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review

Multiple pathways for sorting mitochondrial precursor proteins

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Mitochondria import hundreds of different precursor proteins from the cytosol. More than 50% of mitochondrial proteins do not use the classical import pathway that is guided by amino-terminal presequences, but instead contain different types of internal targeting signals. Recent studies have revealed an unexpected complexity of the mitochondrial protein import machinery and have led to the discovery of new transport pathways. Here, we review the versatility of mitochondrial protein import and its connection to mitochondrial morphology, redox regulation and energetics.

Keywords: MIA; PAM; SAM; TIM; TOM

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Introduction

Mitochondria are dynamic organelles that have a crucial role in cellular energy conversion, metabolism of amino acids, lipids and iron–sulphur clusters, and the regulation of apoptosis. It has been assessed that the mitochondria of baker's yeast contain approximately 1,000 proteins (Sickmann *et al*, 2003; Prokisch *et al*, 2004; Reinders *et al*, 2006). Only few of them are encoded by the mitochondrial genome, whereas 99% of mitochondrial proteins are encoded by nuclear genes and are synthesized as precursors on cytosolic ribosomes.

The cytosolic precursor proteins are recognized by receptors on the mitochondrial surface and are imported by the general entry gate, the preprotein translocase of the outer membrane (TOM complex; Fig 1). Recent studies have revealed a surprising variety of import mechanisms and machineries. At least four classes of precursor proteins exist that contain distinct targeting signals and are directed to different import routes. Here, we review the rapid development of the mitochondrial import field and its connection to areas that so far were assumed not to be directly related to protein import, including redox regulation, respiratory chain and mitochondrial morphology.

Mitochondrial proteome and new import components

In 2001, the mitochondrial import field seemed to have reached a mature state in which two main protein import pathways—the presequence pathway and the carrier pathway (Fig 1)—were believed to be well characterized. It was assumed that most of the critical import components had already been identified. A first surprise came in 2002 when a new component of the presequence translocase of the inner membrane (TIM23 complex) was identified and shown to be essential for protein import and cell viability (Geissler *et al*, 2002; Yamamoto *et al*, 2002). Was this just one component that had been overlooked, or the tip of an iceberg? The latter assumption turned out to be correct; not only were numerous new components of the presequence pathway identified, but also two new import pathways, the sorting and assembly machinery (SAM) of the outer membrane and the machinery for intermembrane space import and assembly (MIA; Table 1).

There are three main reasons why many new import components were identified. First, mild protocols for the purification of translocase complexes were developed (Geissler *et al*, 2002); thus, loosely associated subunits were co-purified and identified by mass spectrometry. Second, native gel systems for monitoring import intermediates were established, leading to the identification of new import stages of precursor proteins (Model *et al*, 2001; Wiedemann *et al*, 2003; Chacinska *et al*, 2004). This technology, in combination with the systematic analysis of yeast mutants, drove the discovery of new protein sorting machineries. Third, a comprehensive proteomic analysis revealed a large number of new mitochondrial proteins, including proteins of unknown function that are essential for cell viability (Sickmann *et al*, 2003; Prokisch *et al*, 2004; Reinders *et al*, 2006). The characterization of these essential proteins yielded numerous new import components.

Thus, a combination of proteomics, yeast genetics and native techniques for protein analysis was responsible for the boost in identification of new import components, and greatly expanded our view of mitochondrial import, leading to the characterization of four mitochondrial import pathways that will be discussed in the following paragraphs.

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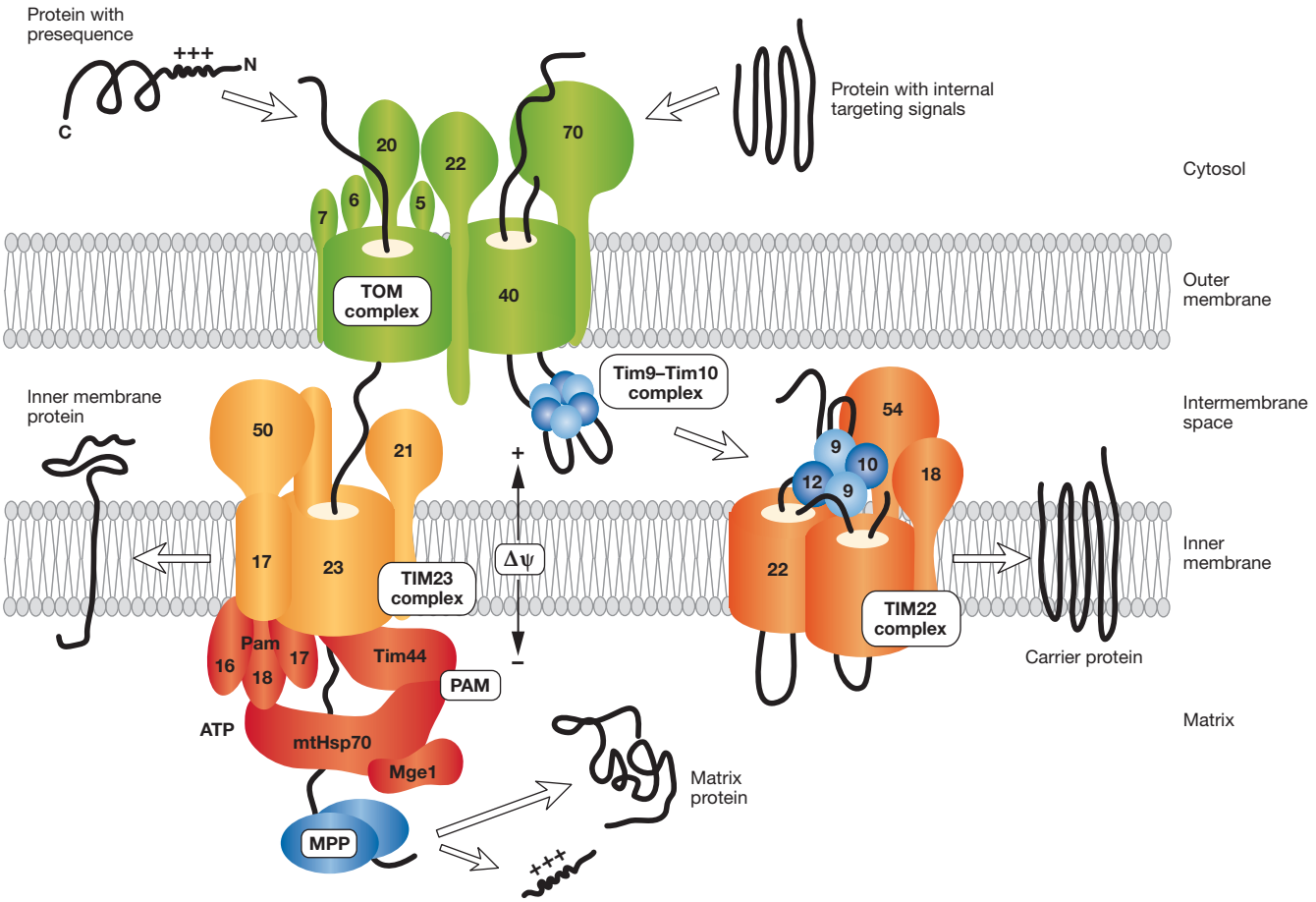

Fig 1 | Two main protein import pathways of mitochondria. Presequences direct proteins through the TOM complex, TIM23 complex and motor PAM to the matrix; the mitochondrial processing peptidase (MPP) removes the presequences. Cleavable inner membrane proteins are laterally released from the TIM23 complex. Carrier precursors with internal targeting signals are recognized by the receptor Tom70, and translocated by the TOM complex and the Tim9–Tim10 chaperone of the intermembrane space. The TIM22 complex promotes insertion of carrier proteins into the inner membrane. MtHsp70, matrix heat shock protein 70; PAM, presequence translocase-associated motor; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

Presequence pathway to matrix and inner membrane

Most mitochondrial matrix proteins are synthesized in the cytosol with a characteristic amino-terminal presequence that forms a positively charged amphipathic α-helix. Presequences direct the proteins to the mitochondria and across both outer and inner membranes into the matrix where they are usually cleaved off by the mitochondrial processing peptidase (MPP; Fig 1).

On recognition by the receptors Tom20 and Tom22, the preproteins are translocated through the channel formed by Tom40 (Abe *et al*, 2000; Becker *et al*, 2005). It has long been debated whether and how the TOM and TIM23 complexes cooperate. Two recently identified proteins with hydrophilic domains in the intermembrane space, Tim50 and Tim21, were found to participate in preprotein transfer from TOM to TIM23 (Geissler *et al*, 2002; Yamamoto *et al*, 2002; Chacinska *et al*, 2005). Tim50 binds to the protein emerging at the *trans* side of the TOM channel and promotes binding of the presequence to the intermembrane space domain of Tom22. Tim21 connects TOM and TIM complexes by direct but transient binding to Tom22. Remarkably, it was shown that Tim21 competes with presequences for binding to Tom22 (Chacinska *et al*, 2005; Albrecht *et al*, 2006). Therefore, as the Tim50-stimulated binding of preproteins to Tom22 does not require Tim21 (Chacinska *et al*, 2005), we propose that Tim21 functions in the subsequent step of preprotein release from Tom22. Owing to its competition with presequences, Tim21 binding to Tom22 is thought to promote release of the preprotein.

Two energy sources drive preprotein translocation across the inner membrane—the electrochemical potential gradient and ATP. The membrane potential Δψ activates the channel-forming protein Tim23 and exerts an electrophoretic effect on the positively charged presequences (Truscott *et al*, 2001; Krayl *et al*, 2007). In addition to its role in cooperation with the TOM machinery, Tim50 has an essential function in regulating the inner membrane barrier. The electrochemical proton gradient generated by the respiratory chain of the inner membrane is crucial for the synthesis of the bulk of cellular ATP by the mitochondrial ATP synthase. Therefore, the inner membrane forms a highly specific barrier to prevent an unspecific leakage of ions. However, Tim23 forms

Table 1 | Components of the mitochondrial protein import machinery*

Name	Systematic yeast name	Aliases	Function/description
Translocase of the outer membrane (TOM complex)			
Tom70	YNL121C	Mas70, Mom72, Omp1	Receptor for non-cleavable precursor proteins
Tom40	YMR203W	Isp42, Mom38	General import pore
Tom22	YNL131W	Mas17, Mas22, Mom22	Central receptor
Tom20	YGR082W	Mas20, Mom19, Pom23, Rir16	Presequence receptor
Tom7	YNL070W	Mom7, Yok22	Stability/assembly factor
Tom6	YOR045W	Isp6, Mom8b	Stability/assembly factor
Tom5	YPR133W-A	Mom8a	Preprotein transfer and assembly factor
Sorting and assembly machinery (SAM) of the outer membrane			
Sam50	YNL026W	Omp85, Tob55	Conserved core component of SAM complex
Sam37	YMR060C	Mas37, Pet3027, Tom37	Subunit of SAM complex
Sam35	YHR083W	Fmp20, Tob38, Tom38	Subunit of SAM complex
Mdm10	YAL010C	Fun37	Cooperates with SAM complex and Mdm12/Mmm1
Mdm12	YOL009C	–	Component of β-barrel assembly pathway
Mmm1	YLL006W	Yme6	Component of β-barrel assembly pathway
Mim1	YOL026C	Tom13	Involved in outer membrane protein biogenesis
Chaperones of intermembrane space (small TIM proteins)			
Tim13	YGR181W	–	Subunit of Tim8–Tim13 chaperone complex
Tim10	YHR005C-A	Mrs11	Subunit of Tim9–Tim10 chaperone complex and TIM22 complex
Tim9	YEL020W-A	–	Subunit of Tim9–Tim10 chaperone complex and TIM22 complex
Tim8	YJR135W-A	DDP1	Subunit of Tim8–Tim13 chaperone complex
Mitochondrial intermembrane space import and assembly machinery (MIA)			
Mia40	YKL195W	Tim40, Fmp15	Intermembrane space receptor for small cysteine-containing precursor proteins
Erv1	YGR029W	–	Sulphydryl oxidase, cooperates with Mia40
Hot13	YKL084W	–	Assembly factor
Carrier translocase of inner membrane (TIM22 complex)			
Tim54	YJL054W	–	Subunit of TIM22 complex
Tim22	YDL217C	–	Central, channel-forming subunit of TIM22 complex
Tim18	YOR297C	–	Subunit of TIM22 complex
Tim12	YBR091C	Mrs5	Peripheral subunit of TIM22 complex, associates with Tim9 and Tim10
Presequence translocase of inner membrane (TIM23 complex)			
Tim50	YPL063W	–	Intermembrane space-exposed subunit of TIM23 complex
Tim23	YNR017W	Mas6, Mim23, Mpi3	Channel-forming subunit of TIM23 complex
Tim21	YGR033C	Fmp17	Interacts with TOM complex and respiratory chain
Tim17	YJL143W	Mim17, Mpi2, Sms1	Regulator of Tim23 channel, sorting of preproteins
Presequence translocase-associated motor (PAM)			
mtHsp70	YJR045C	Ens1, Ssc1	Molecular chaperone, core of PAM
Tim44	YIL022W	Isp45, Mim44, Mpi1	Binding partner of mtHsp70 at inner membrane
Pam18	YLR008C	Tim14	J-protein at inner membrane, complex with Pam16
Pam17	YKR065C	Fmp18	Organizer of Pam18–Pam16 module
Pam16	YJL104W	Mia1, Tim16	J-related regulator of Pam18
Mge1	YOR232W	mtGrpE, Yge1	Nucleotide exchange factor

*Processing enzymes are not listed.

a large channel that can transport precursor polypeptides in α-helical conformation. In fact, reconstitution experiments with purified Tim23 also showed that the channel was mainly in an open state in the absence of preproteins (Truscott *et al*, 2001). As an isolated mitochondrion contains approximately 300 Tim23 molecules, the presence of this channel in an open state would be deleterious to mitochondria by causing a breakdown of the proton gradient. A systematic analysis of the partner proteins of Tim23 revealed that the intermembrane space domain of Tim50 binds to Tim23 and promotes closing of the channel in the absence of preproteins, thus preventing a leakage of ions (Meinecke *et al*, 2006). When a preprotein arrives in the intermembrane space, Tim50 interacts with the preprotein, leading to a conformational change and opening of the channel.

ATP drives the central component of the presequence translocase-associated motor (PAM), the matrix heat shock protein 70 (mtHsp70). MtHsp70 binds to the unfolded preprotein in transit and—in cooperation with membrane-associated co-chaperones—drives the preprotein into the matrix. There are two current hypotheses for the mechanism of action of mtHsp70: trapping of preproteins by a Brownian ratchet that prevents back-sliding of the polypeptide chain, or active pulling (force-generation) by a motor. Experimental and theoretical evidence for both models has been presented and, in addition, a contribution of the membrane potential to the import-driving force has been discussed (Geissler *et al*, 2001; Okamoto *et al*, 2002; Shariff *et al*, 2004; D'Silva *et al*, 2005; Sato *et al*, 2005; Krayl *et al*, 2007). Both models agree that mtHsp70 binds to the inner membrane through Tim44 in an ATP-regulated manner, and not only promotes translocation of the preproteins into the matrix, but is also required for driving the unfolding of preprotein domains that are still located on the cytosolic side. The identification of three new membrane-bound motor components—Pam16, Pam17 and Pam18—revealed that the import motor consists of three modules: a nucleotide-sensitive mtHsp70–Tim44 complex, a regulatory Pam18–Pam16 complex and Pam17 (Li *et al*, 2004; D'Silva *et al*, 2005; van der Laan *et al*, 2005; Mokranjac *et al*, 2006). Pam18 is a DNAJ-like protein that stimulates the ATPase activity of mtHsp70, whereas Pam16 controls the activity of Pam18; Pam17 is needed for the assembly of the Pam18–Pam16 module. These findings suggest that the import motor is a complex machinery containing four membrane-bound co-chaperones, Tim44 and the three Pam proteins, which associate with the TIM23 complex to coordinate the function of mtHsp70 exactly where the preprotein emerges from the Tim23 channel. A mutant form of mtHsp70—which is impaired in binding to the inner membrane, but still able to bind to substrate—is competent in the trapping of unfolded preproteins and driving their import, whereas preproteins with tightly folded domains cannot be imported (Geissler *et al*, 2001). We suggest that this mtHsp70 is able to perform a basic trapping function, whereas the generation of force that unfolds preprotein domains requires an efficient cooperation with the membrane-bound co-chaperones. Although further experimental studies are needed to clarify the molecular mechanism of the mitochondrial import motor, it is tempting to speculate that the integration of mtHsp70 into the multi-subunit PAM machinery leads to a multistep motor that performs both suggested functions, that is, the trapping and pulling of preproteins.

Several integral inner membrane proteins are synthesized with an N-terminal presequence followed by a hydrophobic sorting signal. The sorting signal arrests translocation in the inner membrane and induces lateral release of the protein into the lipid phase (Fig 1). Some intermembrane space proteins are also imported through the presequence route. Those preproteins are cleaved in two steps, by MPP and by inner membrane proteases that remove the sorting signal. A form of the TIM23 complex without mtHsp70 is responsible for the sorting of preproteins into the inner membrane, a process that involves Tim17 and Tim21 (Chacinska *et al*, 2005). Tim17 promotes lateral release from the Tim23 channel, whereas Tim21 performs a remarkable role: it alternates between binding to TOM and a supercomplex of the respiratory chain complexes III and IV. The direct interaction between the TIM23 complex and the respiratory chain stimulates the membrane potential-dependent step of preprotein sorting under conditions in which the overall electrochemical potential of the inner membrane is reduced, for example, when food supply is limited (van der Laan *et al*, 2006). Future studies are needed to elucidate the molecular mechanism of the TIM23–respiratory chain cooperation. At least two mechanisms are conceivable: protein complexes in the direct vicinity of proton pumping complexes might experience a higher proton-motive force, or protons might be transferred directly from the respiratory chain to the TIM23 complex.

Carrier pathway to inner membrane

Metabolite carriers such as the ADP/ATP carrier and the phosphate carrier form a large class of inner membrane proteins. They contain six transmembrane segments and are usually synthesized without cleavable presequence but contain internal targeting signals. Cytosolic chaperones guide the proteins to the receptor Tom70 (Wiedemann *et al*, 2001; Young *et al*, 2003). With the help of other Tom receptors, the carrier precursor is translocated through the Tom40 channel. Thus, presequence-carrying preproteins and carrier precursors are imported through the same channel; however, presequence-carrying proteins are translocated as unfolded linear polypeptide chains, whereas carrier precursors cross the outer membrane in a loop formation (Wiedemann *et al*, 2001; Fig 1).

Chaperone complexes in the intermembrane space—the hexameric Tim9–Tim10 and Tim8–Tim13 complexes—bind to the precursors of carrier proteins and other multi-spanning inner membrane proteins, and guide them through the aqueous space between both mitochondrial membranes (Curran *et al*, 2002; Vial *et al*, 2002; Webb *et al*, 2006; Davis *et al*, 2007; Fig 1). The small Tim proteins dock to the inner membrane-integrated carrier translocase (TIM22 complex). A modified chaperone complex—which contains Tim12 in addition to Tim9 and Tim10—is located at the outer surface of the carrier translocase. The core of the translocase contains the channel-forming Tim22, which inserts the precursors into the inner membrane in a membrane potential-dependent manner (Rehling *et al*, 2003). The exact functions of Tim54 and Tim18—two additional integral membrane proteins associated with Tim22—are not yet known. The problem of keeping the inner membrane channel closed in the absence of precursor proteins—outlined above for Tim23—also applies to the Tim22 channel. We speculate that Tim54, with its large intermembrane space domain, would be an ideal candidate to perform a function analogous to that of Tim50, which is keeping the channel closed in the absence of precursor proteins.

Intermembrane space import and assembly

The mitochondrial intermembrane space contains numerous proteins with a molecular mass less than 20 kDa and characteristic cysteine motifs. Many cysteines are involved in the formation of disulphide bonds, indicating that the intermembrane space is an oxidative environment. It was initially assumed that the small proteins simply

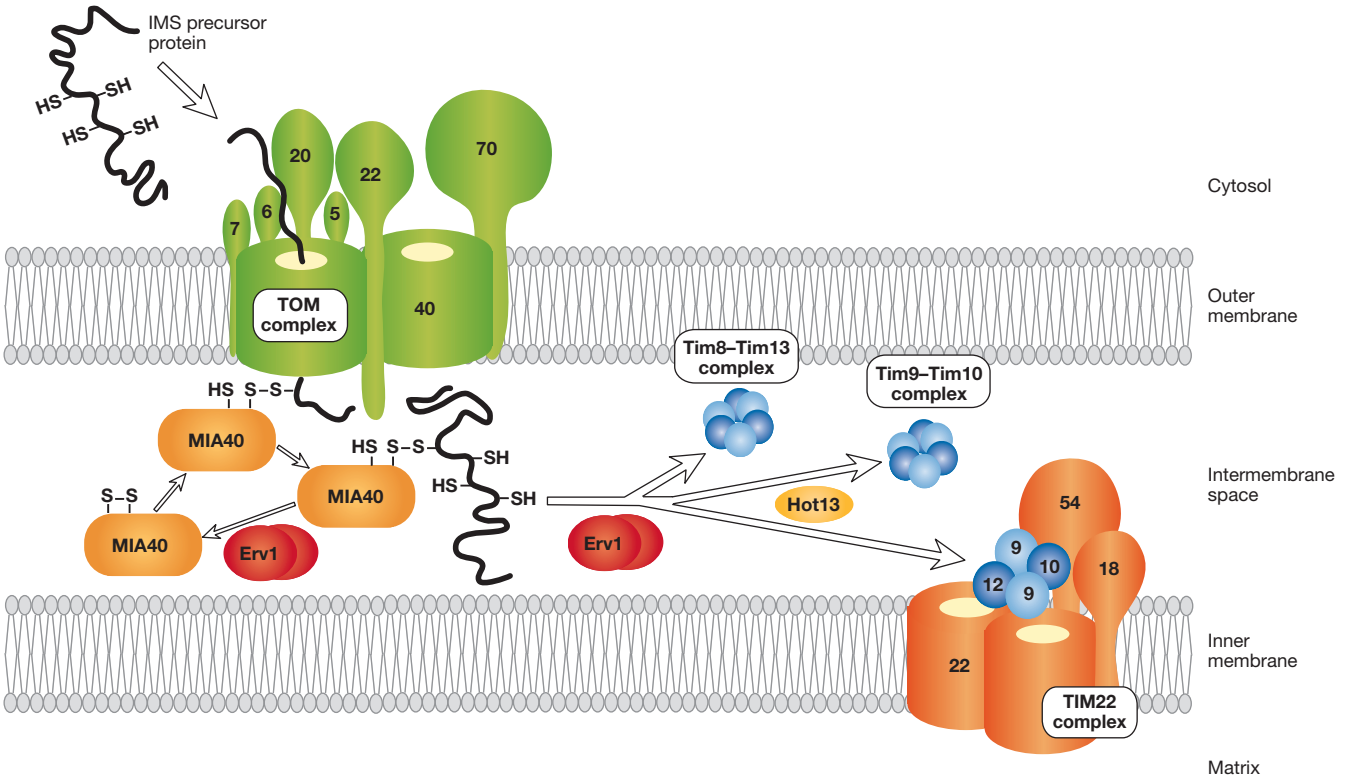

Fig 2 | Mitochondrial intermembrane space import and assembly machinery. Precursors of small intermembrane space (IMS) proteins are translocated through the TOM complex and bound by Mia40 through disulphide bonds. The sulphydryl oxidase Erv1 cooperates with Mia40 in the oxidation of precursor proteins and their assembly into oligomeric complexes. Further factors such as Hot13 support assembly of the protein complexes. Erv1, Essential for respiration and viability 1; Hot13, Helper of TIM13; Mia40, mitochondrial intermembrane space import and assembly; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

diffuse through the TOM channel and are trapped in the intermembrane space by folding to their active form. However, systematic analysis of new essential proteins found in the mitochondrial proteome (Sickmann *et al*, 2003) led to the identification of specific protein import and assembly machinery (MIA; Chacinska *et al*, 2004; Naoé *et al*, 2004). The central component, Mia40, contains characteristic cysteine motifs. Mia40 specifically recognizes the incoming precursor proteins and binds to them through mixed disulphide bonds (Milenkovic *et al*, 2007; Sideris & Tokatlidis, 2007; Fig 2). Mia40 cooperates with the sulphydryl oxidase Erv1 (Essential for respiration and viability 1) in transferring disulphide bonds to precursor proteins, and Erv1 is thought to reoxidize Mia40 (Fig 2). As formation of disulphide bonds requires the removal of electrons, the current model suggests a disulphide relay with electrons being transferred from the precursor protein to Erv1 through Mia40, and from here through cytochrome *c* to the respiratory chain (Allen *et al*, 2005; Mesecke *et al*, 2005; Rissler *et al*, 2005).

Mia40 promotes the sequential formation of intramolecular disulphides in the imported proteins (Milenkovic *et al*, 2007; Sideris & Tokatlidis, 2007) and thus initiates the assembly of imported proteins into oligomeric complexes. Typical examples of this are the hexameric chaperone complexes formed by small Tim proteins. Whether or not Erv1 has further roles, in addition to the reoxidation of Mia40, is now a matter of debate. Studies with yeast mutants suggested that Erv1 was also required for the release of precursor proteins from Mia40, but it is unknown whether this involves a direct interaction of Erv1 with the precursors (Rissler *et al*, 2005). Moreover, further factors—such as the cysteine-rich protein Helper of TIM13 (Hot13; Curran *et al*, 2004)—might have a role in the late steps of assembly of intermembrane space complexes (Fig 2). An alternative view suggests that the oxidation of substrate proteins by Mia40 is sufficient for their assembly into native complexes without a requirement for further mitochondrial factors (Sideris & Tokatlidis, 2007). On the other hand, it has been shown that Erv1 is involved in the maturation of cytosolic iron through sulphur proteins (Lange *et al*, 2001), although whether Erv1 directly participates in the biogenesis of iron–sulphur clusters or if it acts indirectly—for example through Mia40 and the biogenesis of intermembrane space proteins—is still unknown.

Outer membrane sorting and assembly machinery

Although membrane proteins are usually anchored in the lipid phase by transmembrane α-helices, the outer membranes of mitochondria, as well as those of chloroplasts and Gram-negative bacteria, contain a special type of integral membrane proteins, the β-barrel proteins (Schleiff & Soll, 2005). The most abundant mitochondrial outer membrane protein, porin, and the import channel Tom40 are β-barrel proteins. Like all other mitochondrial outer membrane proteins, β-barrel proteins are synthesized in the cytosol. The dissection of their import

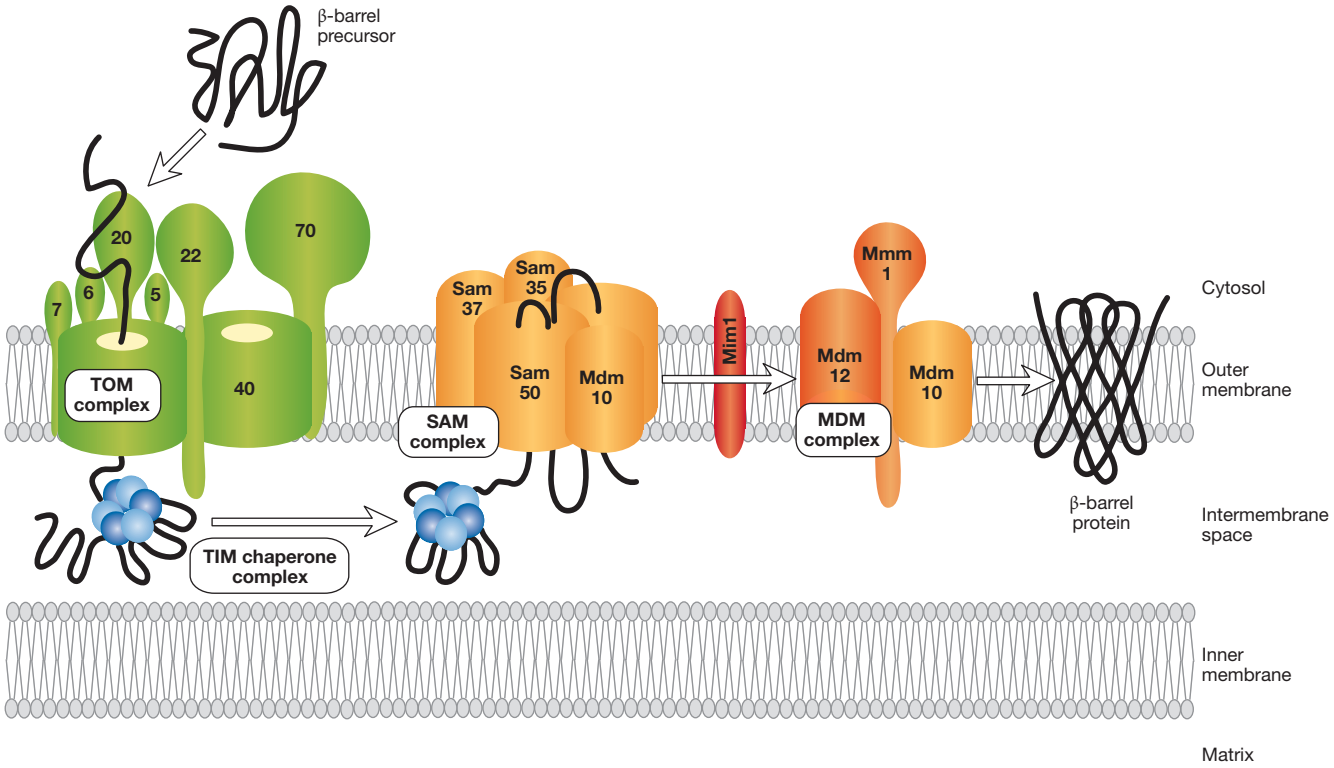

Fig 3 | Sorting and assembly machinery of the outer mitochondrial membrane. The precursors of β-barrel proteins are initially imported through the TOM complex, interact with small TIM chaperones (Tim9–Tim10 complex, Tim8–Tim13 complex) in the intermembrane space, and are inserted into the outer membrane by the SAM complex. Other outer membrane proteins—the MDM complex and Mim1—support assembly of β-barrel proteins. Mdm, mitochondrial distribution and morphology; Mim1, mitchondrial import 1; Mmm1, maintenance of mitochondrial morphology 1; SAM, sorting and assembly machinery; TIM, translocase of the inner memebrane; TOM, translocase of the outer membrane.

pathway by native gel analysis revealed a new protein sorting machinery, the SAM complex (Model *et al*, 2001; Wiedemann *et al*, 2003; Fig 3). The precursors are first transported through the TOM complex to the *trans* side of the outer membrane, where the TIM chaperone complexes guide the hydrophobic precursors to the SAM complex (Hoppins & Nargang, 2004; Wiedemann *et al*, 2004). Sam50, the central component, is homologous to the bacterial outer membrane protein Omp85; therefore, the basic mechanism of β-barrel insertion into the outer membrane has been conserved from bacteria to humans (Kozjak *et al*, 2003; Paschen *et al*, 2003; Gentle *et al*, 2004).

Several partner proteins of Sam50—Sam35, Sam37 and Mdm10 (Mitochondrial distribution and morphology 10)—help in membrane insertion and assembly of the precursors. Of particular interest is Mdm10, initially identified for its role in the maintenance of mitochondrial morphology, but recently found to have a specific role in TOM assembly (Meisinger *et al*, 2004). Mdm10 not only interacts with SAM, but also forms a second complex with two other morphology proteins, Mdm12 and Mmm1 (Maintenance of mitochondrial morphology 1; Fig 3). This MDM complex functions in the assembly pathway of β-barrel proteins downstream of the SAM complex (Meisinger *et al*, 2007). Therefore, these studies connected two apparently distinct areas, protein assembly and mitochondrial morphology. We propose that these MDM proteins function primarily in protein sorting and that their influence on mitochondrial morphology is indirectly mediated by their role in the import of 'true' morphology components. Most proteins required for mitochondrial fusion and fission are not involved in the assembly of mitochondrial outer membrane proteins (Meisinger *et al*, 2007); however, three other morphology proteins—the outer membrane Mmm2 and the inner membrane Mdm31 and Mdm32—show a strong genetic interaction with the subunits of the MDM complex (double mutants are synthetic lethals) and lead to the same morphological phenotype on deletion (Youngman *et al*, 2004; Dimmer *et al*, 2005). Mdm31 and Mdm32 expose large domains to the intermembrane space and thus their involvement in the biogenesis pathway of outer membrane proteins is possible. Future studies will have to address if Mmm2, Mdm31 and Mdm32 represent functional elements of the β-barrel assembly pathway of mitochondria.

Perspectives

The identification of new mitochondrial protein import machineries raises many questions that will be the subject of future research, such as the molecular mechanism of redox-regulation of the MIA pathway, the energetics of protein insertion into the outer membrane and the exact function of different morphology proteins. What is the role of the recently identified Mim1 (mitochondrial import 1; Ishikawa *et al*, 2004)? Which pathways direct

α-helical proteins into the outer membrane (Setoguchi *et al*, 2006)? The relation of protein translocases to the release of intermembrane space factors in apoptosis is also an area in which intensive research is being conducted.

Our view of the presequence pathway into the matrix was also changed substantially by demonstration of a direct but transient cooperation of the TOM and TIM23 complexes. The identification of several new motor subunits added a further level of complexity to the ongoing debate on whether mtHsp70 functions by pulling or trapping, although a final clarification of the molecular mechanism has not been achieved so far. The newly found association between the TIM23 complex and the respiratory chain raises detailed biophysical questions on how the translocase makes use of the energy provided by proton-pumping complexes. An important advance towards a molecular understanding of mitochondrial protein sorting would be to solve high-resolution structures of the membrane-integrated translocases.

The regulation of protein assembly—in particular that of cytosolically and mitochondrially synthesized subunits of the respiratory chain—is a challenging topic. As the mitochondrial proteome revealed many protein kinases and phosphatases (Sickmann *et al*, 2003; Reinders *et al*, 2006), we anticipate that many more mitochondrial functions will be regulated by reversible phosphorylation, and protein biogenesis could be an important one. Finally, we have only limited information on the transfer of precursor proteins from ribosomes to the mitochondrial surface, and a possible coupling of protein synthesis to protein translocation is still being discussed.

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