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Mitochondrial protein import: from transport pathways to an integrated network

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Mitochondria, the powerhouses of the cell, import most of their proteins from the cytosol. It was originally assumed that mitochondria imported precursor proteins via a general pathway but recent studies have revealed a remarkable variety of import pathways and mechanisms. Currently, five different protein import pathways can be distinguished. However, the import machineries cooperate with each other and are connected to other systems that function in the respiratory chain, mitochondrial membrane organization, protein quality control and endoplasmic reticulum-mitochondria junctions. In this Opinion, we propose that mitochondrial protein import should not be seen as an independent task of the organelle and that a network of cooperating machineries is responsible for major mitochondrial functions.

Two classical import routes for mitochondrial proteins

Mitochondria are the powerhouses of eukaryotic cells and play important roles in cellular metabolism and regulation. They consist of two membranes, an intermembrane space and an inner compartment, the matrix. Although mitochondria contain a complete genetic and protein synthesis system in the matrix, derived from their prokaryotic ancestor, ~99% of mitochondrial proteins are encoded in the nucleus of the cell and are synthesized as precursors in the cytosol [1–4]. The precursor proteins contain targeting signals that direct them to the mitochondrial outer membrane and from here to their submitochondrial destination. Until 2003, two mitochondrial protein import pathways were known: the presequence pathway, in which the targeted proteins carry cleavable presequences, and the carrier pathway, for transport of inner membrane carrier proteins (Figure 1) [2,3].

Presequences, the classical mitochondrial targeting signals, are peptide extensions of ~10–60 amino acid residues located at the amino-terminal end of the proteins. They target the mature proteins to the receptors Tom20 and Tom22 of the translocase of the outer membrane (TOM) complex (see Glossary) [5,6]. After passage through the hydrophilic channel formed by Tom40, the precursor proteins engage with the TIM23 complex, which is the presequence translocase of the inner membrane [7–9]. The membrane potential ($\Delta\psi$) activates the Tim23 channel and drives translocation of the positively charged presequences. For translocation into the matrix, a second energy source is required: ATP powers the presequence translocase-associated motor (PAM) that contains a molecular chaperone, the mitochondrial heat shock protein 70 (mtHsp70), as central component [2,3,10]. Processing enzymes remove the presequences and release the mature proteins [11]. Many presequence-containing inner membrane proteins are laterally

Glossary

ERMES: The ER-mitochondria encounter structure (ERMES) tethers the endoplasmic reticulum (ER) to the outer membrane of mitochondria. This complex consists of the ER-localized protein Mmm1 and the mitochondria-bound proteins Mdm10, Mdm12, Mdm34 and Gem1.
MIA: The mitochondrial intermembrane space import and assembly (MIA) machinery mediates oxidative protein transport and folding. Mia40 cooperates with the sulfhydryl oxidase Erv1 in a disulfide relay to drive the import of cysteine-rich proteins into the intermembrane space and catalyzes their oxidative folding.
Mim1: The mitochondrial import protein 1 (Mim1) promotes membrane insertion of many outer membrane proteins that contain α-helical transmembrane segments. Mim1 forms oligomeric structures and can cooperate with the receptor Tom70 or the SAM complex.
MINOS: The mitochondrial inner membrane organizing system (MINOS) maintains the typical cristae structure of the inner membrane and interacts with several protein translocases.
Morphology proteins: Mitochondrial morphology proteins were identified by genetic approaches, revealing dramatic changes of mitochondrial shape in mutants of their genes.
PAM: The presequence translocase-associated motor (PAM) drives the ATP-dependent import of presequence-carrying preproteins into the mitochondrial matrix. The mitochondrial heat shock protein 70 (mtHsp70) forms the core of PAM, and its ATP/ADP cycle is regulated by the co-chaperones Pam18-Pam16 and Mge1. Tim44 docks the PAM module onto the TIM23 translocon.
SAM complex: The sorting and assembly machinery (SAM) is responsible for the insertion of β-barrel proteins into the mitochondrial outer membrane. The SAM core complex consists of the channel-forming protein Sam50 and two proteins that are exposed on the outer membrane surface, Sam35 and Sam37.
TIM22 complex: The carrier translocase of the inner mitochondrial membrane (TIM22) mediates the insertion of non-cleavable precursor proteins into the inner membrane. The TIM22 complex consists of the channel-forming protein Tim22, two further membrane-integrated proteins (Tim18 and Tim54) and the Tim9-Tim10-Tim12 chaperone complex that is located on the intermembrane space side.
TIM23 complex: The presequence translocase of the inner mitochondrial membrane (TIM23) mediates the import of presequence-containing preproteins across the inner membrane. The TIM23 complex consists of the channel-forming protein Tim23 and three further integral membrane proteins, Tim17, Tim21 and Tim50. The TIM23 complex cooperates with the motor PAM for protein translocation into the matrix.
TOM complex: The translocase of the outer mitochondrial membrane (TOM) is the main protein entry gate of mitochondria. The TOM complex consists of three receptor proteins (Tom70, Tom22 and Tom20), the channel-forming protein Tom40 and three small proteins (Tom5, Tom6 and Tom7), which are crucial for the assembly and stability of the complex.

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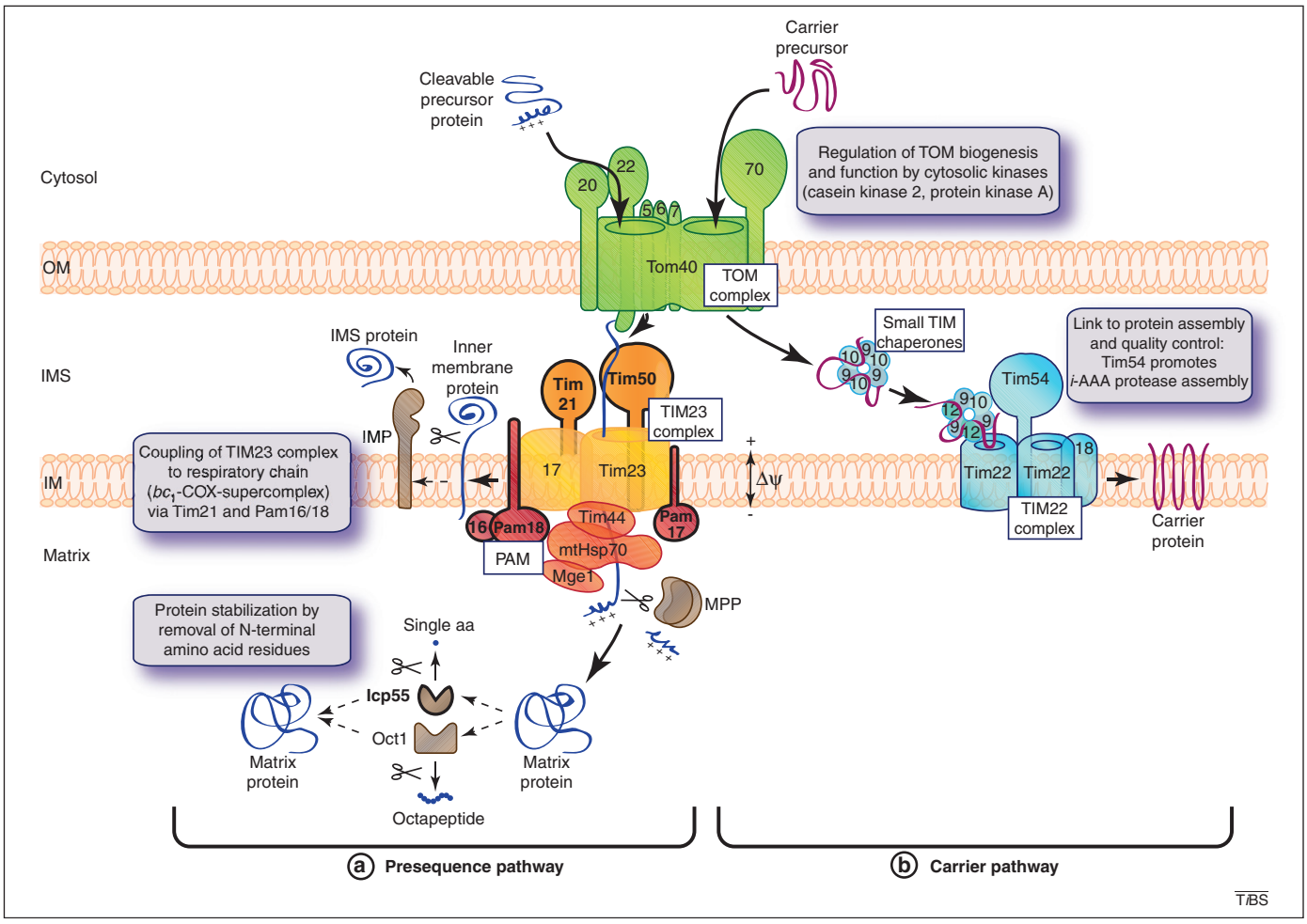

Figure 1. The two classical pathways for mitochondrial protein import. **(a)** The presequence pathway imports preproteins that carry positively charged presequences at the amino-terminus. The preproteins are recognized by the receptors Tom20 and Tom22 and are translocated across the outer mitochondrial membrane (OM) by the Tom40 channel. The TOM complex is regulated by cytosolic kinases. The preproteins are transferred to the TIM23 complex with the help of the intermembrane space (IMS)-exposed proteins Tim50 and Tim21. The membrane potential (Δψ) activates the Tim23 channel and drives translocation of the presequences across the inner membrane (IM). Coupling of the TIM23 complex to respiratory chain complexes supports the Δψ-dependent step. Protein transport into the matrix is completed by the motor PAM with the ATP-dependent chaperone mtHsp70. The presequences are cleaved off by the mitochondrial processing peptidase (MPP). Imported proteins can be stabilized by removal of destabilizing amino-terminal residues by the peptidases Icp55 or Oct1. Presequence-carrying precursors with hydrophobic sorting signals can be inserted into the inner membrane by two different routes. Some preproteins are laterally released from the TIM23 complex. The IM peptidase (IMP) complex removes the hydrophobic sorting signal of some proteins, releasing them into the IMS. Additionally, some proteins are first transported towards the matrix and then inserted into the IM by the Oxa1 export complex [2]. **(b)** The carrier pathway transports non-cleavable precursor proteins with internal targeting signals. The precursors are recognized by the receptor Tom70 and translocated across the OM by Tom40. Small TIM chaperone complexes guide the hydrophobic precursor proteins to the TIM22 complex that mediates Δψ-driven membrane insertion. Additionally, Tim54 promotes assembly of the *i*-AAA protease, linking the translocase to protein quality control. Recently discovered import components are marked in bold.

released from the TIM23 complex, whereas other precursor proteins are first transported into the matrix and subsequently integrated into the inner membrane via the Oxa1 export machinery [1–3].

Many inner membrane proteins, such as metabolite carriers, are synthesized without cleavable presequences and contain several, less defined internal targeting signals. Carrier precursors are bound to cytosolic chaperones and recognized by the receptor Tom70 [5,12]. The core of the TOM complex, including the channel Tom40, is used by both carrier precursors and presequence-carrying precursors, and then the two pathways diverge. The hydrophobic carrier precursors are transferred through the intermembrane space by the Tim9-Tim10 chaperone complex (small TIM chaperone) and are inserted into the inner membrane by the carrier translocase (TIM22 complex) using Δψ as the energy source [2,3,13].

Recent studies have revealed an unexpected complexity of the mitochondrial protein import system, and numerous novel import components and three new import pathways have been identified. Moreover, several results indicate that preprotein translocases do not act as independent units but are linked to machineries involved in different tasks, from energy conversion to maintenance of mitochondrial morphology, interorganellar contact and regulation. Here, we propose that these machineries, which perform seemingly unrelated functions, are connected in a dynamic network and, thus, major mitochondrial activities are functionally and physically linked.

Identification of new import components and pathways

In 2002, the new subunit Tim50 of the presequence translocase (TIM23 complex) was identified. Tim50 is essential for the presequence pathway and for viability of cells, which indicates that it is a major player in mitochondrial biogenesis [7,8]. Later, numerous new mitochondrial import components were identified. For example, important regulatory subunits that function in the TIM23

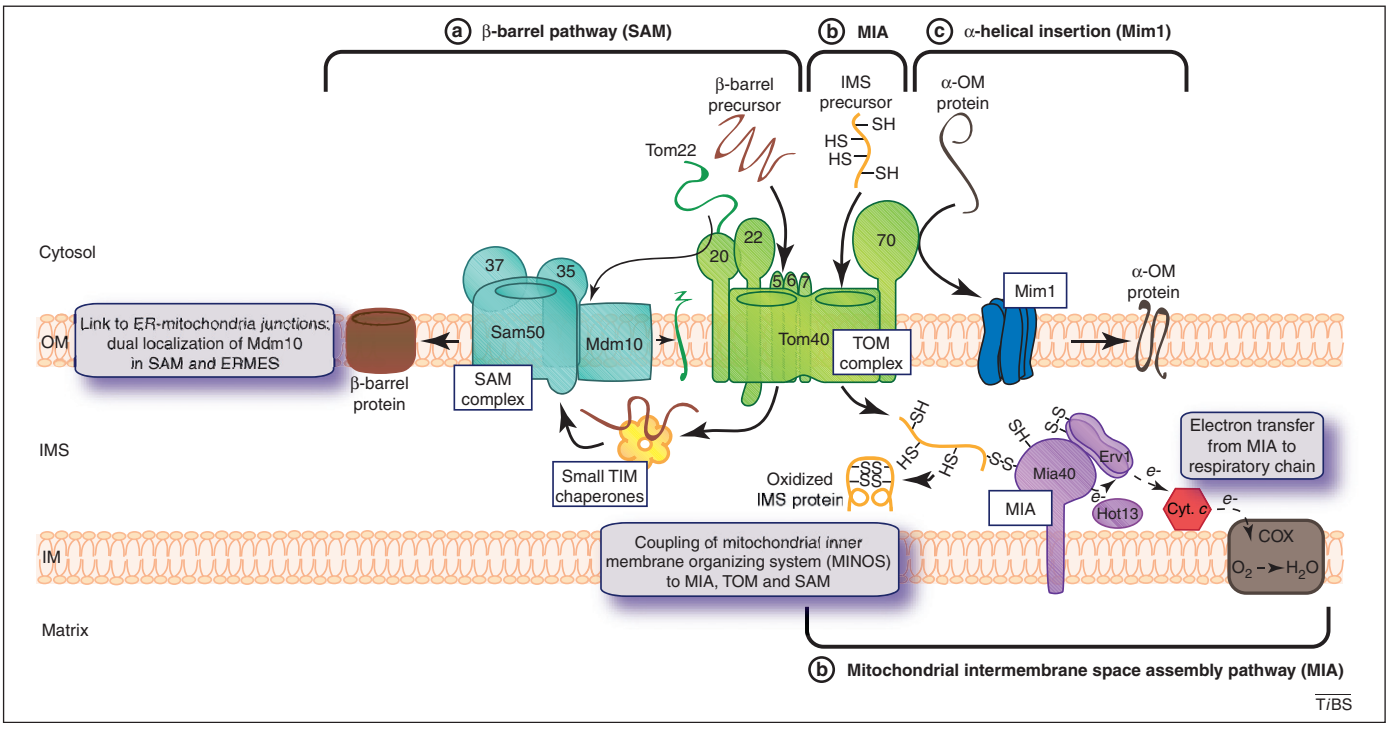

Figure 2. Three new pathways for mitochondrial protein import. **(a)** The β-barrel pathway transports the precursors of β-barrel proteins. The precursors are translocated across the outer membrane (OM) by the TOM complex. Small TIM chaperone complexes of the intermembrane space (IMS) assist in transfer of the precursors to the SAM complex that mediates insertion of the proteins into the outer membrane. The SAM complex can associate with Mdm10 to form a larger complex that facilitates import of the α-helical precursor of Tom22. Mdm10 is localized in two different complexes, SAM and ERMES, and thus links mitochondrial protein biogenesis to ER-mitochondria junctions. **(b)** The mitochondrial intermembrane space assembly pathway (MIA) is used by many cysteine-rich IMS proteins. After passage through the TOM complex, the precursor proteins are bound to Mia40 via disulfide bonds. Mia40 catalyzes the oxidative folding of the proteins. The sulfhydryl oxidase Erv1 mediates re-oxidation of Mia40, which is supported by Hot13. Electrons flow from Mia40 via Erv1 to cytochrome *c* and the cytochrome *c* oxidase (COX). The mitochondrial inner membrane organizing system (MINOS) is transiently coupled to Mia40, TOM and SAM, linking protein import and mitochondrial membrane morphology. **(c)** α-Helical insertion (Mim1) pathway. Outer membrane proteins with multiple α-helical transmembrane segments are recognized by Tom70 and transferred to the Mim1 complex for membrane insertion. Mim1 also promotes membrane insertion of several outer membrane proteins with a single transmembrane helix. Mim1 can transiently associate with the SAM complex to support assembly of small (α-helical) Tom proteins with Tom40.

complex, the motor PAM or in preprotein processing were identified for the presequence pathway (Figure 1) [9,11, 14–17]. In addition, entirely new protein import routes were discovered, such as the sorting and assembly machinery (SAM) for β-barrel proteins of the outer membrane [18–20], the mitochondrial intermembrane space assembly pathway (MIA) [21–23] and an insertion pathway for α-helical proteins of the outer membrane that uses the mitochondrial import protein 1 (Mim1) (Figure 2) [24–26].

Several experimental approaches have been fundamental for the identification of new components and transport routes for mitochondrial protein biogenesis. For example, blue native gel electrophoresis, originally developed for the analysis of mitochondrial respiratory chain complexes [27], has proved to be a remarkably powerful technique to follow intermediate steps of preprotein import. Since blue native gels allow the separation of intact protein complexes, even with bound preproteins in transit, distinct import stages of precursor proteins and their interaction with translocases can be directly monitored [28,29]. Combined with a systematic analysis of yeast strains with defects in mitochondrial protein import, native gel systems led to the identification of the SAM pathway [18] and have contributed to the functional characterization of the MIA [21,30] and Mim1 pathways [24]. In addition, the purification of translocase complexes under mild conditions (such as in the presence of the non-ionic detergent digitonin), combined with high-resolution mass spectrometry, have led to the identification of several new translocase subunits, such as Tim50, Tim21 and PAM subunits [7,9,14,16,17]. Large-scale proteomics studies of purified mitochondria have provided a huge number of uncharacterized mitochondrial proteins [31–34], which can be studied using the available collections of mutant yeast strains. Given that mitochondrial protein import is essential for cell viability [3], it is not surprising that functional screens for import components have identified several mitochondrial proteins that are essential for life; indeed, several of these proteins, such as new subunits of SAM and PAM, function in the protein import machinery [3]. The MIA pathway was discovered by analyzing an essential protein of unknown function, now known as Mia40 [21,22].

Therefore, a combination of functional biochemical and genetic assays, including large-scale genomics and proteomics studies, has yielded a major progress in mitochondrial research. Other experimental approaches have contributed to the characterization of the machineries and mechanisms of mitochondrial protein biogenesis and are summarized in Box 1. New import components and pathways probably remain to be discovered.

Two pathways for protein insertion into the mitochondrial outer membrane

The mitochondrial outer membrane contains two classes of proteins: β-barrel proteins, which are integrated into the membrane by multiple β-strands and are derived from the

Box 1. Approaches for identification and characterization of the mitochondrial protein import machinery

Classical approaches to study protein import into mitochondria include *in vitro* import assays using radiolabeled precursor proteins and isolated mitochondria; *in vivo* studies in mutant cells, particularly in yeast; localization of proteins by subfractionation of cells and mitochondria; and interaction studies using co-immunoprecipitation. The establishment and application of the following experimental approaches were important for the identification of new import components and the analysis of protein import mechanisms:

- Blue native gel electrophoresis for the characterization of protein complexes and precursor import intermediates [18,27].
- Mild conditions, such as the use of non-ionic detergents (e.g. digitonin), for isolation of protein complexes [7,9].
- Systematic analysis of the mitochondrial proteome [31–34]. Analysis of gene products with unknown function by using yeast deletion mutants or temperature-sensitive mutants in the case of essential genes.
- Fusion proteins of mitochondrial precursors and passenger proteins (with folded domains) for accumulation of transport intermediates and formation of supercomplexes between translocases, e.g. TOM-TIM23 supercomplex [9,28].
- Site-specific photo-crosslinking using modified amino acid residues for mapping interaction sites of preproteins with translocase components during import [8].
- Efficient *in vitro* synthesis of import-competent precursor proteins as a tool for the purification and analysis of import intermediates and the rescue of import capacity of mutant mitochondria [24,42].
- Mass spectrometry techniques for large-scale determination of the N-termini of imported proteins for studying presequences and protein processing [11] and for determination of protein modifications for the analysis of signaling processes [77]. Combination of mass spectrometry with stable isotope labeling with amino acids in cell culture (SILAC) for the identification of novel partner proteins of mitochondrial import components [62].
- Characterization of translocation channels by electrophysiological measurements using planar lipid bilayer techniques [13].
- Single-particle electron microscopy analysis of translocase complexes and high-resolution structures of individual components provide structural and mechanistic insight [2,4].

bacterial ancestor of mitochondria, and proteins with α-helical transmembrane segments, which probably derive from the eukaryotic host cell. Although the TOM complex is the main entry gate for the large majority of mitochondrial precursor proteins, further machineries are needed to insert proteins into the outer membrane.

In the so-called β-barrel pathway, β-barrel proteins are inserted into the outer membrane via the SAM complex (Figure 2). First, the precursors of β-barrel proteins use TOM receptors and the Tom40 channel for translocation to the intermembrane space side of the outer membrane [18,29]. Here, small TIM chaperone complexes bind to the precursors and guide them to the SAM complex. The sorting signal in β-barrel proteins consists mainly of a carboxy-terminal β-strand that directs the precursor proteins to Sam50 (also known as Tob55) and Sam35 (Tob38), which are core components of the SAM complex [35]. Sam50 is conserved from bacteria (in which it is known as Omp85 or BamA) to humans [19,20,36] and the basic mechanisms of β-barrel sorting are shared by bacteria and mitochondria [35,37]. In fact, some bacterial β-barrel proteins can be assembled into the mitochondrial outer membrane when expressed in yeast [38].

The α-helical insertion machinery of the outer membrane is the most recently defined import pathway into mitochondria (Figure 2). Mim1, the core of the machinery [24,25], contains one α-helical transmembrane segment and forms a large oligomeric complex that is the functional unit interacting with precursor proteins. Most Tom proteins (which contain a single α-helical transmembrane segment) and multi-spanning outer membrane proteins use Mim1 on their insertion pathway [24–26]. The precursors for the two types of proteins are probably not transported via the Tom40 channel and thus do not use the main translocation route across the outer membrane that is common to the other import pathways [24,25,39,40]. However, Mim1 transiently interacts with the receptor Tom70 in the import of multi-spanning outer membrane precursors (Figure 2) [24]. The exact function of Mim1 is not known, but it might form channel-like structures or serve as scaffold for the insertion of precursor proteins at the protein–lipid interphase.

Although the SAM and Mim1 complexes function independently during the membrane insertion of precursor proteins, they can cooperate in the assembly of some outer membrane complexes. The best-studied case is the biogenesis of the TOM complex, which consists of the central β-barrel protein Tom40 and several α-helical subunits (three receptors and three small Tom proteins). A fraction of Mim1 complexes interact with a subpopulation of the SAM complex and thereby deliver newly inserted small Tom proteins to SAM. The small Tom proteins then assemble with the precursor of Tom40 directly on the SAM complex [41,42]. We propose that, in addition to its basic and conserved function in β-barrel insertion, the SAM complex acquired further functions during evolution to become an assembly platform for the TOM complex and possibly other outer membrane complexes. The SAM complex does not function as a single stable unit but shows a dynamic behavior with several modular forms. An example for the versatility of the SAM complex is provided by the biogenesis of Tom22. This α-helical protein is not inserted into the outer membrane by the Mim1 complex but by a special form of the SAM complex that contains the morphology protein Mdm10 (Figure 2) [43–47].

Thus, the mitochondrial outer membrane contains distinct machineries for insertion of β-barrel and α-helical precursors. For the import of some precursor proteins and, in particular, for the assembly of protein complexes, the machineries can cooperate, which supports the view of a dynamic organization of the mitochondrial protein import machinery.

Redox-regulated protein import into the intermembrane space

A few intermembrane space proteins are imported via the presequence pathway. The preproteins are laterally released from the TIM23 complex, and the mature proteins are cleaved off by an intermembrane space-exposed peptidase (Figure 1). However, the majority of intermembrane space proteins use the MIA pathway, during which the Mia40 protein interacts with the precursor proteins via disulfide bonds [21,23]. Many intermembrane space proteins contain characteristic cysteine motifs, and the mature proteins are typically oxidized, i.e. contain intramolecular disulfide bonds. The precursors are transported from the cytosol and through the TOM complex in a

reduced state. As soon as the precursors emerge on the intermembrane space side, Mia40 binds to a cysteine-containing signal of the precursor and thus forms a transient disulfide bond with the precursor during translocation (Figure 2) [48,49]. Mia40 forms the core of an oxidative import and folding machinery that functions as disulfide relay. The sulfhydryl oxidase Erv1 catalyzes the formation of disulfide bonds in Mia40, which then oxidizes cysteines in the precursor proteins, resulting in new disulfide bonds. The electrons that are removed by oxidation from the substrate proteins flow via Mia40 to Erv1, and from here to the respiratory chain via cytochrome *c* [23,30,50–53]. Mia40, Erv1 and the substrate protein can form a ternary complex, which may promote the efficient transfer of disulfide bonds (disulfide channeling) [30].

The basic principle of oxidative protein folding resembles the disulfide relay systems that are found in the periplasm of bacteria and in the endoplasmic reticulum (ER). However, mitochondria use the disulfide relay not only for protein folding but also for import of the precursor proteins. Mia40 thus functions as a receptor that recognizes the precursors and drives completion of translocation into the intermembrane space.

Integration of protein import machineries into a network of organellar and cellular functions

In addition to discovering new protein import routes into mitochondria, recent studies suggested unexpected connections of the preprotein translocases to various other mitochondrial systems. In this section, we summarize these observations and then propose a hypothesis on the organization of the protein import system.

Regarding the presequence pathway, the TIM23 complex is physically connected to the mitochondrial respiratory chain (Figure 1) [54–56]. Tim21 binds to a supercomplex formed by respiratory complex III (bc_1-complex) and complex IV (cytochrome *c* oxidase). In addition, Pam16 (also known as Tim16) and Pam18 (Tim14), essential cochaperones of mtHsp70, bind to this supercomplex, yet independently of Tim21. The interaction of the presequence translocase with respiratory complexes promotes the membrane potential-driven step of preprotein insertion, which suggests an energetic coupling [54], although the exact molecular mechanism is unknown.

Upon arrival in the matrix, presequences are proteolytically removed from the imported proteins by the mitochondrial processing peptidase (MPP). This process affects the half-life of the proteins [11] because the amino-terminal amino acid residue of proteins is closely related to the regulation of proteolytic degradation (the N-end rule) [57]. Since MPP removes the amino-terminal presequences, new amino-termini of the imported proteins are generated that may contain a stabilizing or destabilizing amino acid (bulky hydrophobic residues are typically destabilizing). Destabilizing residues can be removed by two peptidases that function after MPP, the intermediate cleaving peptidase Icp55 (which removes a single amino acid) or the mitochondrial intermediate peptidase Oct1 (which removes an octapeptide) (Figure 1) [11,58]. Thus, the processing of imported proteins is connected to protein turnover and quality control.

A link to protein turnover was also reported for the carrier pathway. Tim54, which functions as docking site for the Tim9-Tim10-Tim12 chaperone complex at the carrier translocase [59] (Figure 1), is also crucial for the assembly of the *i*-AAA protease, a large ATP-dependent protease of the inner membrane that is exposed to the intermembrane space [60]. The monomeric precursor of the *i*-AAA protease (Yme1) is imported via the presequence translocase, but its subsequent assembly to form a large oligomeric form requires Tim54. Because the *i*-AAA protease is important for quality control of inner membrane proteins [61], Tim54 connects pathways of protein import, assembly and turnover [60].

As we mentioned before, the MIA system is connected to the respiratory chain by the flow of electrons to cytochrome *c* oxidase (Figure 2). In addition, Mia40 is linked to the inner membrane protein mitofilin [62]. Mitofilin is part of a large protein complex, the mitochondrial inner membrane organizing system (MINOS, also known as MitOS or MICOS), that is required to maintain the typical tubular invaginations (cristae) of the inner membrane [62–65]. MINOS components transiently interact with Mia40 and the protein translocases TOM and SAM of the outer membrane, thus linking protein import and membrane morphology [62,64–67].

An unexpected link to mitochondrial morphology and membrane organization has also been reported for the SAM complex. Mdm10, a subunit of the SAM complex that is active in the biogenesis of Tom22, is present in another protein complex together with other morphology proteins [43,45,68,69]. Whereas initial genetic approaches showed that these morphology proteins are functionally linked to inner membrane components and to the stability of mitochondrial DNA [68,70,71], it has recently been shown that they form ER-mitochondria junctions. One of the proteins, Mmm1, is anchored in the ER membrane and forms a stable complex with several mitochondria-bound proteins, such as Mdm10 and the calcium-binding GTPase Gem1. The complex has thus been termed ER-mitochondria encounter structure (ERMES) (Figure 2) [72–74]. The ER–mitochondria junctions are possibly involved in transfer of lipids and calcium ions between the two organelles and may also be connected to the mitochondrial inner membrane and matrix [75,76]. Thus, ERMES and SAM may be part of a large organizing center that integrates diverse functions such as biogenesis of proteins and lipids, ion transport, membrane dynamics and mitochondrial DNA (mtDNA) organization. The mitofilin/MINOS system of the inner membrane may also be involved in this organizing center [62,64–67].

In addition, mitochondrial preprotein translocases are regulated by post-translational modification. The TOM complex and Mim1, which together are responsible for the import of the vast majority of mitochondrial proteins, are regulated through phosphorylation by cytosolic kinases [77]. Casein kinase 2 plays an important stimulatory role, whereas protein kinase A causes an inhibition of Tom receptor activity. Further cytosolic kinases are probably involved [77]. Thus, the main mitochondrial entry gate does not function autonomously, but is controlled by a network of cytosolic kinases.

At first glance, the multiple connections of the mitochondrial protein import machinery to so many different systems are rather confusing. So, we propose the hypothesis of an integrative mitochondrial network. During evolution of mitochondria from their bacterial ancestor, many mitochondrial genes were transferred to the nucleus and thus the proteins had to be imported from the cytosol together with new proteins that were provided by the eukaryotic cell. Therefore, mitochondria and the eukaryotic cell had to develop efficient protein import systems [1–3]. In parallel, mitochondria had to integrate into the eukaryotic cell, which involved numerous changes in the regulation of bioenergetic activities, membrane dynamics, mtDNA organization, protein quality control and establishment of mitochondrial contacts with other membranes and organelles of the cell. We propose that these various processes did not develop independently but co-evolved, and thus protein import became connected with seemingly unrelated systems. The machineries that perform major mitochondrial activities became integrated into a functional network of dynamic interactions. Many of these systems can function independently in biochemical *in vitro* assays but they are intimately linked *in vivo* and only their combination yields a fully active mitochondrion.

Concluding remarks

The analysis of mitochondrial protein import has provided exciting new insights into a highly complex system of organellar biogenesis and function. However, many more unexpected findings can be anticipated, as many open questions remain (Box 2). We speculate that further import pathways may exist; for example, some small proteins of the inner membrane are imported without a clear requirement for a membrane potential $\Delta\psi$ [78], whereas a $\Delta\psi$ is strictly needed for the two known translocases of the inner membrane (presequence translocase and carrier translocase). Our hypothesis of an integrative mitochondrial network predicts that the protein transport system does not act autonomously but is embedded in a large, dynamic network of mitochondrial and cellular systems. Such an integrative network may serve as a paradigm for the development and organization of protein transport systems in other cell organelles.

Box 2. Questions for future research

- Which cytosolic factors are crucial for guiding precursor proteins or mRNAs to mitochondria?
- How many other mitochondrial protein transport pathways await discovery?
- Are all protein translocases subject to post-translational regulation, and which kinases, phosphatases and other modifying enzymes are involved?
- How are protein translocases and ER-mitochondria junctions connected at a molecular and mechanistic level?
- How are structures involved in organization of the mitochondrial inner membrane coupled to protein transport?
- By which molecular mechanism does coupling of the presequence translocase to respiratory chain complexes promote protein translocation?
- Which role do lipids play in mitochondrial protein import, and are protein biogenesis and lipid biosynthesis coordinated?

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