harboring YadA-MA were treated with PK and the samples were analyzed on a urea-containing SDS–PAGE system optimized for detection of small polypeptides. Although a marker protein as small as 3.5 kDa could be detected with this gel system, a band at 6 kDa was not observed after treatment with PK (Figure 4B, right panel). Of note, the IMS isoform of Mcr1 was resistant under these conditions, confirming that the outer membrane was intact (Figure 4B). Taken together, our results demonstrate that, similarly to the topology in bacteria, YadA-MA is integrated into the outer membrane of mitochondria in a conformation in which the N terminus of each monomer is facing the external surface.

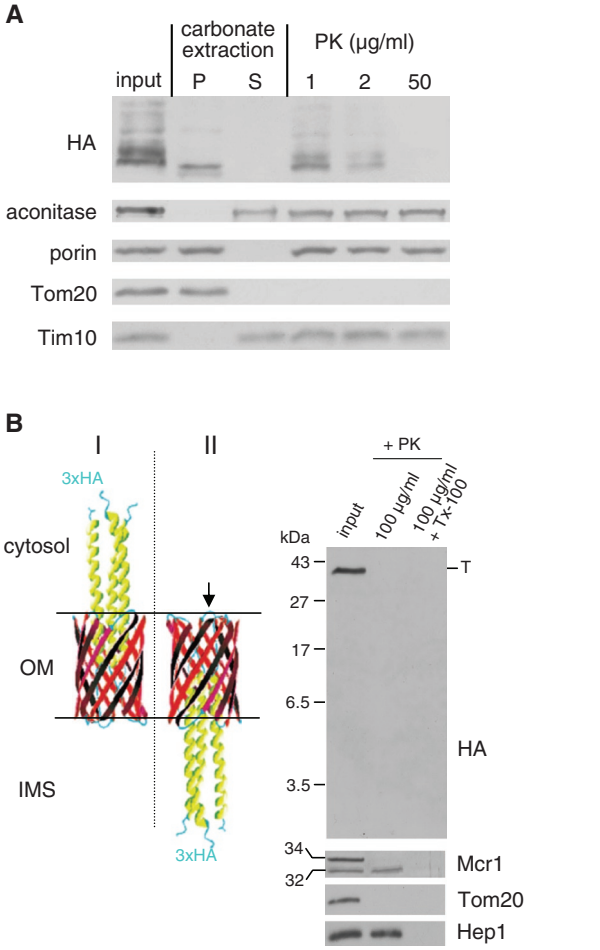

FIGURE 4: YadA-MA is integrated into the mitochondrial outer membrane in the correct topology. (A) Mitochondria isolated from cells expressing YadA-MA were loaded directly on SDS–PAGE gel (input), or were first subjected to carbonate extraction and then centrifuged to discriminate between membrane proteins in the pellet (P) and soluble proteins in the supernatant (S). Additional aliquots of mitochondria were treated with the indicated amounts of PK. Proteins were analyzed by SDS–PAGE and immunodecorated with antibodies against the indicated proteins: aconitase, a mitochondrial matrix protein; porin, a protein embedded in the outer membrane; Tom20, an outer membrane protein exposed to the cytosol; Tim10, a soluble IMS protein. (B) Left, models of two putative conformations of HA-tagged YadA-MA in the mitochondrial outer membrane. The native (I) and the upside-down conformation (II) are displayed with an arrow pointing putative PK-sensitive loops for the upside-down conformation. Right, PK protection assay of mitochondria isolated from yeast cells expressing HA-tagged YadA-MA. Mitochondria were left untreated (input) or were treated with PK in the absence or presence of Triton X-100 (Tx-100). The samples were analyzed by SDS–PAGE on a gel optimized for detection of small polypeptides followed by immunodecoration with antibodies against HA, Tom20 (outer membrane), Hep1 (matrix), and Mcr1 (outer membrane and IMS). The latter protein has two isoforms: a 34 kDa form exposed at the outer membrane and a 32 kDa form in the IMS. Molecular mass markers are indicated on the left. The trimeric form of YadA-MA is indicated to the right with T.

YadA-MA assembly into mitochondria requires the small Tim chaperones and the TOB complex

We previously observed that bacterial β-barrel proteins expressed in yeast cells require the import receptor Tom20 for their initial

recognition at the organelle (Walther *et al.*, 2009a). This requirement is shared with mitochondrial β-barrel proteins like Tom40, porin, and Tob55 (Rapaport and Neupert, 1999; Krimmer *et al.*, 2001; Model *et al.*, 2001; Habib *et al.*, 2005). Thus we asked whether the import receptors of the TOM complex play a role in the import of YadA-MA. To address this point, we expressed YadA-MA in cells deleted for either Tom20 or Tom70/Tom71 and monitored its level in these cells. Surprisingly, highly pure mitochondria from strains lacking either import receptor had similar (*tom20*Δ) or even slightly higher (*tom70*Δ) amounts of YadA-MA as compared to those in wild-type organelles (Figure 5, A and B). As previously reported, the level of bacterial PhoE was reduced in mitochondria lacking Tom20 (Figure 5A). Taken together, it appears that, in contrast to their contribution to the import of precursors of mitochondrial and other bacterial β-barrel proteins, the import receptors are not involved in the membrane integration of YadA-MA.

Next we investigated whether YadA-MA requires the small chaperones in the IMS for its assembly in mitochondria. To that end, both PhoE and YadA-MA were transformed into a strain lacking both Tim8 and Tim13. Crude mitochondria were isolated from these cells and subjected to SDS–PAGE and immunodecoration. It can be observed that the steady-state levels of both PhoE and YadA-MA are reduced in cells lacking the Tim8/Tim13 complex (Figure 5C). Hence it seems that these small chaperones are playing an important role in the assembly of YadA-MA in mitochondria.

Does the TOB complex facilitate the membrane insertion of YadA-MA? Cells lacking the peripheral subunit of the TOB complex, Mas37, were transformed with a plasmid encoding YadA-MA. Mitochondria were isolated from these cells and subjected to SDS–PAGE and immunoblotting. Whereas the wild-type control shows significant expression of YadA-MA, the protein is hardly detectable in *mas37*Δ cells (Figure 5D). Interestingly, the effect of the absence of Mas37 on endogenous β-barrel proteins like porin or Tom40 is less severe (Figure 5D). Nevertheless, as the steady-state levels of Tom40 are also reduced in *mas37*Δ mitochondria, we wanted to exclude the possibility that the compromised insertion of YadA-MA is solely due to reduced levels of this central Tom component. To that goal we transformed a plasmid for the expression of YadA-MA under the control of the *TPI* promoter into cells in which the essential component Tob55 is under the control of the inducible *GAL* promoter (Paschen *et al.*, 2003). When these cells are grown on glucose, the level of Tob55 is gradually reduced and, as a result, the cells´ growth is slowed down (Paschen *et al.*, 2003; Walther *et al.*, 2009a). Mitochondria were isolated at various time points from these Tob55-depleted cells, and the levels of various mitochondrial proteins were analyzed. Noticeably, Tob55 was gradually depleted upon growth on glucose-containing medium. The compromised amounts of Tob55 caused a clear reduction in the levels of additional mitochondrial β-barrel proteins like porin and Tom40 as well as in those of YadA-MA (Figure 5E). Importantly, the decline in the amounts of YadA-MA preceded that of Tom40, suggesting that the depletion of YadA-MA is not initiated by the reduction in the levels of Tom40.

Of note, cells grown in galactose-containing medium contain excess amounts of Tob55 molecules (Figure 5E, time 0). These unassembled surplus molecules interfere with the assembly of YadA-MA; therefore we initially observed only YadA-MA monomers. Similarly, we previously observed that overexpression of Tob55 resulted in severely compromised assembly of newly synthesized Tom40 and porin molecules into isolated organelles (unpublished data). Upon shifting the cells to glucose-containing medium, Tob55 is gradually depleted and returns to its normal levels. This initial reduction resulted in assembly of YadA-MA, whereas further depletion of Tob55

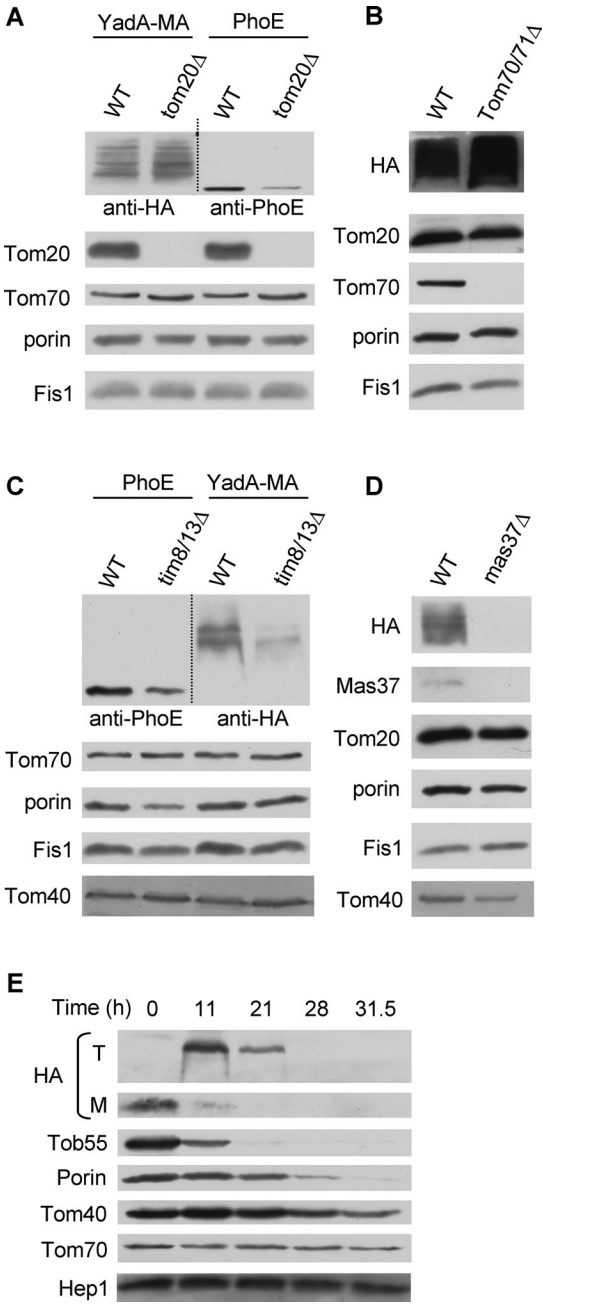

FIGURE 5: The assembly of YadA-MA depends on Tim8/Tim13 and Mas37. (A and C) Mitochondria isolated from either *tom20*Δ (A) or *tim8*Δ/*tim13*Δ (C) and their corresponding wild-type strains transformed with either YadA-MA or PhoE were analyzed by SDS–PAGE and immunodecoration with antibodies against either HA-tag or PhoE, respectively. In addition, immunodecoration with antibodies against the indicated mitochondrial proteins was performed. (B and D) Mitochondria isolated from either *tom70*Δ/*tom71*Δ (B) or *mas37*Δ (D) and their corresponding wild-type cells transformed with YadA-MA were analyzed as in (A). (E) YadA-MA was transformed into cells expressing Tob55 under the control of the *GAL10* promoter. Cells were harvested at the indicated time points after a shift from galactose- to glucose-containing medium. Crude mitochondria were isolated, and proteins were analyzed by SDS–PAGE and immunodecoration with antibodies against HA-tag and the indicated mitochondrial proteins. The monomeric and trimeric forms of YadA-MA are indicated with M and T, respectively. Tob55, Tom40, and porin are β-barrel proteins.

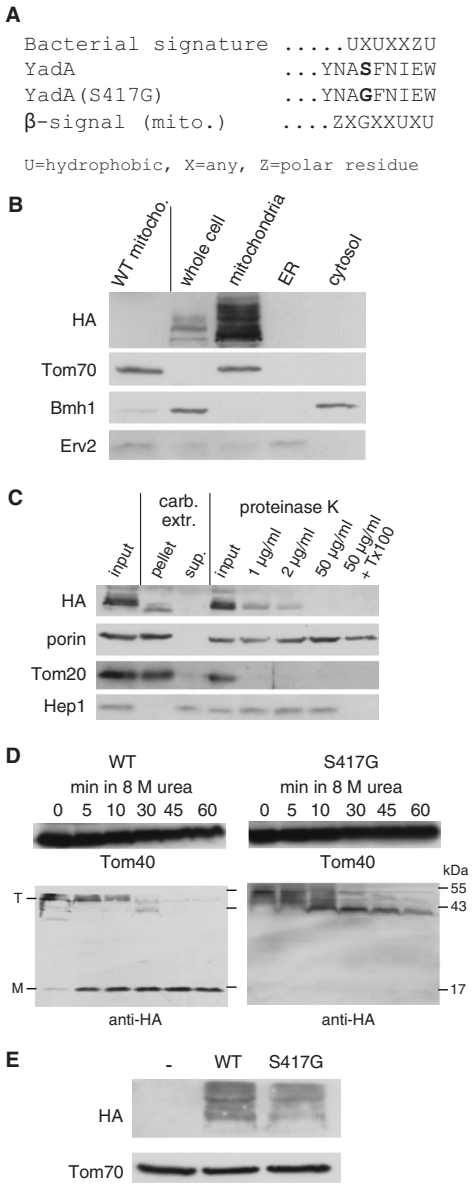

FIGURE 6: A eukaryotic-like β-signal improves the stability but not the overall assembly of YadA-MA. (A) Comparison of the bacterial and mitochondrial β-barrel assembly signals and the C termini of the YadA-MA variants used in this study. X, any amino acid; U, hydrophobic residue; Z, polar residue. (B) YadA-MA-S417G is located in mitochondria. Whole cell lysate of cells expressing the YadA-MA-S417G variant and fractions corresponding to highly pure mitochondria, ER, and cytosol were analyzed by SDS–PAGE and immunodecoration with antibodies against HA-tag, the mitochondrial protein Tom70, a marker protein for the cytosol (Bmh1), and the ER protein Erv2. To demonstrate the specificity of the HA antibody, crude mitochondria isolated from wild-type, untransformed cells were coanalyzed as a control. (C) Mitochondria isolated from cells expressing YadA-MA-S417G were loaded directly on SDS–PAGE gel (input), or were subjected first to carbonate extraction and then centrifuged to discriminate between membrane proteins in the pellet and soluble proteins in the supernatant (sup). Additional aliquots of mitochondria were left intact (input) or were treated with the indicated amounts of PK in the absence or presence of Triton X-100 (Tx-100). Samples were analyzed by SDS–PAGE and immunodecoration with antibodies against the indicated proteins. Porin, protein embedded in the outer membrane; Tom20, outer membrane protein exposed to the cytosol; Hep1, a mitochondrial matrix protein. (D) Monomerization assay of YadA-MA. Mitochondria were isolated from yeast cells expressing either native YadA-MA (WT) or the S417G variant. Mitochondria were boiled for 5 min in Laemmli buffer without urea (0) or in Laemmli buffer containing 8 M urea for the indicated time periods. The samples were analyzed by SDS–PAGE and immunodecoration with antibodies against the HA-tag and against Tom40 as a loading control. The monomeric and trimeric forms of YadA-MA are indicated with M and T, respectively. (E) The S417G variant is present in lower steady-state levels. Mitochondria isolated from yeast cells transformed with an empty plasmid (–) or with a plasmid encoding either native YadA-MA (WT) or its variant (S417G) were analyzed by SDS–PAGE and immunodecoration with antibodies against HA and Tom70 as a loading control.

caused diminished assembly of the former protein. Collectively, these results demonstrate the involvement of the TOB complex in the membrane integration of YadA-MA.

A eukaryotic-like β-signal improves the stability but not the overall assembled levels of YadA-MA

Recently a signature motif termed β-signal, located at the C-terminal β-strand of mitochondrial β-barrel precursors, was identified. This signal, which contains a highly conserved glycine residue, was suggested to be important for the interaction of β-barrel substrates with the TOB complex (Kutik *et al.*, 2008; Figure 6A). To resemble this eukaryotic β-signal and to test the importance of such a signal for the assembly of YadA-MA in mitochondria, a serine residue in position 417, which resides within the last of the four encoded β-strands, was replaced by a glycine residue resulting in the S417G variant (Figure 6A). We verified by subcellular fractionation that, similarly to the native protein, the YadA-MA-S417G variant is localized to mitochondria (Figure 6B). Furthermore, proteolytic assay and alkaline extraction revealed that the mutated protein is embedded in the correct topology in the mitochondrial outer membrane (Figure 6C). Next we compared the stability of both forms. The wild-type trimeric form was partially converted after 5 min of boiling in the presence of 8 M urea to the monomeric one. In contrast, the trimeric form of the variant was turned into the smaller form much more slowly, and, even after 60 min, the monomeric band could hardly be observed (Figure 6D). These experiments show that the point mutation S417G leads to the formation of a trimer that is even more stable than the native protein. When we compared the steady-state levels of both proteins in yeast cells, however, we observed lower amounts of the mutant protein (Figure 6E). Hence it seems that increased stability of a β-barrel structure does not necessarily lead to overall improved biogenesis of such a protein.

DISCUSSION

Mitochondrial β-barrel proteins are synthesized in the cytosol and therefore must bear targeting signals to direct them to the right organelle. Their bacterial counterparts contain an N-terminal signal sequence that mediates their translocation from the bacterial cytoplasm across the inner membrane. This signal shows some similarity to signal sequences that direct eukaryotic proteins to the ER. During evolution mitochondrial β-barrel proteins lost such an extension, and our results show that indeed bacterial PhoE with a signal sequence is assembled in reduced levels into mitochondria as compared to a construct without this extension. The presence of a signal sequence results in a protein with two competing targeting signals, one for the mitochondria (within the β-barrel domain) and one for the ER (signal sequence). Neither of these signals is dominant, resulting in a dual localization of the protein. Those molecules that

reach the mitochondria integrate into the outer membrane in a stable manner. In contrast, we propose a scenario in which the signal sequence directs the other population to the SEC system in the ER, where Sig-PhoE is translocated into the lumen because there is no hydrophobic membrane-spanning segment that stops the translocation. This process is similar to the transport of the protein into the periplasm through the bacterial SEC machinery in the inner membrane (Bos *et al.*, 2007a). Because there is no BAM complex (or eukaryotic equivalent) in the ER, these molecules cannot get assembled into the membrane and remain in the ER lumen. Comparable accumulation of β-barrel precursors is observed in the periplasm of BamA-depleted bacterial cells (Bos *et al.*, 2007a). In the ER lumen, PhoE can become glycosylated and eventually destined for degradation because the yeast cell probably recognizes it as an unfolded, nonfunctional protein. Analogously, unassembled β-barrel precursors are degraded in the bacterial periplasm (Bos *et al.*, 2007a). Taken together, as the signal sequence appears to be counterproductive for the assembly into the mitochondrial outer membrane, these observations provide an experimental explanation for the absence of bacterial-like signal sequences in precursors of modern mitochondrial β-barrel proteins.

Rather than the presence of a linear sequence, it was suggested that the ability of a protein to adopt a membrane-embedded β-barrel-like conformation could be sufficient for its specific targeting to mitochondria (Rapaport, 2003). Recent results supported this hypothesis by demonstrating that bacterial β-barrel proteins, like PhoE, expressed in yeast cells are targeted to mitochondria, although these proteins show no significant sequence similarity with mitochondrial β-barrel proteins (Walther *et al.*, 2009a). To better understand this putative structural signal, we tested if specific targeting to mitochondria requires a complete β-barrel precursor structure or whether even a fragment of such a structure would be sufficient. For this purpose, we used YadA, a member of the class of trimeric autotransporters that is found only in bacteria. These proteins are synthesized in the cytoplasm as monomers and form β-barrel-like trimers with their membrane-embedded, C-terminal domain. Recent work demonstrated that BamA, similarly to its function in the biogenesis of other β-barrel proteins, interacts directly with YadA and is essential for its membrane integration (Lehr *et al.*, 2010).

Our data demonstrate that YadA was exclusively targeted to mitochondria where it formed native trimeric structure. Thus it appears that even fragments of a β-barrel structure are sufficient for the recognition of a β-barrel protein and its correct targeting to mitochondria. The usage of the heterologous expression system can also help to address the yet open question: In which step of the protein biogenesis is the trimeric structure formed? To investigate whether YadA monomers can form a trimeric structure already in the eukaryotic cytosol, we performed cell-free translation experiments using rabbit reticulocyte lysate. Our results suggest that a formation of cytosolic trimer is unlikely because only signals corresponding to monomeric YadA-MA were observed under these conditions (unpublished data).

The finding that YadA-MA is specifically targeted to mitochondria raised this question: Which components of the mitochondrial import machinery are used? The initial interaction between endogenous β-barrel proteins like porin or Tom40 and the general entry gate, the TOM-complex is mediated by Tom20 (Rapaport and Neupert, 1999; Krimmer *et al.*, 2001; Yamano *et al.*, 2008). The same appears to be true for β-barrel proteins of bacterial origin (this study and Walther *et al.*, 2009a), but surprisingly we found that this is not the case for YadA-MA. Similarly, Tom70 is also not required for the import of YadA, and even a slight increase in YadA-MA levels was observed in its absence. Tom70 exposes a large domain on the cytosolic surface of the outer membrane. As this receptor is part of the TOM holo complex, this bulky domain can be in the vicinity of the import pore and thus form a steric hindrance for precursor proteins that are translocated via this pore. Thus, for those proteins that are not recognized by Tom70, the absence of this receptor can even result in a slight improvement of their import efficiency. A similar observation was made by Hines et al. regarding the import of CoxIV-dihydrofolate reductase (DHFR) into mitochondria lacking Tom70 (Hines *et al.*, 1990).

Of note, Tom import receptors are not absolutely required for the translocation in vitro of bona fide mitochondrial precursor proteins. Import can still occur, albeit with low efficiency, after destroying protease-sensitive receptors (Pfaller *et al.*, 1989). The import via this so-called "bypass" route occurs most probably by a direct interaction of the precursor proteins with the Tom40 import pore. Alternatively, Tom22 can function as a secondary receptor and thus might be involved in the recognition of the YadA precursor. The receptor domain of Tom22 was shown recently to be required for the in vitro import of porin. Furthermore, Tom22 and Tom20 were suggested to be involved in the same step or sequential steps in similar import pathways (Yamano *et al.*, 2008). Hence we propose that YadA is recognized on the surface of the organelle either by Tom22 or directly by Tom40. Naturally, these two alternatives are not mutually exclusive.

The finding that the import of YadA-MA is independent of the import receptors could have evolutionary reasons. Whereas the TOB complex is most probably derived from a bacterial translocase, the TOM complex has no bacterial ancestor (Dolezal *et al.*, 2006) and only three of the TOM-complex components (Tom40, Tom7, and Tom22) are commonly found in eukaryotes (Macasev *et al.*, 2004). It is thought that the TOM complex developed on the way of converting the endosymbiont into an organelle. Thus, although it is not clear when trimeric autotransporters emerged, it could be hypothesized that the class of these proteins was lost in early eukaryotes before the development of the primary import receptors (Tom20 and Tom70). In such a scenario, there was never a need for the import receptors to recognize such proteins, and thus import of YadA is independent of the two receptors just mentioned. Astonishingly, the evolutionary origin of mitochondria from bacteria allows the organelle to assemble a class of proteins that are not present in modern eukaryotic organisms.

Upon leaving the TOM complex, YadA is probably exposed in its assembly pathway to the IMS as its overall import efficiency is reduced in cells lacking the small chaperones Tim8/Tim13. This reduction, however, is somewhat less significant as compared to that observed for PhoE. One possible explanation of this difference is the smaller size of hydrophobic elements in YadA as compared to those in PhoE. This proposal is supported by a previous report that larger bacterial β-barrel proteins were more dependent on the presence of all five polypeptide-transport-associated (POTRA) domains of *Neisseria meningitidis* BamA as compared to small β-barrel proteins (Bos *et al.*, 2007b). From the IMS, precursor molecules of YadA-MA are most likely relayed to the TOB complex, and our results clearly show a strong dependence of YadA-MA assembly on the TOB subunits, Tob55 and Mas37. These findings are in accordance with our previous findings for PhoE the import of which into mitochondria is also severely affected by the deletion of Mas37 or the depletion of Tob55 (Walther *et al.*, 2009a). Although both PhoE and YadA-MA can be assembled by the TOB complex, they probably represent suboptimal substrates for this complex. Hence an efficient membrane integration of these

proteins necessitates most likely the presence of a fully functional TOB complex. Therefore in the absence of Mas37, the Tob55-Tob38 subcomplex cannot deal efficiently with bacterial precursors, whereas it can still process mitochondrial β-barrel substrates.

Assembly of mitochondrial β-barrel proteins appears to be facilitated by the presence of a eukaryotic-specific β-signal present in the most C-terminal β-strand (Kutik *et al.*, 2008). Interestingly we found that mutation of Ser-417 to glycine, a mutation that allows the last β-strand of YadA-MA to resemble the eukaryotic β-signal, led to a much higher stability of the trimer. This mutation enhances also the stability of the bacterially expressed trimeric form of full-length YadA (Lehr *et al.*, 2010). Nevertheless, wild-type YadA-MA was present in higher steady-state levels than was the mutant construct. Thus it can be speculated that, although β-signal-like sequences improve the final stability of β-barrel proteins, some structural flexibility is actually an advantage in other stages in the assembly pathway of these proteins, most probably in the integration into the lipid core of the membrane.

In conclusion, our findings shed new light on the biogenesis of mitochondrial β-barrel proteins. They demonstrate that rather than a specific linear sequence, the structural information contained in four β-strands is sufficient for it to be recognized and processed by the mitochondrial import machinery.

MATERIALS AND METHODS

Yeast strains and growth methods

Standard genetic techniques were used for growth and manipulation of yeast strains. The wild-type strains YPH499 and W303 were used. For construction of the *tom20*Δ mutant strain, the *TOM20* gene was deleted by replacement with a *HIS3* gene cassette. The *mas37*Δ and *tim8*Δ*/tim13*Δ strains were described before (Habib *et al.*, 2005, and Paschen *et al.*, 2000, respectively). The *tom70*Δ*/tom71*Δ double deletion strain was a gift from K. Okamoto (Kondo-Okamoto *et al.*, 2008). The *imp1*Δ strain was purchased from Euroscarf (Frankfurt, Germany). For drop-dilution assays, yeast cells were grown to an OD_{600} of 1.0 in synthetic medium and diluted in 10-fold increments, and then 5 μl of each dilution was spotted onto solid medium with different carbon sources. In some experiments, cycloheximide (100 μg/ml) was added to the yeast culture.

Recombinant DNA techniques

Sequences encoding *E. coli* PhoE with or without its signal sequence (first 21 amino-acid residues) were cloned by PCR amplification from a plasmid encoding the full-length protein. An additional N-terminal methionine was added in constructing PhoE without its signal sequence. The PCR products were inserted into the yeast expression vector pYX113, in which the *GAL1* promoter was replaced by the *S. cerevisiae POR1* promoter.

Sequences encoding YadA-MA or YadA-MA-S417G were obtained by PCR amplification using pASK-IBA2 encoding the *Yersinia enterocolitica* YadA as a template. The S417G mutation was introduced by using a reverse primer containing the desired mutation. Both sequences were inserted into either pYX113 or pYX242 vectors using *Eco*RI and *Sal*I restriction sites. For constructing HA-tagged YadA-MA, the 3xHA cassette was PCR amplified from pFA6a-3HA-KanMX4 plasmid and inserted into the target vectors pYX113-GAL1pro-URA or pYX242-TPIpro-LEU using *Eco*RI and *Nco*I restriction sites. For expression of HA-tagged YadA-MA in *E. coli*, we modified our published procedure for expressing Strep-tagged YadA-MA (Wollmann *et al.*, 2006). In short, HA-tagged YadA-MA was PCR-amplified using the yeast expression vector as template. The PCR product was then cloned using the *Bsa*I restriction site into the pASK-IBA2 vector, which already encodes an N-terminal signal sequence derived from the *E. coli* outer membrane protein OmpA.

RT-PCR

Total RNA from yeast was isolated by phenol/chloroform/isoamylalcohol (ratio 25:24:1, vol/vol) extraction and subsequent ethanol precipitation. Isolated RNA (2 μg) was treated with RQ1-DNase (Promega, Madison, WI). The samples were split in half and used for RT-PCR in the presence or absence of RevertAid Premium Reverse Transcriptase (Fermentas, Glen Burnie, MD) using oligo-dT and random hexamer primers. PCR amplification from the cDNA was performed using Taq-Polymerase (Fermentas) and primers specific for *phoE* or *ACT1* (as a control).

Biochemical procedures and computational biology

Mitochondria were isolated from yeast cells by differential centrifugation as described (Daum *et al.*, 1982). Subcellular fractionation was performed according to published procedures (Walther *et al.*, 2009a). Treatment of samples with Endoglycosidase H_f (New England BioLabs, Ipswich, MA) was for 1 h at 37°C according to the manufacturer's recommendations and in the presence of a cocktail of protease inhibitors (Roche, Basel, Switzerland). Radiolabeled YadA-MA was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Perkin-Elmer, Rodau, Germany) after in vitro transcription by SP6 polymerase from pGEM4 vectors (Promega). A monomerization assay was performed by resuspending 50 μg of isolated mitochondria in sample buffer containing 8 M urea. Samples were then boiled at 95°C for various time periods before their analysis by SDS–PAGE.

Three-dimensional models of YadA-MA were produced using the Swiss PDB Viewer in combination with Persistence of Vision Raytracer (PovRay) rendering software, based on published model coordinates (Grosskinsky *et al.*, 2007).

ACKNOWLEDGMENTS

We thank K. Rehn and E. Kracker for technical support. This work was supported by the Deutsche Forschungsgemeinschaft (RA 1048/4–1 to D.R., SFB766/B1 to I.A., and SFB766/B4 to D.L.), the Evolution and Ecology Forum Tübingen (to J.E.N.M.), a postdoctoral fellowship from the Carl Zeiss Stiftung (to K.S.D.), and the Max Planck Society (to D.L. and I.G.).

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