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Review

Mitochondrial protein import: Common principles and physiological networks ☆

Jan Dudek [a], Peter Rehling [a,b,*], Martin van der Laan [c,d,**]

[a] Abteilung Biochemie II, Universität Göttingen, 37073 Göttingen, Germany
[b] Max-Planck-Institut für biophysikalische Chemie, 37077 Göttingen, Germany
[c] Institut für Biochemie und Molekularbiologie, ZBMZ, Universität Freiburg, 79104 Freiburg, Germany
[d] BIOSS Centre for Biological Signalling Studies, Universität Freiburg, 79104 Freiburg, Germany

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ABSTRACT

Most mitochondrial proteins are encoded in the nucleus. They are synthesized as precursor forms in the cytosol and must be imported into mitochondria with the help of different protein translocases. Distinct import signals within precursors direct each protein to the mitochondrial surface and subsequently onto specific transport routes to its final destination within these organelles. In this review we highlight common principles of mitochondrial protein import and address different mechanisms of protein integration into mitochondrial membranes. Over the last years it has become clear that mitochondrial protein translocases are not independently operating units, but in fact closely cooperate with each other. We discuss recent studies that indicate how the pathways for mitochondrial protein biogenesis are embedded into a functional network of various other physiological processes, such as energy metabolism, signal transduction, and maintenance of mitochondrial morphology. This article is part of a Special Issue entitled: Protein Import and Quality Control in Mitochondria and Plastids.

1. Introduction

Mitochondria are ubiquitous organelles surrounded by two membranes, the outer and the inner membrane, which confine two aqueous compartments, the matrix and the intermembrane space (IMS). Tubular invaginations of the inner mitochondrial membrane form the cristae, which harbor the enzyme complexes of the oxidative phosphorylation system. In addition to their central role in ATP synthesis mitochondria accommodate central metabolic pathways, like the Krebs cycle and the β-oxidation of fatty acids. They provide cells with a large number of metabolites, such as amino acids and steroids, and are involved in the formation of heme and iron-sulfur clusters. Based on proteomic analyses it has been estimated that mitochondria contain ~1500 different proteins in mammals and ~1000 different proteins in yeast [1–3]. Because of their endosymbiotic origin mitochondria still contain their own small genome encoding for a limited amount of proteins that are mostly subunits of respiratory chain complexes and the F_1F_o-ATPase synthase. Thus, nearly all mitochondrial proteins are encoded by nuclear genes, synthesized as precursor forms on cytosolic ribosomes and subsequently transported into the organelle. Our knowledge on the general principles of protein import into mitochondria mainly originates from genetic and biochemical studies with the model organism baker's yeast (*Saccharomyces cerevisiae*). Most of the mechanisms described in the following were initially discovered in *S. cerevisiae*, but the vast majority of the protein machineries involved were later found to be highly conserved in higher eukaryotes (Fig. 1). It is widely accepted that import of precursor proteins into mitochondria generally occurs in a post-translational manner. For some proteins, like Sod2 or fumarase, however, there are clear indications for a co-translational import mechanism [4,5]. Cytosolic ribosomes translating mRNAs for mitochondrial precursor proteins have been found in proximity to the outer mitochondrial membrane [6–8]. Specific signals within both the 3′ untranslated and the coding regions of these mRNAs have been shown to mediate their targeting [9–12]. In yeast, recruitment of mRNAs to the mitochondrial surface involves the Puf3 protein and the outer membrane precursor protein receptors Tom20 and Tom70 [13–16].

Precursor protein targeting to mitochondria and sorting to distinct mitochondrial subcompartments requires the presence of specific import signals within the transported polypeptides (Fig. 1). The most frequently found mitochondrial import signal is an N-terminal extension termed presequence. These presequences are amphipathic α-helical segments with a net positive charge and show a prevalent length distribution of 15 to 55 amino acids [17]. In general, N-terminal presequences are proteolytically removed after import by the mitochondrial processing peptidase and other proteases [18,19]. An interesting exception is the helicase Hmi1, which is channeled into mitochondria by a presequence-like structure at its C-terminus [20]. Often less well defined internal signal

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* Correspondence to: P. Rehling, Abteilung Biochemie II, Universität Göttingen, 37073 Göttingen, Germany. Tel.: +49 551 39 5947; fax: +49 551 39 5979.
** Correspondence to: M. van der Laan, Institut für Biochemie und Molekularbiologie, ZBMZ, Universität Freiburg, 79104 Freiburg, Germany. Tel.: +49 761 203 5270; fax: +49 761 203 5261.
E-mail addresses: peter.rehling@medizin.uni-goettingen.de (P. Rehling), martin.van.der.laan@biochemie.uni-freiburg.de (M. van der Laan).

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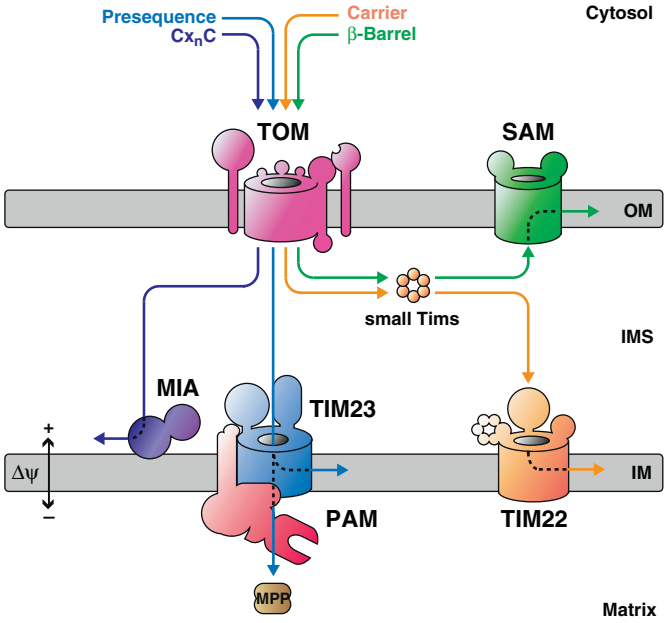

Fig. 1. Different targeting signals direct nuclear encoded precursor proteins on specific transport routes to their final localization within mitochondria. After translocation of precursors through the general translocase of the outer membrane (TOM complex), distinct downstream import pathway diverge in the intermembrane space (IMS): Biogenesis of β-barrel proteins of the outer membrane (OM) requires the small Tim chaperones of the IMS and the sorting and assembly machinery (SAM). Proteins of the IMS that contain cysteine-rich signals (Cx_nC) are imported via the mitochondrial intermembrane space import and assembly (MIA) pathway. Carrier proteins of the inner membrane (IM) are transported with the help of the small Tims and the translocase of the inner membrane 22 (TIM22 complex). Presequence-containing proteins are inserted into the inner membrane or imported into the matrix by the translocase of the inner membrane 23 (TIM23 complex; presequence translocase). Matrix translocation requires the activity of the presequence translocase-associated import motor (PAM). Presequences are proteolytically removed by the mitochondrial processing peptidase (MPP) upon import. Δψ, membrane potential across the inner mitochondrial membrane.

sequences direct the transport of other mitochondrial proteins. Different forms of these internal import signals target the precursors to diverse destinations within mitochondria and will be further discussed in the respective sections of this review.

To prevent misfolding and aggregation, hydrophobic segments of mitochondrial precursor proteins are shielded from the aqueous cytosolic milieu by dedicated chaperones that escort them to the organelle's surface [21–23]. The outer membrane receptors Tom20 and Tom70 serve as initial docking sites for precursor proteins and function as quality control checkpoints, which only permit access to mitochondria, if a given protein contains an appropriate targeting signal. Except for some α-helical outer membrane proteins, virtually all precursors initially enter mitochondria by passing a common entry gate formed by the Translocase of the Outer Membrane (TOM complex). Upon translocation across the outer membrane different transport routes exist that are specifically required for transport of a subset of precursor proteins to their final destination (Fig. 1). Many IMS proteins are imported by the Mitochondrial Intermembrane space import and Assembly (MIA) machinery, which couples sorting of client proteins to their oxidative folding through a disulfide relay mechanism. Outer membrane β-barrel proteins are bound by small Tim chaperones of the IMS that transfer precursors to the Sorting and Assembly Machinery (SAM), where they are integrated into the outer membrane. Mitochondrial metabolite carrier proteins are also guided by small Tim chaperones through the IMS and subsequently integrated into the inner membrane by the Translocase of the Inner Membrane 22 (TIM22 complex) in a membrane potential (Δψ)-driven manner. Presequence-containing precursor proteins are directly passed on from the TOM complex to the Translocase of the Inner Membrane 23 (TIM23 complex; presequence translocase) without the need for soluble IMS chaperones. Dependent on the presence or absence of additional import signals, the TIM23 complex mediates either the translocation of presequence-carrying precursors into the matrix or their lateral sorting into the inner membrane. Whereas lateral membrane integration depends on Δψ as the sole energy source, complete import into the matrix additionally requires the ATP-driven Presequence translocase-Associated import Motor (PAM).

2. Protein translocation across the outer membrane: the TOM complex

The TOM complex is a particularly fascinating protein translocase, as it mediates the transport of various different types of precursors with highly diverse import signals across the outer membrane and then selectively distributes them to multiple downstream protein sorting machineries. Tom40, the central component of the TOM complex, is integrated into the outer membrane in a β-barrel conformation and forms aqueous pores, through which mitochondrial precursor proteins pass [24–26]. Additional subunits support or modulate the quaternary structure of the TOM complex and/or function as receptors: Tom20, Tom70/Tom71, Tom22, and the small Tom proteins Tom5, Tom6 and Tom7. The primary receptors Tom20 and Tom70/Tom71 selectively bind to different subsets of mitochondrial precursor proteins [27]. Tom20 mainly recognizes N-terminal presequences by binding the hydrophobic face of their amphipathic α-helical conformation [28,29]. Precursor proteins with hydrophobic internal targeting signals are preferentially bound by Tom70 and Tom71 [30,31]. Tom70 and Tom71 have high sequence homology and overlapping functions, however Tom71 is expressed only in small amounts [32–34]. Apart from their role in protein import, the Tom70 and Tom71 receptors have been suggested to participate in mitochondrial morphology maintenance by recruiting the morphogenesis factor Mfb1 to the organellar surface [35]. The central receptor Tom22 is critical for the integrity of the TOM complex and exposes presequence binding domains to both the cytosol and the IMS [36–39]. Tom5 is thought to assist the transfer of precursor proteins from Tom22 to the Tom40 channel and to support the biogenesis of Tom40 [40,41]. Tom6 and Tom7 antagonistically regulate the dynamic assembly of the TOM complex: Whereas Tom6 promotes TOM biogenesis through its association with early assembly intermediates, Tom7 destabilizes both intermediate and mature TOM complex forms, likely to facilitate the incorporation of newly imported subunits [42–45]. Interestingly, the assembly and activity of the TOM complex was recently shown to be controlled by cytosolic protein kinases: Whereas casein kinase 2 (CK2) phosphorylates Tom22 to facilitate its biogenesis, the receptor activity of Tom70 is decreased through phosphorylation by protein kinase A [46,47]. These findings demonstrate that the functional state of the TOM complex is intimately linked to large-scale regulatory circuits of cellular physiology.

The pathway of precursor passage through the TOM complex is best understood for presequence-containing proteins. After their initial recognition by the Tom20 receptor, precursors bind to cytosolic domains of Tom22 and Tom5, engage a polar slide formed within the pore of Tom40 and finally contact an acidic binding site on the trans side of the TOM complex formed by Tom40, Tom7 and the IMS domain of Tom22 [36,37,48–51]. The increasing affinity of these interactions is considered to drive the inward-directed movement of precursors. For presequence-containing proteins, the transport across the outer membrane is tightly coupled to the translocation across or into the inner membrane via the TIM23 machinery through a direct hand-over of substrates as soon as they emerge from the TOM complex [52,53].

3. Biogenesis of outer membrane β-barrel proteins: the SAM complex

The presence of membrane proteins with a β-barrel conformation is a key feature of the outer membrane of Gram-negative bacteria. In

eukaryotic cells β-barrel proteins are exclusively found in the outer membranes of endosymbiotic organelles, as they originate from prokaryotic ancestors [54]. Porin, Tom40, Sam50 and Mdm10 are β-barrel proteins of the outer mitochondrial membrane. β-barrel precursors are recognized by the receptors of the TOM complex and guided through the Tom40 pore by a series of hydrophobic binding sites that are thought to prevent precursor aggregation in a chaperone-like manner [25,55,56]. Subsequently, precursor proteins are handed over to soluble chaperone complexes of the IMS that are formed by the small Tim proteins (Fig. 2). Two types of small Tim chaperone complexes consisting of two homologues small Tim proteins each are found in the IMS: the Tim9–Tim10 and the Tim8–Tim13 complex. Tim9–Tim10 plays a crucial role in the translocation of β-barrel proteins and metabolite carriers [57–60], whereas Tim8–Tim13 has mainly been implicated in the biogenesis of the Tim23 protein (see below) [61–64]. Both small Tim complexes are organized as hexameric, ring-like structures of alternating subunits that resemble a six-bladed propeller with a central cavity [60,65].

Integration of β-barrel proteins from the IMS side into the outer membrane is initiated by a specific import signal within the last β-strand of the precursors. This targeting sequence has been termed β-signal and consists of a large polar amino acid (lysine or glutamine), an invariant glycine and two hydrophobic amino acids [66]. Guided by the β-signal precursor proteins are delivered from the small Tim chaperones to the SAM complex (TOB complex) in the outer mitochondrial membrane for folding and insertion into the lipid bilayer (Fig. 2) [56,66–68]. The core components of the SAM complex are conserved amongst eukaryotes and comprise the integral outer membrane protein Sam50 (Tob55), which is a member of the Omp85 protein family, and two peripheral membrane proteins, termed Sam35 (Tob38) and Sam37 in yeast, that are exposed to the cytosolic side of the SAM complex [54,56,67–73]. Sam50 is integrated into the outer membrane with its C-terminal β-barrel domain. Electrophysiological measurements have demonstrated that recombinant Sam50 forms aqueous pores across membranes [66,67]. Current models suggest that Sam35 protrudes into these pores from the cytosolic side of the SAM complex to provide a binding site for β-signal-containing precursors [66]. It has been suggested that Sam50 oligomers constitute a hydrophilic, proteinaceous chamber within the outer membrane that promotes the folding of β-barrel precursors [66,67]. Finally, release of folded substrates into the lipid bilayer is supported by Sam37 and the N-terminal polypeptide-transport-associated (POTRA) domain of Sam50 [74,75]. The molecular mechanism of substrate release from the SAM complex is unknown. Major conformational rearrangements seem to be required to allow the escape of a β-barrel protein from the postulated protein folding chamber largely composed of Sam50 molecules. Notably, also in the case of the TOM complex the existence of a large central pore formed by several Tom40 molecules has been suggested that appears to be capable of lateral release of α-helical precursor proteins into the outer membrane [76].

For the biogenesis of the Tom40 precursor the SAM complex associates with the Mdm10 protein (Fig. 2) [77,78]. A recent study indicated that the SAM-Mdm10 complex supports the assembly of Tom40 with the α-helical Tom22 protein [79]. Mdm10 not only associates with the SAM complex, but is also a subunit of the Endoplasmic Reticulum (ER)–Mitochondria Encounter Structure (ERMES). The distribution of Mdm10 between the SAM and ERMES complexes is regulated by the small Tom protein Tom7 [45,80,81]. The ERMES complex is composed of the mitochondrial outer membrane proteins Mdm10, Mdm34 and Gem1, the integral ER membrane protein Mmm1 and the adaptor protein Mdm12, and thus forms a direct physical connection between mitochondria and the ER [78,82–86]. Distinct mitochondria-ER contact sites have been implicated in the coordination of phospholipid biosynthesis, intracellular calcium homeostasis and programmed cell death (apoptosis) (summarized in ref [87]). Gem1 is a calcium-dependent GTPase that has been suggested to modulate mitochondria-ER contacts by regulating the size and quantity of ERMES complexes [86]. However, the exact role of ERMES in the coordination of mitochondrial and ER activities and the role of Gem1 are still under debate [88]. Earlier studies had shown that mutations affecting ERMES components lead to alterations of mitochondrial morphology with the formation of giant globular mitochondria and defects in mitochondrial inheritance [82,89,90]. Moreover, not only Mdm10, but also other ERMES subunits are required for the biogenesis of mitochondrial β-barrel proteins (Fig. 2) [78]. Upon conditional inactivation of Mmm1, β-barrel protein assembly defects are observed before morphological alterations of mitochondria become evident [78]. Moreover, several mutations affecting the TOM and SAM complexes were found to cause morphology defects similar to those of ERMES mutants [77,78]. These findings suggest that ERMES is primarily involved in protein biogenesis at mitochondria-ER contact sites. Such a model raises the question, why protein import and mitochondria-ER contact sites should be linked. There is currently no

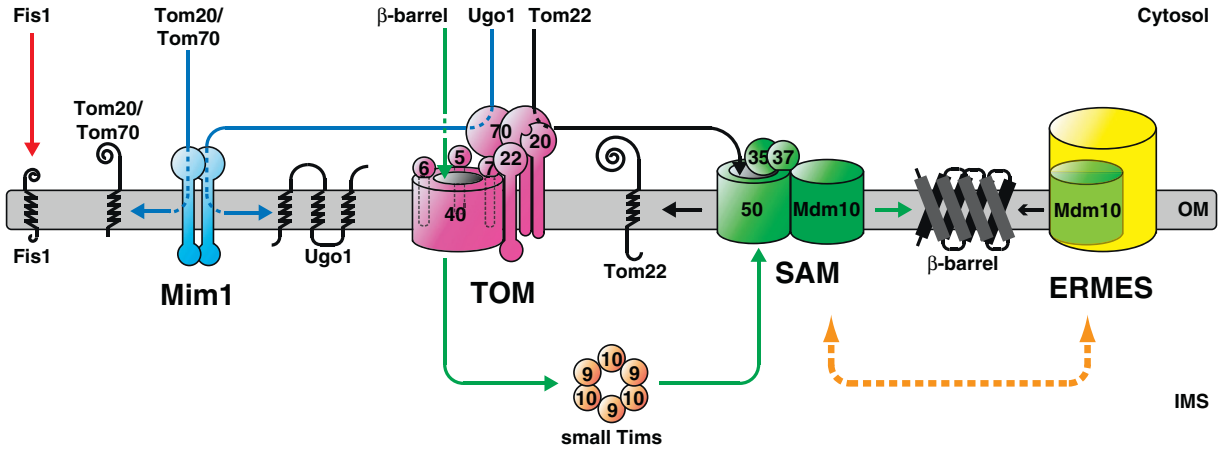

Fig. 2. Multiple mechanisms exist to integrate proteins with different transmembrane topologies into the outer mitochondrial membrane (OM). A few α-helical proteins of the outer membrane, like Fis1, seem to insert without the help of proteinaceous translocases. OM proteins with an N-terminal α-helical membrane anchor, like Tom20 or Tom70, and multi-spanning α-helical OM proteins, like Ugo1, depend on Mim1 for membrane integration. Tom22 is recognized by the TOM complex receptors and subsequently inserted into the OM via the SAM complex. The SAM complex also mediates the membrane integration of β-barrel OM proteins, which are handed over from the TOM to the SAM complex by the small Tim chaperones Tim9–Tim10. Mdm10 has a dual function in the SAM complex and in the endoplasmic reticulum (ER)-mitochondria encounter structure (ERMES). Further ERMES proteins have been implicated in the biogenesis of β-barrel proteins as well. IMS, intermembrane space.

experimental data that gives a clear answer, but it seems plausible that protein insertion into the outer mitochondrial membrane may be coupled to the import of membrane phospholipids from the ER. In any case, these intriguing relationships indicate that ERMES connects mitochondrial protein assembly to other pathways of mitochondrial biogenesis and function and may represent a cornerstone of a larger organizing system that coordinates protein transport and membrane architecture at the interface of mitochondria and ER [91].

Further evidence for the presence of an ER-mitochondria organizing network is provided by the finding that both TOM and SAM complexes associate with components of the recently discovered Mitochondrial Inner membrane Organizing System (MINOS), which has also been termed mitochondrial contact-site (MICOS) complex or mitochondrial organizing structure (MitOS) and is required for the maintenance of mitochondrial inner membrane architecture [91–98]. The evolutionary conserved core of MINOS is formed by the mitochondrial inner membrane proteins mitofilin/Fcj1 and Mio10 (Mos1, Mcs10; MINOS1 in mammals). MINOS appears to represent a central integrating factor that links mitochondrial protein import to membrane architecture: The formation of contact sites between outer and inner mitochondrial membranes through the interaction of mitofilin/Fcj1 with the TOM complex was shown to facilitate protein import into the IMS via the MIA pathway (see below) [91,92]. The mechanistic implications of the MINOS-SAM interaction, e.g. for the biogenesis of outer membrane proteins, are not understood so far. Several further observations, however, support a model, which functionally links the MINOS, TOM/SAM and ERMES complexes (summarized in ref. [91]): (i) Mdm10 shuttles between SAM and ERMES complexes and promotes the biogenesis of the TOM complex. (ii) ERMES mutations affect mitochondrial protein biogenesis, mitochondrial morphology and phospholipid transfer between ER and mitochondria. Notably, the levels of the negatively charged phospholipid cardiolipin, which is synthesized at the inner mitochondrial membrane, is reduced by mutations that inactivate the outer membrane ERMES complex [83]. (iii) MINOS and ERMES are connected to each other and to the mitochondrial phospholipid biosynthesis machinery through multiple genetic interactions [94]. Taken together, the MINOS, TOM/SAM and ERMES complexes are likely part of an extended network that structurally and functionally connects different intracellular membranes, regulates membrane morphology and may facilitate the transfer of both molecules and signal information between the mitochondrial matrix and the ER lumen.

4. Biogenesis of α-helical outer membrane proteins

Diverse targeting and insertion pathways have been identified for the biogenesis of different classes of outer membrane proteins with α-helical membrane-spanning segments. Tom20 and Tom70 are membrane-anchored via single, N-terminal transmembrane domains (signal-anchored). The outer membrane protein Mim1 supports the insertion of these proteins into the lipid bilayer (Fig. 2) [99–101]. Like the central Tom receptor Tom22, Mim1 is phosphorylated by CK2 [46]. Elimination of two serine residues of Mim1 that are targeted by CK2 inhibits the biogenesis of Tom20 and Tom70 [46]. Mim1 also assists the integration of multi-spanning outer membrane proteins, like Ugo1 (Fig. 2) [102,103]. This process depends on the Tom70 receptor, whereas the remainder of the TOM complex is dispensable [102–104]. No proteinaceous insertion machinery has been identified so far for the integration of outer membrane proteins with a single C-terminal transmembrane segment (tail-anchored), like the fission protein Fis1 [105,106]. Instead, the lipid composition of the target membrane seems to be critical [106]. The transmembrane domains of both, signal-anchored and tail-anchored outer mitochondrial membrane proteins are relatively short and moderately hydrophobic [107]. Together with their flanking positively charged residues they are thought to act as mitochondrial import signals. Why specifically tail-anchored proteins would not require a proteinaceous membrane insertion machinery, is still unclear. Both, the TOM complex and the SAM-Mdm10 complex were shown to be involved in the biogenesis of Tom22 that comprises a central, α-helical membrane-spanning segment (Fig. 2) [79,108]. In contrast to the assembly of outer membrane β-barrel proteins, no factors of the IMS have been found to be involved in the biogenesis of the topologically different α-helical proteins, which indicates that they are integrated from the cytosolic side of the outer membrane.

5. Protein transport into the intermembrane space: the MIA pathway

Many IMS proteins contain multiple cysteine residues that are implicated in the formation of disulfide bridges, like for Tim9 and Tim10, or the binding of cofactors and metal ions, like for Cox17. These cysteine residues are generally found in characteristic Cx_3C or Cx_9C motives (Fig. 1). A mitochondrial IMS sorting signal (MISS) around these cysteine motives has been identified that targets such proteins to the destined compartment via the MIA pathway [109,110]. The import receptor Mia40 and the sulfhydryl oxidase Erv1 are the essential core components of the MIA machinery that operates as a disulfide relay system in the IMS [111–116]. Mia40 forms transient intermolecular disulfides with incoming precursor proteins as soon as they emerge from the outer membrane TOM complex thereby trapping client proteins in the IMS [92,111,117]. The initial binding of precursor proteins to Mia40 occurs at a hydrophobic cleft that contains a redox-active CPC (cysteine–proline–cysteine) motive, which engages in mixed disulfide formation [118–121]. In yeast, this early substrate interaction of Mia40 is facilitated by its transient interaction with mitofilin/Fcj1, which is an integral inner membrane protein [92]. Mitofilin/Fcj1 is implicated in multiple contact sites between inner and outer mitochondrial membranes, thereby recruiting Mia40 to the IMS side of the TOM complex and thus into the proximity of the arriving precursors [92].

In the next step, Mia40 catalyzes the formation of intramolecular disulfide bridges within the precursors, which leads to substrate release into the IMS and reduction of Mia40 [111,115–117]. Interestingly, substrate oxidation by Mia40 in the IMS was demonstrated to regulate the subcellular distribution of the chaperone protein Ccs1, which is found both in mitochondria and the cytosol [122–125]. Re-oxidation of Mia40 for another round of import is mediated by Erv1 that shuttles electrons to cytochrome *c* and finally to the cytochrome *c* oxidase complex of the respiratory chain [113,121,126–128]. The active form of Erv1 is a homodimer, and an exchange of electrons between the two monomers has been identified as a central aspect of the reaction mechanism [129]. Erv1 also associates directly with Mia40 and precursor proteins in a ternary complex that has been suggested to assist the formation of multiple disulfide bonds in a concerted manner [121,128].

6. Biogenesis of inner membrane metabolite carriers: the TIM22 complex

The mitochondrial inner membrane is one of the most protein-rich membranes known. Integral inner membrane proteins span the phospholipid bilayer in an α-helical conformation and are mainly distinguished by the presence or absence of N-terminal presequences in their precursor forms. Multi-spanning inner membrane proteins that lack such presequences contain several internal import signals that overlap with the transmembrane segments of the mature proteins and closely cooperate to govern mitochondrial targeting and membrane integration. The majority of these proteins belong to the family of mitochondrial metabolite carriers, like the ADP/ATP carriers (AAC) or the phosphate carrier (PiC). They are characterized by a modular structure with three pairs of transmembrane domains connected by hydrophilic loops. Additional substrates of this pathway are the three homologues proteins Tim17, Tim22 and Tim23 that are essential

core subunits of inner membrane protein translocation machineries. These proteins exhibit a structural organization similar to metabolite carriers, but contain only four transmembrane segments.

Experimental dissection of the carrier import pathway led to the identification of five distinct stages [130,131]. Due to the particularly hydrophobic nature of carrier proteins chaperones of the Hsp70 and Hsp90 families are required to bind the precursors in the cytosol, to prevent their aggregation, and to assist their targeting to mitochondria (stage I) [21,23]. Precursor–chaperone complexes are recruited to the mitochondrial surface through binding to multiple tetratricopeptide repeats in the cytosolic domain of the Tom70 receptor [21,132,133]. A hydrophobic groove in the C-terminal domain of Tom70 was suggested to comprise a binding site for precursors [31,134]. It is assumed that the three structural modules of a carrier precursor recruit three Tom70 dimers (stage II) [30]. The receptor function of Tom70 was recently shown to be regulated by reversible phosphorylation via protein kinase A in yeast [46,47]. In the presence of glucose, when yeast cells produce ATP mainly in the cytosol by fermentation, protein kinase A phosphorylates Tom70 and inhibits the recruitment of cytosolic precursor–chaperone complexes. In this way, the import of carrier proteins into mitochondria may be adapted to the energetic status of the cell in response to global catabolic switches between fermentative and respiratory metabolism.

After binding to Tom70, carrier precursors are handed over to the protein-conducting channel of the TOM complex. At the IMS site of this pore, the precursors are bound by the small Tim chaperones Tim9 and Tim10 (Fig. 1) [135–139]. Binding of the Tim9–Tim10 complex to the hydrophobic segments protects carrier precursors from aggregation in the aqueous environment of the IMS (stage IIIa) [57]. The Tim9–Tim10 complex is organized as a helical wheel-like structure with protruding tentacles that have been suggested to associate with two adjacent transmembrane segments of an incoming carrier precursor [60]. The homologous Tim8–Tim13 complex was shown to promote the biogenesis of Tim23, the central subunit of the presequence translocase (see below), likely through a similar mechanism [63–65,140]. Kinetic measurements indicated a cooperative binding of peptides derived from the Tim23 precursor to hydrophobic patches in the Tim8–Tim13 complex [65]. Targeting of precursors to the TIM22 complex (Fig. 1) for inner membrane insertion is initiated by the association of a further small Tim protein, Tim12, with the Tim9–Tim10 complex. Tim12 mediates the subsequent tethering of the IMS translocation intermediate to the inner membrane translocase (stage IIIb) [136,141]. In the absence of a $\Delta\psi$ across the inner mitochondrial membrane, carrier biogenesis does not proceed beyond this stage [130].

The membrane-embedded core of the TIM22 complex is composed of the central pore-forming subunit Tim22 together with Tim54, Tim18 and Sdh3 [130,142–147]. Tim54 exposes a large domain into the IMS providing a binding site for the Tim9–Tim10–Tim12 complex [148,149]. Additionally, the function of Tim54 was found to be required for the assembly of the Yme1 complex, an inner membrane protease of the AAA superfamily that is a key player of the mitochondrial protein quality control system [148]. Tim18 supports the assembly of Tim54 into the TIM22 complex [149]. Tim18 is homologous to Sdh4, a membrane-integral subunit of the respiratory chain complex II (succinate dehydrogenase) [144,145,150]. Within complex II Sdh4 is tightly associated with the Sdh3 subunit. A recent study demonstrated the Sdh3 is not only a component of complex II, but also a genuine subunit of the TIM22 complex, where it interacts with Tim18 [147]. This surprising finding sheds an intriguing light on the co-evolution of mitochondrial protein transport machineries and respiratory chain complexes: Apparently, a common structural module composed of the Sdh3 protein and Sdh4/Tim18 has been used as a building block to form both, complex II and the TIM22 machinery.

After docking of the substrate-loaded Tim9–Tim10–Tim12 complex to the Tim54 receptor, the carrier precursor is inserted into the TIM22 complex, which contains two pores with an estimated diameter that is large enough to accommodate two α-helices each (stage IV) [130]. How the import signals in carrier precursors are recognized by the TIM22 complex remains to be defined. The opening of the protein-conducting pores requires a $\Delta\psi$ and the internal targeting signals within the precursor. It is further assumed that the $\Delta\psi$ exerts an electrophoretic force on positively charged residues located in the loops that connect the carrier's hydrophobic transmembrane segments. Finally, the precursor is laterally released from the TIM22 complex into the inner membrane by a yet unknown mechanism and assembles to its mature, functional form, which is most likely a homo-dimer in the case of metabolite carriers (stage V).

7. Biogenesis of presequence-carrying proteins: the TIM23 complex

The majority of mitochondrial precursor proteins in yeast carries N-terminal, cleavable presequences that govern targeting and sorting of these proteins to the mitochondrial inner membrane or matrix [17]. Presequence-containing precursor proteins (hereafter named preproteins) pass the outer membrane TOM complex and associate with the IMS domain of the central receptor Tom22 [36,37]. At this early stage of import the preproteins are already in close proximity to the TIM23 complex in the inner mitochondrial membrane. Transfer of the N-terminal portion of a preprotein to the protein-conducting pore of the TIM23 complex in fact occurs, when the C-terminal region is still inside the TOM complex. In this manner, two-membrane-spanning translocation intermediates composed of a TOM complex and a TIM23 complex connected by a translocating polypeptide are formed that localize to contact sites between outer and inner mitochondrial membranes [151–155]. Such TOM–TIM23 supercomplexes can be accumulated both *in vivo* and *in organello* by the fusion of a stably folded protein domain, like dihydrofolate reductase in complex with methotrexate, to the C-terminus of a preprotein [52,53,156–162]. At the inner membrane preproteins are either translocated into the mitochondrial matrix or inserted into the lipid bilayer depending on their specific import signals (Fig. 1). Hydrophobic sorting signals downstream of the N-terminal presequences induce translocation arrest and the lateral release of preproteins into the inner mitochondrial membrane by a stop-transfer mechanism [163–166].

Three essential membrane-integral subunits form the catalytic core of the TIM23 complex ($TIM23^{CORE}$): Tim23, Tim17 and Tim50 (Fig. 3). Tim23 forms a $\Delta\psi$-dependent protein-conducting pore across the inner membrane [165,167–169]. The N-terminal IMS region of Tim23 ($Tim23_{IMS}$) is part of the preprotein receptor domain of the TIM23 complex [37,170]. Tim17 is involved in the stabilization and regulation of the channel formed by Tim23 and the differential sorting of preproteins [162,171–173], however, its function is not understood at the molecular level. Tim50 has been proposed to maintain the Tim23 channel across the inner membrane in a closed state to prevent ion leakage and dissipation of $\Delta\psi$ in the absence of a preprotein [168]. The large IMS domain of Tim50 ($Tim50_{IMS}$) associates with $Tim23_{IMS}$ to form the preprotein receptor module of the TIM23 complex [161,174–179]. Crosslinking studies indicate that $Tim50_{IMS}$ is located in close proximity to the Tom22 subunit of the TOM complex [39]. This spatial arrangement allows $Tim50_{IMS}$ to interact with preproteins at early stages of import, when the bulk of the preprotein in still inside the TOM complex [161,174,175]. Two distinct preprotein binding sites within $Tim50_{IMS}$ have been proposed: one in the central core domain and one in the C-terminal region [180,181]. The crystal structure of the conserved core domain of $Tim50_{IMS}$ shows the presence of an extended groove on the surface with negatively charged amino acid residues at the bottom that could accommodate an amphipathic α-helical peptide [180]. Adjacent to this groove a β-hairpin that is crucial for the binding of $Tim50_{IMS}$ to $Tim23_{IMS}$ protrudes from the surface of the molecule [180]. Binding

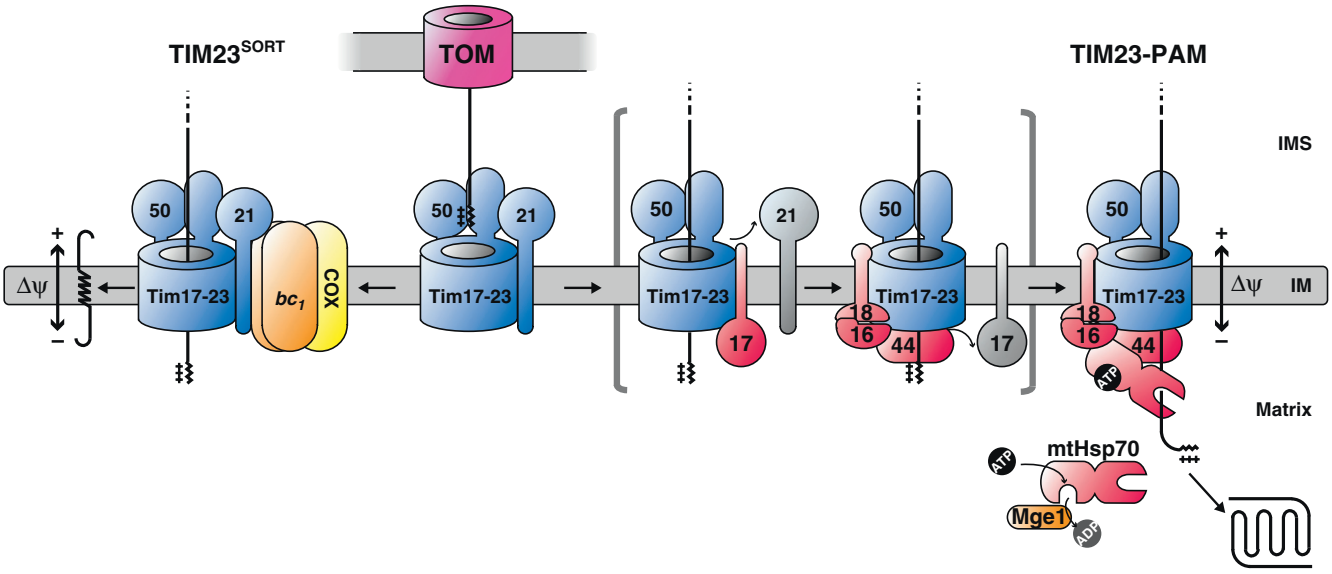

Fig. 3. Modular rearrangements and coupling to different partner protein complexes trigger functional switches in the presequence translocase (TIM23 complex). The membrane potential (Δψ) across the inner mitochondrial membrane (IM) drives the transfer of positively charged presequences from the TOM complex into the protein-conducting pore of the TIM23 complex in a process that involves Tim21. For membrane integration of preproteins the TIM23–Tim21 complex ($TIM23^{SORT}$) associates with respiratory chain supercomplexes composed of cytochrome bc_1 complexes and cytochrome *c* oxidase (COX). Matrix translocation requires the recruitment and activation of the presequence translocase-associated import motor (PAM). In the working model illustrated here, Pam17 displaces Tim21 from the TIM23 core complex and together with Tim44 triggers the binding of the Pam16–Pam18 module. Pam17 itself is released again during later assembly steps of PAM. In the fully active PAM machinery, mtHsp70 cooperates with Pam16–Pam18, Tim44 and Mge1 to mediate ATP-driven import of preproteins into the mitochondrial matrix. TIM23 complex: Tim17–Tim23 (Tim17–23), Tim50 (50), Tim21 (21); PAM: Tim44 (44), Pam16 (16), Pam17 (17), Pam18 (18); IMS, intermembrane space.

of presequences to the C-terminal portion of $Tim50_{IMS}$ was demonstrated by a photo-affinity labeling approach [181]. Taken together, these data suggest that the Tim23/Tim50 receptor module is engaged in multiple interactions with an incoming preprotein that cooperatively function to mediate the transfer of the preprotein from the outer mitochondrial membrane to the protein-conducting channel of the inner membrane. This transfer reaction appears to be facilitated by Tim21, an additional, membrane-integral component of the TIM23 machinery that dynamically associates with $TIM23^{CORE}$ (Fig. 3). Tim21 was shown to directly interact with the Tom22 subunit of the TOM complex *in vitro* and to compete with presequences for binding to Tom22 [171,182,183].

From the energetic perspective, direct handover of preproteins from the TOM complex to the TIM23 complex and the initiation of preprotein translocation across the inner membrane are driven by the Δψ, which triggers opening of the TIM23 protein-conducting channel and exerts an electrophoretic force on the positively charged presequence [167,184]. The reconstitution of TIM23 complexes (purified via tagged Tim21) into proteoliposomes directly demonstrated that the energy derived from Δψ is sufficient for the membrane insertion of preproteins with a hydrophobic stop-transfer signal adjacent to the presequence [165]. This TIM23 complex form composed of $TIM23^{CORE}$ and Tim21 has therefore been termed $TIM23^{SORT}$. The Δψ-dependent step of preprotein insertion via $TIM23^{SORT}$ is supported by the recruitment of the proton-pumping respiratory chain complexes III (cytochrome bc_1) and IV (cytochrome *c* oxidase) (Fig. 3) [185–187]. These respiratory chain complexes associate with each other to form different types of supercomplexes in the inner mitochondrial membrane [188–190]. Binding of such supercomplexes to $TIM23^{SORT}$ is at least in part mediated by an interaction of Tim21 with the Qcr6 subunit of complex III [185,186]. In the absence of Tim21 the connection between $TIM23^{SORT}$ and respiratory chain complexes is impaired and preprotein insertion into the membrane via the stop-transfer pathway shows an increased sensitivity to partial uncoupling of Δψ [185,186].

For the full translocation of soluble preproteins (or large hydrophilic domains of membrane-anchored preproteins) into the matrix the Δψ-dependent activity of $TIM23^{SORT}$ is not sufficient. Additional energy derived from ATP hydrolysis in the mitochondrial matrix is required.

8. Matrix translocation of preproteins: the import motor PAM

The ATP-dependent steps of preprotein import via the TIM23 machinery require the presequence translocase-associated import motor (PAM). The inward-directed force on the incoming preprotein is provided by the ATPase activity of mitochondrial heat shock protein 70 (mtHsp70). A nucleotide-dependent sequence of concerted conformational changes in both the ATPase domain and the peptide binding domain of mtHsp70 provides the mechanistic basis for the translocation of preproteins into the matrix [191–195]. The mtHsp70 is recruited to the TIM23 channel and tightly regulated by several co-chaperones: Pam18 (Tim14), Pam16 (Tim16), Mge1, Tim44, and Pam17 (Fig. 3). Pam18 is a member of the J-protein family and activates the ATPase activity of mtHsp70 at the TIM23 complex [196–200]. Pam18 is composed of an N-terminal IMS domain, a single transmembrane segment and a matrix-localized J-domain. All three domains contribute to the binding of Pam18 to $TIM23^{CORE}$ [171,201–206]. Pam18 forms a stable complex with the J-like protein Pam16, which is crucial for the recruitment of Pam18 to import sites [201–208]. Additionally, Pam16 was suggested to control the activity of Pam18 and thus to directly participate in the regulation of the mtHsp70 reaction cycle [199]. Mge1 is also crucial for matrix preprotein import, as it mediates nucleotide exchange on mtHsp70 [209–212]. Tim44 interacts with mtHsp70 in an ATP-dependent manner to mediate the dynamic association of mtHsp70 with the $TIM23^{CORE}$ complex [213–219]. Mutational analyses and crosslinking studies indicate that Tim44 is also implicated in the interaction of the Pam16/Pam18 complex with $TIM23^{CORE}$ and modulates the functional cooperation of Pam16/Pam18 with mtHsp70 [198,202,208,220]. Taken together, Tim44 may provide a platform that brings together the different components of the PAM machinery and orchestrates their activities at the matrix site of the TIM23 complex. To fulfill this role Tim44 cooperates with Pam17, which is involved in the recruitment of the Pam16/Pam18

complex to TIM23^{CORE} and is thought to trigger the assembly of the active import motor [208,221–223].

9. Modular switches in the TIM23 machinery coordinate different activities

Over the last years, many studies have illustrated that the TIM23 complex is a highly versatile molecular machine with different activities: Δψ-dependent lateral sorting of inner membrane proteins and ATP-dependent matrix translocation. How this functional dualism is brought about is still a matter of debate. One model suggests that a persistent TIM23–PAM machinery mediates the transfer of all preproteins from the outer to the inner mitochondrial membrane and their subsequent sorting to either the inner membrane or the matrix [177,222,224,225]. However, there are several lines of evidence that the TIM23 machinery is more dynamic: Switches between the different activities are controlled by the import signals of preproteins and accompanied by the coupling of distinct partner protein complexes to TIM23^{CORE} (Fig. 3). For the transfer of preproteins from the outer membrane, components of the TIM23 complex, including Tim21, associate with the TOM complex [39,171,177,182,183]. It is therefore conceivable that the Tim21-containing TIM23^{SORT} complex mediates the initial steps of preprotein import at the inner mitochondrial membrane (Fig. 3). Indeed, early translocation intermediates also of matrix-targeted preproteins have been found associated with TIM23^{SORT} [162]. Only small substoichiometric amounts of PAM components have been co-isolated with TIM23^{SORT} upon purification via tagged Tim21 [162,165,171,185,221,222]. Instead, for the Δψ-driven insertion of preproteins into the inner membrane, TIM23^{SORT} associates with respiratory chain supercomplexes in a Tim21-dependent manner [185,186]. Import of preproteins into the matrix, however, requires ATP and thus the stepwise recruitment of PAM to TIM23^{CORE} (Fig. 3). This process appears to be facilitated by Pam17 and leads to the release of Tim21 from Tim23^{CORE} [162,171,208,222]. Taken together, these findings suggest that the TIM23 complex dynamically switches between a Tim21-bound state that has a low affinity for PAM and a PAM-coupled state with a low affinity for Tim21. However, more work is certainly needed to understand in detail the mechanisms of preprotein sorting to different mitochondrial subcompartments by the TIM23 machinery and the modular rearrangements that control functional switches in this sophisticated translocase.

A remarkable example for a complex sequence of functional switches in the TIM23 machinery can be deduced from the recently unraveled biogenesis pathway of the ABC transporter Mdl1, a presequence-carrying multi-spanning inner membrane protein [166]. Mdl1 exhibits a modular structure with three pairs of two transmembrane segments, which were shown to be inserted into the inner membrane by different mechanisms. Whereas membrane integration of the first and the third module occurs independently of PAM via a lateral sorting mechanism, the second central module is initially translocated across the inner membrane in a PAM-dependent manner and subsequently membrane-inserted with the help of the evolutionary conserved export translocase Oxa1 [166]. The latter mechanism is known as “conservative sorting” [226]. Oxa1 is also a crucial factor for the Δψ-dependent, co-translational export of mitochondrially encoded subunits of respiratory chain complexes [227–229]. For the biogenesis of nuclear encoded multi-spanning inner membrane proteins Oxa1 closely cooperates with the TIM23–PAM machinery [166,230,231]. Thus, the biogenesis of the topologically complex inner membrane protein Mdl1 requires multiple switches between distinct TIM23 activities and the cooperation with different partner protein complexes.

10. Concluding remarks and future perspectives

Sorting of proteins to the correct subcellular compartment is essential for the survival of every eukaryotic cell. The structural complexity of mitochondria as double-membrane-bound organelles has led to the evolution of diverse protein sorting and translocation machineries that must thoroughly coordinate their activities. In the last decade a striking number of novel mitochondrial protein sorting pathways and additional components of known protein transport machineries has been identified by many different research groups. These discoveries have been greatly enhanced by the comprehensive analysis of the mitochondrial proteome in the widely used model organism *S. cerevisiae* [1] and have had a massive impact on our understanding of the biogenesis and organization of mitochondria. However, there are still many unsolved questions regarding the molecular mechanisms that ensure the reliable and efficient sorting of proteins to and within mitochondria. How are import signals within precursor proteins read out and how is the containing information translated into large-scale conformational changes in protein translocases? How are precursor proteins passed on from one translocation machinery to the next? What are the principles that allow protein sorting machineries to catalyze very different reactions, like translocation across and insertion into a membrane, that require controlled transversal and lateral openings of protein-conducting pores? Finally, only a few studies so far have addressed how membrane phospholipids modulate the activities of the different mitochondrial protein sorting devices [165,232–234].

Apart from questions regarding the function of the individual mitochondrial protein translocation systems, it will be a major challenge for the future to understand how protein import into mitochondria is regulated in space and time on a global scale. Thinking about these problems inevitably leads to questions about, how mitochondrial biogenesis is coordinated with other cellular activities including metabolism as well as developmental programs, like cell division and differentiation. A number of recent studies that have been highlighted in this review have provided first hints how mitochondrial protein import is embedded into extended physiological frameworks: i) the regulation of the biogenesis and the receptor activity of the TOM complex through cytosolic kinases [46,47]; ii) the multiple branched links between mitochondrial membrane architecture, mitochondria-ER communication, phospholipid metabolism and protein biogenesis by an organizing network that includes the MINOS, TOM/SAM and ERMES complexes [77,78,80,81,83–86,91–98]; iii) the direct coupling of proton-pumping respiratory chain complexes to preprotein insertion into the inner mitochondrial membrane by the TIM23 machinery [185–187]. It will be most exciting to learn more about these intriguing connections and further yet unrecognized processes that also impact mitochondrial biogenesis.

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