Mitochondrial protein import: from proteomics to functional mechanisms

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Abstract | Mitochondria contain ~1,000 different proteins, most of which are imported from the cytosol. Two import pathways that direct proteins into the mitochondrial inner membrane and matrix have been known for many years. The identification of numerous new transport components in recent proteomic studies has led to novel mechanistic insight into these pathways and the discovery of new import pathways into the outer membrane and intermembrane space. Protein translocases do not function as independent units but are integrated into dynamic networks and are connected to machineries that function in bioenergetics, mitochondrial morphology and coupling to the endoplasmic reticulum.

α-proteobacterium
A Gram-negative (outer membrane-containing) bacterium, such as *Rickettsia* spp., that is probably the closest living bacterial relative of mitochondria.

According to the endosymbiont hypothesis, mitochondria are derived from an ancient α-proteobacterium that was taken up by a primordial eukaryotic cell ~1.5–2 billion years ago[1]. Mitochondria have a central role in energy conversion and are thus termed the powerhouses of eukaryotic cells. The complexes of the respiratory chain in the mitochondrial inner membrane use energy, which is gained from the oxidation of food molecules, to pump protons across the membrane and generate a membrane potential ($\Delta\psi$). The proton gradient is then used to drive the mitochondrial ATP synthase that produces the bulk of ATP for the cell. Additionally, however, eukaryotic cells have intimately integrated mitochondria into multiple metabolic and signalling pathways[2–4]. Mitochondria have crucial roles in the metabolism of amino acids and lipids and the biosynthesis of haem and iron–sulphur clusters. Studies in recent years identified an unexpectedly large number of signalling molecules located in or at mitochondria and revealed a central function of mitochondria in pathways leading to programmed cell death.

Like Gram-negative bacteria, mitochondria contain two membranes (an outer membrane and an inner membrane) and two aqueous spaces (the intermembrane space and the matrix) (FIG. 1). Mitochondria have retained a complete genetic system in the matrix; however, only ~1% of mitochondrial proteins are encoded by the mitochondrial genome and synthesized in the matrix. Mitochondrion-encoded proteins form a few subunits of the respiratory chain complexes and are typically inserted into the inner membrane by an export and assembly machinery, called the oxidase assembly (OXA) machinery[1,5].

99% of mitochondrial proteins are encoded by nuclear genes. These include genes that were transferred from the endosymbiont to the nucleus and genes for new mitochondrial proteins that arose during eukaryotic evolution. In present-day organisms, the nuclear genome codes for all mitochondrial outer membrane and intermembrane space proteins, as well as for most inner membrane and matrix proteins[1,2,5].

Nucleus-encoded mitochondrial proteins are synthesized as precursor proteins on cytosolic ribosomes and imported into the organelle[1,5]. Until 2003, only two main protein import pathways into the inner membrane and matrix were known. Since then, the studies on mitochondrial protein biogenesis have received a major boost with the identification of numerous new import components and import pathways to the intermembrane space and outer membrane. The rapid development of the mitochondrial protein import field is an excellent example of how a systematic proteomic analysis can provide novel insight into a complex cellular process when combined with genetics and functional biochemical studies. In this Review, we first give an overview of the protein import pathways and summarize which approaches led to the discovery of new mitochondrial import components and entire import pathways. Then we discuss the functional implications for the mechanisms of protein sorting and the integration of protein translocases into a dynamic network of interactions.

Overview of mitochondrial protein import

Nucleus-encoded mitochondrial precursor proteins possess targeting signals that are recognized by receptors on the mitochondrial surface. The targeting signals then direct the precursors to their functional destination in the mitochondrial subcompartments. Two main groups of targeting signals can be distinguished[1,5]. The first group is the amino-terminal extensions of precursors,

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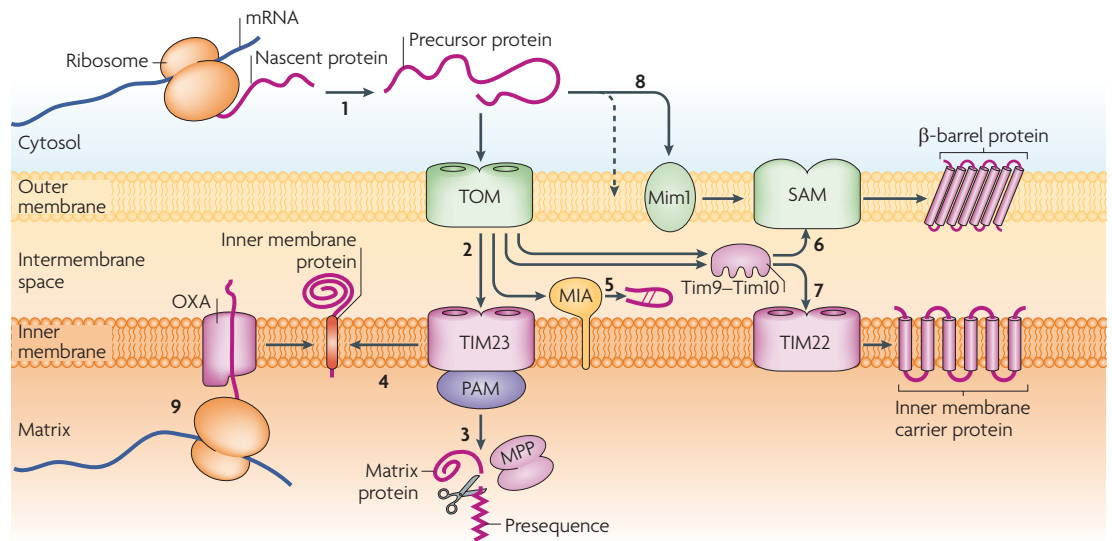*e-mails: nikolaus.pfanner@biochemie.uni-freiburg.de; chris.meisinger@biochemie.uni-freiburg.de*

Figure 1 | **Biogenesis pathways of mitochondrial proteins.** Most mitochondrial proteins are synthesized on cytosolic ribosomes and imported through the translocase of the outer mitochondrial membrane (TOM) complex (1). After passage through the TOM channel, the precursor proteins can use different sorting machineries (2). Presequence-carrying proteins destined for the matrix are imported by the presequence translocase of the inner membrane (TIM23) complex and presequence translocase-associated motor (PAM). Mitochondrial processing peptidase (MPP) removes the presequences (3). Some proteins are laterally released from the TIM23 complex into the lipid phase of the inner membrane (4). The mitochondrial intermembrane space assembly (MIA) machinery drives the import and oxidative folding of many intermembrane space proteins (5). The Tim9–Tim10 chaperone complex transfers hydrophobic precursor proteins through the intermembrane space to either the sorting and assembly machinery (SAM) complex, in the case of β-barrel proteins of the outer membrane (6), or through the carrier pathway to the TIM22 complex (7). The precursors of α-helical outer membrane proteins do not use the TOM channel but are inserted into the membrane by different pathways, some involving mitochondrial import 1 (Mim1; 8). A few proteins are synthesized on matrix ribosomes and are exported into the inner membrane by the oxidase assembly (OXA) machinery (9).

which are the classical mitochondrion-targeting signals. These presequences are usually proteolytically removed after import into mitochondria. Second, many precursor proteins are not synthesized with cleavable extensions but contain internal targeting signals that remain part of the mature protein. This second group includes different types of precursor proteins and targeting signals.

Currently, at least five main classes of precursor proteins are known, each class following a different import route into mitochondria (FIG. 1). The common entry gate for most precursors is formed by the translocase of the outer membrane (TOM) complex[1,5–8], but after passing through the TOM complex, the precursors use different pathways to the mitochondrial subcompartments. The four main pathways are described below, but the insertion of α-helical proteins into the outer membrane probably involves several other pathways that have only been partly characterized.

Presequence pathway to the matrix and inner membrane. Cleavable precursors (also termed preproteins) are transferred from the TOM complex to the presequence translocase of the inner membrane (TIM23) complex[1,5,7]. From here they are either laterally released into the inner membrane or are completely imported into the matrix with the help of presequence translocase-associated motor (PAM). Most matrix proteins, including many metabolic enzymes, are synthesized with cleavable presequences.

Carrier pathway to the inner membrane. Many inner membrane proteins that contain multiple transmembrane segments are synthesized with internal targeting signals. The main group is formed by metabolite carriers such as the ADP and ATP carrier. These hydrophobic proteins are imported through the TOM complex, chaperone complexes of the intermembrane space and the carrier translocase, the TIM22 complex[9–11].

Oxidative folding pathway of the intermembrane space. Many proteins of the intermembrane space contain internal targeting signals and characteristic Cys motifs. The precursors are imported by the TOM complex and the mitochondrial intermembrane space assembly (MIA) machinery that oxidizes the Cys residues[12–14].
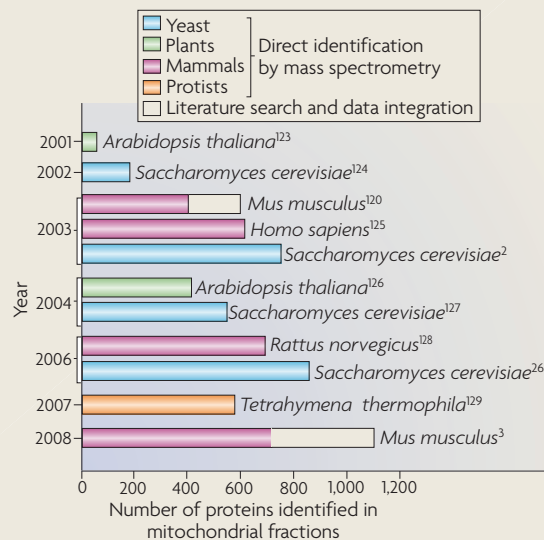
Transport pathways of outer membrane proteins. All proteins of the outer mitochondrial membrane are synthesized as non-cleavable precursors carrying internal targeting signals. The outer membrane possesses two types of membrane-integrated proteins: α-helical proteins (for example receptors), which are anchored in the outer membrane by one or more transmembrane α-helical segments, and pore-forming β-barrel proteins that consist of multiple transmembrane β-strands. β-barrel membrane proteins have been found only in the outer membranes of bacteria, mitochondria and chloroplasts; all other membranes, including the mitochondrial inner membrane,

Box 1 | **New import components and the mitochondrial proteome**

Three lines of research were mainly responsible for the identification of new components of the mitochondrial protein import machinery. First, translocase complexes were isolated under mild conditions such that loosely associated subunits remained associated. Sensitive mass spectrometry techniques together with the availability of completely sequenced genomes then led to the direct identification of new subunits, in particular of the presequence translocase of the inner membrane complex, TIM23, and presequence translocase-associated motor (PAM)[19–21]. Second, native gel techniques such as blue native electrophoresis[22] are powerful tools for the efficient separation of intact membrane protein complexes. Import intermediates of precursor proteins could thereby be visualized, leading to the identification of the β-barrel pathway[15] and the dynamic composition of the TIM23 complex[23–25]. Third, the large-scale identification of the mitochondrial proteome yielded many proteins of unknown function that were analysed by a combination of yeast genetics and functional biochemical assays.

Purified mitochondria proved to be an excellent substrate for a systematic mass spectrometry analysis. Within a few years, most *Saccharomyces cerevisiae* and several mammalian mitochondrial proteins were identified. Although previous studies had the problem that proteins of low abundance and membrane proteins were underrepresented, a systematic analysis of the most comprehensive proteome of *S. cerevisiae* mitochondria with ~850 identified proteins revealed that the use of different separation techniques (such as chromatographic steps, and one- and two-dimensional gels) and the high sensitivity of mass spectrometry overcame the bias against those proteins, and all classes of proteins analysed were successfully identified[26]. In addition, some proteomic studies included detailed literature searches and integration of data from different sources to maximize the number of identified proteins[3,120] (see the figure).

The next level of proteomic analysis of mitochondria includes several approaches, such as the analysis of mitochondrial subcompartments (such as the outer membrane proteome)[109,121], the determination of protein modifications (for example, the mitochondrial phosphoproteome)[118,119] and the identification of the amino termini of proteins[28,122], and thus a large-scale determination of presequences (the mitochondrial N-proteome)[28].

contain α-helical membrane proteins only. The mitochondrial import pathway for β-barrel proteins has been identified and involves the TOM complex, intermembrane space chaperones and the sorting and assembly machinery (SAM) complex of the outer membrane[15–18]. The β-barrel pathway thus constitutes the fourth protein import pathway into mitochondria.

Functional proteomics of mitochondria

How were so many new mitochondrial import components identified in recent years? A combination of several approaches, including sensitive mass spectrometry, yeast genetics, isolation of translocase complexes under mild conditions and analysis of import intermediates by native gel systems, have yielded most identifications[19–26]. Mitochondria are the first cell organelle for which a comprehensive proteomic analysis has been achieved[27] (BOX 1). Currently, ~85% of the ~1,000 yeast mitochondrial proteins[26] and 70–75% of the ~1,500 mammalian mitochondrial proteins have been identified[3]. The functional classification of the mitochondrial proteome and the remarkable variety of functions carried out by mitochondria are discussed in BOX 2.

The mitochondrial proteome includes several proteins of unknown function. How is it possible to find potential new components of the protein import machinery among them? The genetic possibilities of yeast proved to be of great value. After sequencing the genome of *Saccharomyces cerevisiae* as the first eukaryotic genome, a joint effort of yeast laboratories yielded a large collection of gene deletion mutants with phenotypic analyses. Organisms like yeast that are capable of fermentative growth can live without a respiratory chain, but the mitochondrial protein import machinery is essential for cell viability[4,5]. Thus, a particular focus was put on the mitochondrial proteins of unknown function that are encoded by genes essential for life. Conditional mutants of the candidates were analysed for protein import into mitochondria and several new import components were identified, including SAM and PAM subunits[16–21]. A major finding of these functional proteomic analyses was the identification of the MIA pathway of the intermembrane space[12–14].

Presequence pathway and processing

The presequence pathway is responsible for the import of nearly all mitochondrial matrix proteins and a considerable fraction of inner membrane proteins. The presequences are located at the N termini of preproteins and typically consist of ~15–50 amino acids (FIG. 2a). Presequences are positively charged and form amphipathic α-helices that are recognized by TOM and TIM23 complexes in a sequential manner[1,5–7]. Thus, presequences direct the preproteins to mitochondria and across outer and inner membranes into the matrix. Preproteins destined for the inner membrane
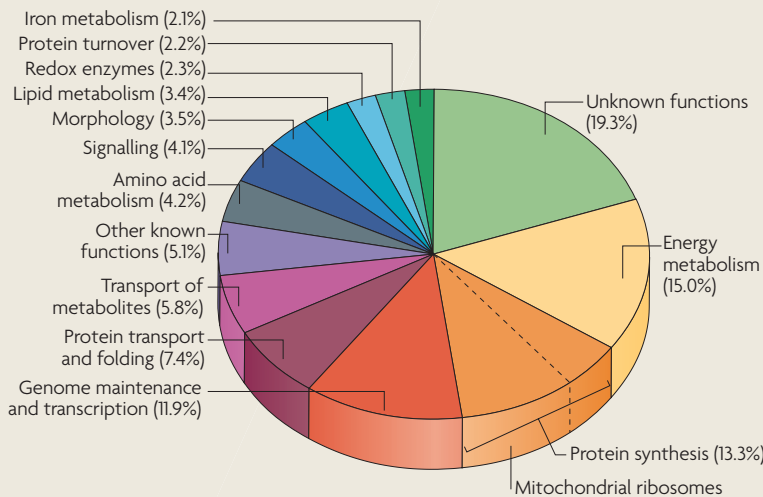
Box 2 | **Functional classification of the mitochondrial proteome**

The presumed functions of the ~850 proteins of the yeast mitochondrial proteome[26] are assigned according to the *Saccharomyces* genome database (SGD; January 2010)[130] (see the figure, part **a**). Remarkably, the classical textbook function of mitochondria in energy metabolism, including the respiratory chain complexes, mitochondrial ATP synthase and citric acid cycle (Krebs cycle), is carried out by only ~15% of the different proteins. Mitochondria carry out various functions, from many metabolic processes to protein transport and turnover, maintenance of membrane morphology, signalling and redox processes. For more than 160 proteins (19%), no reliable information on their function is available.
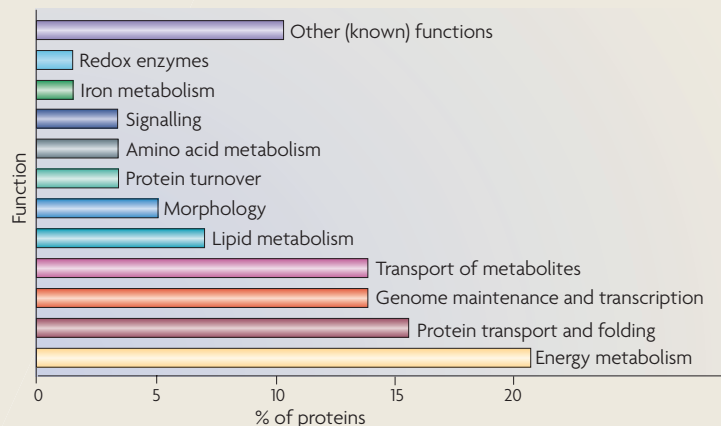
A comparison of the functional classification of ~750 mitochondrial proteins in 2003 (REF. 2) to the ~850 proteins classified in 2010 shows the areas of active research on new mitochondrial proteins. From 2003–2010, functions could be assigned to ~60 mitochondrial proteins (see the figure, part **b**). Areas of particularly high activity were energy metabolism, protein transport and folding, genome maintenance and transport of metabolites.

Because of the rapid identification of new mitochondrial import components in several laboratories, some of the components were given more than one name. Examples are topogenesis of outer membrane β-barrel proteins (TOBs) for the sorting and assembly machinery (SAM) complex components and translocase of the inner membrane 14 (Tim14)–Tim16 for the presequence translocase-associated motor 18 (Pam18)–Pam16 module of the import motor PAM. The components were typically identified in *Saccharomyces cerevisiae*, and the SGD[130] provides an excellent platform for the exact assignment and curation of yeast genes and proteins, including all alias names. To avoid any confusion, we use the SGD standard nomenclature in this Review. The SGD names also serve as an unambiguous basis for the standard nomenclature of mitochondrial import components in other organisms.

a *Saccharomyces cerevisiae* mitochondrial proteome (2010)

Iron metabolism (2.1%)
Protein turnover (2.2%)
Redox enzymes (2.3%)
Lipid metabolism (3.4%)
Morphology (3.5%)
Signalling (4.1%)
Amino acid metabolism (4.2%)
Other known functions (5.1%)
Transport of metabolites (5.8%)
Protein transport and folding (7.4%)
Genome maintenance and transcription (11.9%)
Unknown functions (19.3%)
Energy metabolism (15.0%)
Protein synthesis (13.3%)
Mitochondrial ribosomes

b Novel functions of *Saccharomyces cerevisiae* mitochondrial proteins (2003–2010)

Other (known) functions
Redox enzymes
Iron metabolism
Signalling
Amino acid metabolism
Protein turnover
Morphology
Lipid metabolism
Transport of metabolites
Genome maintenance and transcription
Protein transport and folding
Energy metabolism

Function

% of proteins

additionally contain a hydrophobic sorting signal that arrests translocation in the inner membrane. A large-scale determination of the N termini of mature mitochondrial proteins (the N-proteome) suggested that 60% or more of all mitochondrial proteins may be synthesized with N-terminal extensions[28].

Import by TOM and TIM23 complexes. The TOM complex comprises the central component Tom40, three preprotein receptors, Tom20, Tom22 and Tom70, and several small TOM proteins[5–7,29] (FIG. 2a). The initial recognition of presequences on the mitochondrial surface occurs by the receptors Tom20 and Tom22. Whereas Tom20 binds to the hydrophobic surface of the presequence, Tom22 recognizes the positively charged surface. The preproteins are translocated through the import channel formed by the β-barrel protein Tom40. Preproteins using the presequence import pathway cross the membranes as linear polypeptide chains that may adopt an α-helical and/or extended conformation. On the intermembrane space side of the outer membrane, the preproteins interact with the intermembrane space tail of the receptor Tom22.

Translocation across the inner mitochondrial membrane is mediated by the presequence translocase, the TIM23 complex. Whereas the channel-forming Tim23 and associated partner Tim17 have been known to be a part of this complex for over 15 years, the identification of Tim50 and Tim21 by proteomic approaches provided important insight into the cooperation of the TIM23 and TOM complexes during preprotein transfer. Tim21, Tim50 and Tim23 expose domains to the intermembrane space that transiently connect the TOM and TIM23 complexes[19,30–32] (FIG. 2a).

Tim50 is the first component of the inner membrane that binds to the preprotein emerging in the intermembrane space. Tim50 is tightly associated with the intermembrane space domain of Tim23 (REFS 31,32) and thus the information about the entry of a preprotein into the intermembrane space can be directly transferred to the import channel. Moreover, Tim50 also plays an important part in the absence of preproteins[33]. The channel formed by Tim23 is large enough to permit the translocation of a polypeptide chain in an α-helical conformation[34]. In the absence of a preprotein in transit, an open Tim23 channel would lead to a major leakage of ions across the inner membrane and thus to a dissipation of the Δψ. Therefore, the opening and closing of the Tim23 channel has to be tightly regulated. The intermembrane space domain of Tim50 induces channel closure[33]. Tim50 releases the channel block only on arrival of a presequence allowing preproteins to pass through the channel. When the polypeptide chain has traversed the channel, Tim50 induces its closing and the next round of translocation will be initiated when a new preprotein emerges in the intermembrane space[31–35].

Tim21 binds to the intermembrane-space tail of Tom22 and thus, like Tim50 and Tim23, contributes to the direct but transient connection between the translocases of the outer and inner membranes[23,31,36,37]. The currently available evidence suggests that Tim21 does not bind preproteins but rather plays a regulatory part in preprotein
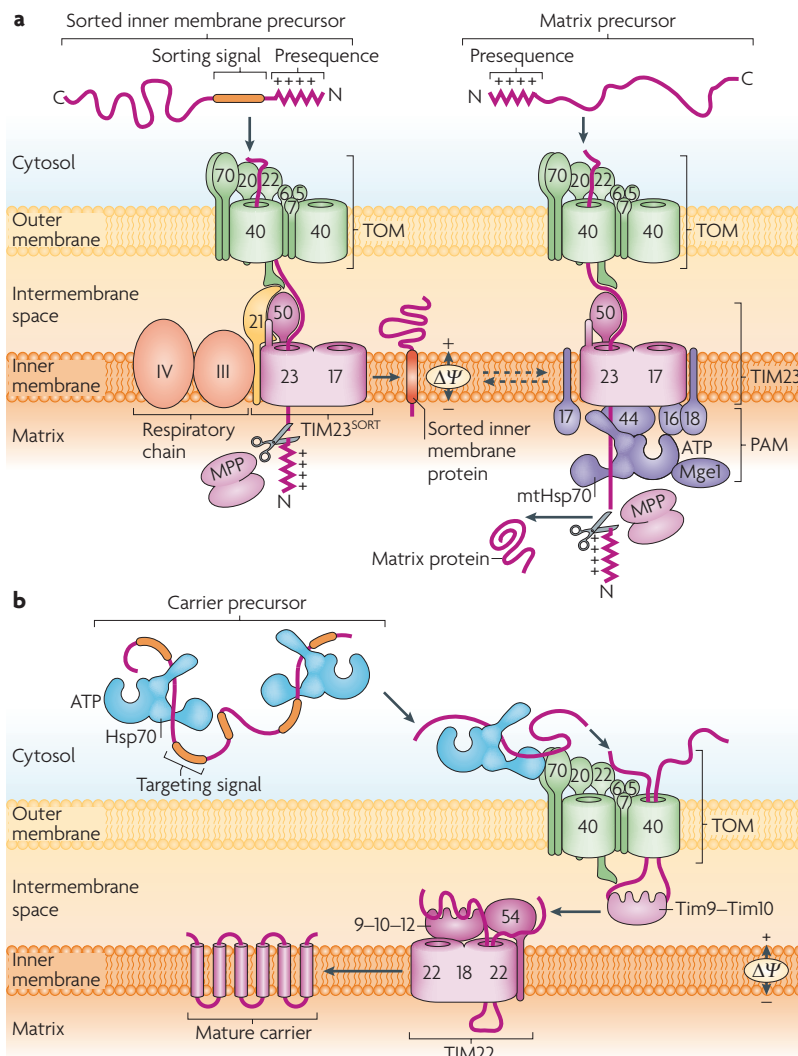
Figure 2 | **Classical routes of protein import: the presequence pathway and the carrier pathway. a** | The presequence pathway. The precursor proteins are imported by the translocase of the outer membrane (TOM) complex, which involves recognition by the receptors Tom20 and Tom22 and translocation through the Tom40 channel. The presequence translocase of the inner membrane complex, TIM23, which comprises Tim23, Tim17 and Tim50, functions in two modular forms. TIM23^{SORT} additionally contains Tim21, which transiently binds to the TOM complex and a supercomplex of the respiratory chain (left). TIM23^{SORT} mediates the lateral release of preproteins with hydrophobic sorting signals into the inner membrane. The other TIM23 form lacks Tim21 but interacts with presequence translocase-associated motor (PAM) and transports proteins into the matrix (right). This form is called TIM23–PAM. The membrane potential ($\Delta\psi$) drives translocation of the presequence through the TIM23 complex and ATP powers the chaperone mtHsp70 (matrix 70 kDa heat shock protein) of PAM. Mitochondrial processing peptidase (MPP) removes the presequences. **b** | The carrier pathway. The precursors of metabolite carriers contain internal targeting signals. Cytosolic chaperones (such as Hsp70) prevent aggregation of the precursors. The receptor Tom70 possesses binding sites for precursors and chaperones. After translocation through Tom40, the precursors are transferred by the Tim9–Tim10 chaperone through the intermembrane space. The carrier translocase of the inner membrane, the TIM22 complex, drives the membrane insertion of imported proteins in a $\Delta\psi$-dependent reaction.

transfer from the outer membrane to the inner membrane. Tim21 competes with presequences for binding to Tom22. Thus, the binding of Tim21 to Tom22 leads to a release of the preprotein and promotes its further transfer towards the Tim23 channel.

The $\Delta\psi$ across the inner membrane is crucial for translocation of the presequences through the Tim23 channel. Two roles have been assigned to $\Delta\psi$: activation of Tim23 and an electrophoretic effect that drives the import of presequences ($\Delta\psi$ is negative on the matrix side of the inner membrane and presequences are positively charged)[7,33,34].

Coupling of TIM23 to motor and respiratory chains.
Whereas it has been shown that the purified and reconstituted TIM23 complex itself can carry out the basic function of protein membrane insertion[38], the complex cooperates with three further machineries *in organello*. In addition to the transient interaction with the TOM complex, the TIM23 complex cooperates with the import motor PAM and respiratory chain complexes[7,24,25].

On translocation of the presequence across the inner membrane, preproteins can follow one of two routes. They are either laterally released into the inner membrane or completely imported into the matrix[5,7]. Whereas the matrix-targeted preproteins are hydrophilic and the N-terminal presequence usually contains the necessary information for import (FIG. 2a; right), inner membrane-sorted preproteins possess at least one hydrophobic transmembrane segment (FIG. 2a; left). The hydrophobic segment functions as a sorting signal that arrests translocation in the TIM23 complex (stop–transfer) and induces lateral release of the preprotein into the lipid phase of the membrane.

Two forms of the TIM23 complex have been identified. One form, termed TIM23^{SORT}, contains Tim21 in addition to the three core components, Tim50, Tim23 and Tim17. TIM23^{SORT} is involved in the early stage of preprotein transfer from the TOM to the TIM23 complex, and in the lateral release of preproteins with an inner membrane-sorting signal[23,38,39] (FIG. 2a; left). The other form, termed TIM23–PAM, functions in the translocation of preproteins into the matrix. This form is associated with the subunits of PAM, but lacks Tim21 (REFS 23,39) (FIG. 2a; right).

Tim21 has a dual role[23,36,39,40]. It not only binds to the TOM complex but is also involved in the interaction of the TIM23^{SORT} complex with the respiratory chain (FIG. 2a; left). Tim21 alternates between binding Tom22 and a supercomplex of the respiratory chain that contains two proton-pumping complexes, complex III (also called the cytochrome bc_1 complex) and complex IV (also called cytochrome oxidase)[40]. The exact molecular function of TIM23^{SORT}–respiratory chain coupling has not been elucidated, but the available results suggest that the close vicinity of TIM23^{SORT} to proton-pumping complexes ensures an efficient use of the electrochemical proton gradient for preprotein translocation[24,25,40].

For translocation of hydrophilic preproteins into the matrix, Tim21 (and the respiratory chain complexes) is released from the TIM23 complex, whereas the motor PAM associates with the translocase. The core of PAM is formed by the molecular chaperone mtHsp70 (matrix 70 kDa heat shock protein), which binds the unfolded polypeptide chain and drives its translocation into the matrix in an ATP-powered manner[5,7]. mtHsp70 cooperates with several

co-chaperones. For a long time, only two co-chaperones of mtHsp70 were known: the nucleotide exchange factor Mge1 (also known as mitochondrial GrpE), which stimulates the release of ADP from mtHsp70; and Tim44, which is a docking site for mtHsp70 at the TIM23 complex (FIG. 2a; right). Based on this limited set of motor components, two models for the mechanism of the import motor were proposed and discussed in a controversial manner: active pulling of the preprotein by conformational changes of Tim44-associated mtHsp70, and passive trapping of the preprotein simply by the binding of mtHsp70 molecules (and thus preventing a back-sliding of the polypeptide chain). Subsequently, proteomic studies led to the identification of three new membrane-associated co-chaperones, Pam16, Pam17 and Pam18, that are required to coordinate the function of mtHsp70 at the translocase of the inner membrane. Pam18 is a J-type co-chaperone that stimulates the ATPase activity of mtHsp70. The J-related Pam16 forms a complex with Pam18 and functions as a negative regulator[20,21,41–43]. Pam17 is involved in the organization of the TIM23–PAM interaction[44,45]. Thus, the mitochondrial HSP70 system is regulated by five different co-chaperones, revealing a much higher complexity of PAM function than anticipated. A combination of both mechanisms, trapping and pulling, may best explain the dynamic activity of this multisubunit motor.

The existence of two forms of the TIM23 complex has been discussed and an alternative model of a single-entity translocase in which the TIM23 complex and PAM are permanently associated was proposed[45]. The analysis of the reaction cycle of the TIM23 complex in cooperation with TOM, the respiratory chain and PAM shows that neither a single-entity translocase nor two permanently separated translocase forms are sufficient to describe the reaction mechanism, but that the different TIM23 forms are in dynamic exchange with each other[23,24,39] (FIG. 2a). For example, matrix-targeted preproteins initially use the TIM23^{SORT} complex for transfer from the TOM to the TIM23 complex and then the TIM23–PAM machinery for translocation into the matrix[39].

Processing enzymes and protein stability. On arrival in the matrix, most presequences are proteolytically removed by a heterodimeric enzyme, mitochondrial processing peptidase (MPP)[46]. Both subunits of MPP are essential for cell viability, underscoring the crucial function of MPP in mitochondrial biogenesis. Several preproteins are processed a second time by inner membrane-bound enzymes or matrix-located enzymes (BOX 3).

Some preproteins that are sorted to the intermembrane space carry a bipartite presequence, consisting of a positively charged matrix-targeting signal and a hydrophobic sorting signal that arrests translocation in the inner membrane. The matrix-targeting signal is removed by MPP, but the sorting signal is cleaved off by inner membrane peptidase (IMP), which has its active centre on the intermembrane-space side of the inner membrane, and thus the mature protein is released into the intermembrane space. In a few cases, the sorting signal is not processed by IMP but by the rhomboid protease processing of cytochrome *c* peroxidase 1 (Pcp1), which

cleaves in the membrane[47–50] (BOX 3). Cleavage by Pcp1 is of particular importance for the formation of an isoform of the inner membrane fusion protein, mitochondrial genome maintenance 1 (Mgm1)[51].

Two processing enzymes can carry out a second cleavage step in the matrix. The mitochondrial intermediate peptidase Oct1 typically removes an octapeptide after cleavage by MPP[47,50], but it is unknown why this second cleavage occurs. Recently, a mitochondrial aminopeptidase was identified[28,52] — the intermediate cleaving peptidase Icp55 — that typically removes a single amino acid residue after processing by MPP. Icp55 was discovered in a systematic analysis of the N termini of mitochondrial proteins (the N-proteome), revealing a difference of one amino acid for numerous mitochondrial proteins when the mature N terminus and the MPP-cleaved product were compared[28] (BOX 3). Why should one amino acid be removed when the preceding cleavage by MPP leads to the removal of ~15–50 amino acids? It turned out that Icp55 plays an important part in the stabilization of mitochondrial proteins, leading to the discovery of an 'N-end rule pathway' of protein turnover in mitochondria. The N-end rule has been established for protein turnover in bacteria and the eukaryotic cytosol; it connects the N-terminal amino-acid residue of proteins with their half-life[53,54]. Certain large amino-acid residues at the N terminus of a protein favour its degradation by the N-end rule pathway (destabilizing residues), and other amino acids at the N terminus indicate a longer half-life (stabilizing residues). In cases where the cleavage by MPP generates an N terminus with a destabilizing amino acid, Icp55 removes this residue. Thereby, an N terminus carrying a stabilizing residue is typically generated[28]. The activity of Icp55 thus favours the stabilization of the mitochondrial proteome.

Carrier import pathway

The carrier pathway transports the hydrophobic precursors of non-cleavable proteins of the mitochondrial inner membrane. These proteins are integrated into the membrane by multiple α-helical transmembrane segments. Main representatives of these proteins are the metabolite carriers such as the ADP and ATP carrier, and the phosphate carrier, which belong to a large family of inner membrane carrier proteins that mediate the translocation of metabolites between the matrix and intermembrane space. Further examples of multi-spanning inner membrane proteins that are imported by the carrier pathway include the core subunits Tim23, Tim17 and Tim22 of the inner membrane translocases. Proteins using the carrier pathway are synthesized without cleavable presequences but contain internal segments that target the hydrophobic precursors to mitochondria and into the inner membrane[5,7]. A carrier protein contains approximately three to six discontinuous internal targeting elements that have only been partly characterized.

Chaperone-guided transport of carrier precursors. The carrier import pathway uses the same mitochondrial entry gate, the TOM complex, as the presequence pathway. However, the mechanisms of translocation differ greatly.

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70 kDa heat shock protein
An ATP-dependent molecular chaperone that is essential in unstressed and stressed cells. The chaperones bind hydrophobic segments of unfolded proteins, preventing protein aggregation and promoting protein transport and folding.

Box 3 | Mitochondrial processing enzymes and protein stability
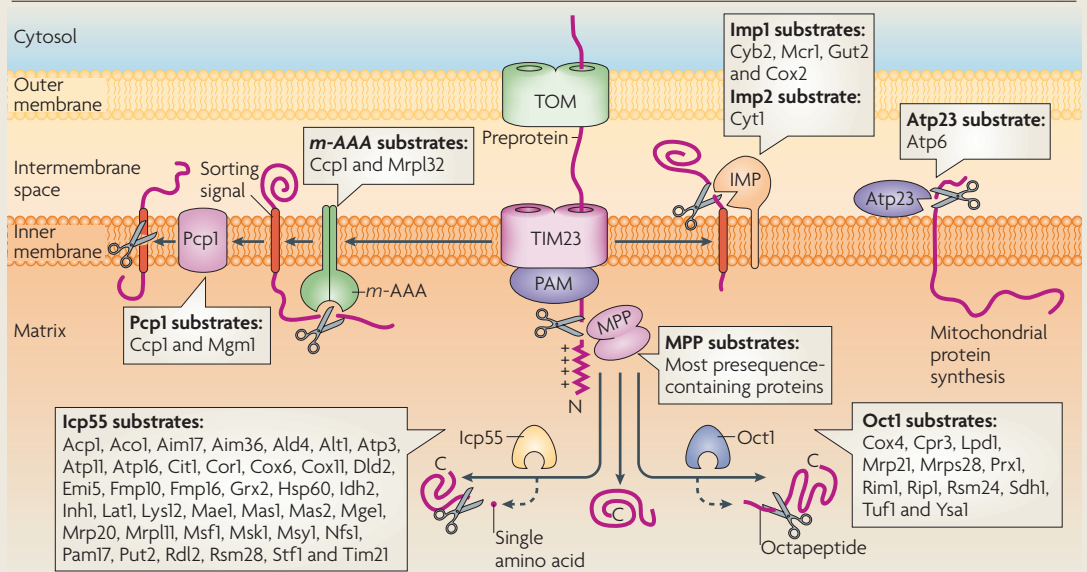
Presequence-carrying proteins are proteolytically cleaved by matrix processing peptidase (MPP), which removes the positively charged matrix-targeting sequences of most proteins (see the figure; precursor cleavage is indicated by scissors). Several proteases, such as the membrane-bound inner membrane peptidase (IMP) and the rhomboid intramembrane protease processing of cytochrome *c* peroxidase 1 (Pcp1), can carry out a second processing step (see the figure). These proteases cleave in or after hydrophobic sorting signals. The official names from the *Saccharomyces* genome database (SGD) have been used here for the known substrates of the proteases (see the figure).

In the matrix, a second processing step can be carried out by the intermediate cleaving peptidase Icp55, which removes single amino-acid residues and the matrix intermediate peptidase Oct1, which removes segments of approximately eight amino acids. Although Icp55 is the most recently identified mitochondrial peptidase, the long list of substrates indicates its importance in mitochondrial biogenesis[28]. Indeed, the aminopeptidase Icp55 removes destabilizing amino acids according to the 'N-end rule pathway' of protein degradation[53,54] and thus typically leads to the mature protein carrying a stabilizing amino acid at its amino terminus. Icp55 is peripherally associated with the inner mitochondrial membrane.

MPP does not remove all presequences as at least two preproteins are cleaved by the ATP-dependent *m*-AAA protease of the inner membrane[48,131]. Although preprotein processing by the *m*-AAA protease is not required for cell viability, it is important for mitochondrial function as one of its substrates is a subunit of the mitochondrial ribosome. Inactivation of the protease impairs ribosome assembly and leads to the human neurodegenerative disorder hereditary spastic paraplegia[131].

Proteolytic processing has also been shown for a few proteins that are encoded by the mitochondrial genome and inserted into the inner membrane: ATP synthase subunit 6 (Atp6), which is processed by the intermembrane space-exposed protease Atp23 (REFS 132,133), and cytochrome *c* oxidase subunit 2 (Cox2), which is processed by Imp1 (REF. 134) (see the figure).

Acp1, acyl carrier protein 1; Aco1, aconitate hydratase 1; Alt1, alanine aminotransferase 1; Ccp1, cytochrome *c* peroxidase 1; Cit1, citrate synthase 1; Cor1, cytochrome *b*-c1 complex subunit 1; Cyb2, cytochrome *b*2; Cyt1, cytochrome *c*1; Dld2, D-lactate dehydrogenase 2; Emi5, early meiotic induction protein 5; Fmp, found in mitochondrial proteome; Grx2, glutaredoxin 2; Hsp60, 60 kDa heat shock protein; Idh2, isocitrate dehydrogenase subunit 2; Inh1, ATPase inhibitor 1; Mae1, NAD-dependent malic enzyme 1; Mrp, mitochondrial ribosomal protein; PAM, presequence translocase-associated motor; Rsm24, mitochondrial ribosomal small subunit protein 24; Sdh1, succinate dehydrogenase flavoprotein subunit 1; Stf1, stabilizing factor 1; TOM, translocase of the outer membrane; TIM, translocase of the inner membrane.

Deafness dystonia syndrome
An X chromosome-linked neurodegenerative disease that includes deafness, cortical blindness and dystonia. It was the first human disease caused by a defect in the mitochondrial protein import machinery (specifically, in Tim8 (known as TIMM8A in humans)). It is also called Mohr–Tranebjaerg syndrome.

Molecular chaperones in the cytosol and intermembrane space are crucial to prevent aggregation of the hydrophobic carrier precursors in the aqueous environment. Chaperones of the HSP70 and HSP90 classes bind to the precursors upon their synthesis on cytosolic ribosomes. The chaperones not only guide the precursors to mitochondria but also specifically bind to the receptor Tom70 and thus ensure a direct transfer of the precursors to the import machinery of mitochondria[29,55,56] (FIG. 2b). Tom70 possesses binding sites for precursor proteins and chaperones[29,55]. ATP is needed to release the precursor

proteins from the chaperones. With the help of the receptors Tom20 and Tom22, the precursor is inserted into the Tom40 channel. In contrast to presequence-carrying preproteins, carrier precursors are not translocated as linear polypeptide chains but traverse the outer membrane in a loop structure[57]. Hexameric chaperone complexes in the intermembrane space — Tim9–Tim10 and the homologous Tim8–Tim13 — bind to the precursor proteins and transfer them through the aqueous intermembrane space to the inner membrane[10,58,59]. Defects in human TIM8 lead to deafness dystonia syndrome[60].
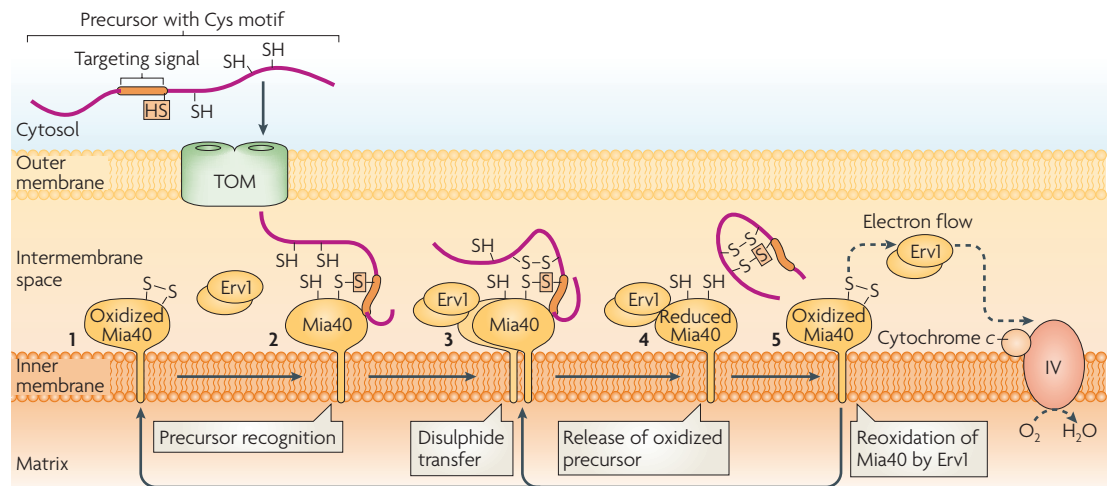
Figure 3 | **Oxidative import and folding in the intermembrane space.** The mitochondrial intermembrane space assembly (MIA) machinery is responsible for the import of small intermembrane-space proteins that contain characteristic Cys motifs (with sulphhydryl groups (SH)). MIA includes the protein disulphide carrier Mia40 and the sulphhydryl oxidase Erv1 (essential for respiration and viability 1). Erv1 oxidizes Mia40 by transferring a disulphide bond (S–S) to it (1). Oxidized Mia40 functions as a receptor for the incoming precursor protein and binds its targeting signal, forming a transient disulphide bond (2). Mia40 transfers the disulphide bond to the precursor (3). Thus, Mia40 becomes reduced (4) and is re-oxidized by Erv1 (5). For transferring several disulphide bonds to the precursor, Mia40 may shuttle between Erv1 and the precursor (known as a disulphide relay), or Erv1, Mia40 and the precursor protein may be associated in a transient ternary complex (in a process known as disulphide channelling and probably involving several molecules of Erv1 and Mia40). Protein oxidation (the formation of disulphide bonds) is coupled to the removal of electrons that flow from the precursor via Mia40 to Erv1 and from here, via cytochrome c, to the respiratory chain.

Protein insertion into the inner membrane. The carrier translocase of the inner membrane, the TIM22 complex, forms a twin-pore translocase[9] (FIG. 2b). A modified form of the intermembrane space chaperone, comprising Tim9, Tim10 and the related protein Tim12, docks onto the TIM22 complex[5,11]. The translocation channel is formed by the integral membrane protein Tim22. Two further integral membrane proteins, Tim54 and Tim18, are associated with Tim22. Tim54 exposes a large domain to the intermembrane space and probably serves as a docking point for the Tim9–Tim10–Tim12 complex[61]. Tim18 is involved in the assembly of the TIM22 complex[61].

Precursor proteins interact with the TIM22 complex in two stages[9]. They are first bound to the Tim9–Tim10–Tim12 chaperone complex on the surface of the translocase. Activation of the Tim22 channel involves the internal targeting signals of the precursors and the $\Delta\psi$. The precursors are probably inserted into the translocase in a loop structure. Finally, the proteins are laterally released into the lipid phase of the inner membrane, although the mechanism of release has not yet been clarified. Tim22 is homologous to Tim23 and Tim17 of the presequence translocase, indicating that these core components of the inner membrane translocases were derived from a primordial machinery by gene duplication[62].

Intermembrane space assembly

The identification of the MIA machinery completely changed the view of how the mitochondrial intermembrane space is assembled. For a long time, it was assumed that, like the cytosol, the intermembrane space possesses a reducing environment and thus the oxidation of proteins (the formation of disulphide bonds) would be disfavoured. A systematic analysis of new proteins that were found in the mitochondrial proteome led to the identification of Mia40 (REFS 12–14). This protein is essential for cell viability and contains characteristic Cys motifs. Mia40 became the core component of a new machinery that inserts disulphide bonds into proteins imported into the intermembrane space, leading to the unexpected finding that many intermembrane space proteins contain disulphides; that is, they are in an oxidized state.

Mia40 as a receptor and protein disulphide carrier. Most intermembrane space proteins are small proteins that are synthesized without cleavable presequences but contain Cys motifs[63,64]. The precursors of these proteins are synthesized on cytosolic ribosomes and translocated across the outer membrane in a reduced, unfolded conformation (FIG. 3). Mia40 functions as a receptor on the intermembrane space side of the outer membrane. It recognizes an internal signal of the precursor proteins that includes a conserved hydrophobic residue and a Cys residue[65,66]. Mia40 binds the precursor in a hydrophobic cleft and by the formation of a transient disulphide bond[67,68]. Mia40 then acts as a protein disulphide carrier that transfers disulphide bonds to the imported proteins and thus promotes their oxidation to mature forms.

Disulphide relay and channelling. Mia40 does not form disulphide bonds *de novo* but functions as an oxidoreductase that is part of a disulphide-transferring reaction chain (the disulphide relay)[69–72]. The sulphhydryl oxidase essential for respiration and viability 1 (Erv1)

generates disulphide bonds and transfers them to Mia40 by the formation of transient intermolecular disulphide bonds. Mia40 then transfers the disulphides onto the substrate; that is, the imported protein. As the formation of disulphides is coupled to the removal of electrons, electrons flow from the imported proteins via Mia40 to Erv1, and from here via cytochrome *c* to the respiratory chain[71,73,74] (FIG. 3).

The substrates of Mia40 typically receive more than one disulphide bond. Different models are conceivable for how multiple disulphides are transferred. On the one hand, the protein disulphide carrier Mia40 may function in an alternating relay by shuttling between binding to the oxidase Erv1 and the substrate, and thus the transfer of two or more disulphides will require several cycles of binding and release[69]. On the other hand, it has been reported that Erv1, Mia40 and the substrate are physically associated in a transient ternary complex[75]. In the case of mitochondria, the interactions between disulphide carrier and substrate, as well as between disulphide carrier and oxidase, are much more stable and long-lived than the transient interactions in the disulphide relays of the endoplasmic reticulum (ER) and bacterial periplasm. Although the redox conditions of the mitochondrial intermembrane space are more oxidizing than in the cytosol, they are probably less oxidizing than in the ER and periplasm. In an alternating relay, partially oxidized substrates are released and thus the conditions in the intermembrane space may favour a reduction of the substrates. In a ternary complex containing all three partners, disulphides can be directly transferred without the intermittent release of substrate (disulphide channelling)[75] (FIG. 3). The exact molecular mechanism of disulphide transfer and the role of individual Cys residues involved are only partly understood. It is likely that more than one molecule of Erv1 and Mia40 participate in the formation of the ternary complex and that non-covalent interactions between the partner proteins will be important to stabilize such a complex and for the formation of transient disulphide linkages[72,75].

Protein sorting to the outer membrane

All proteins of the mitochondrial outer membrane are imported from the cytosol. The membrane contains two different types of integral proteins, α-helical proteins and β-barrel proteins. These proteins are not simply inserted into the membrane by the TOM complex. A combination of proteomic studies and functional analysis of transport intermediates have led to the identification of at least two different sorting pathways of outer membrane proteins.

β-barrel pathway through TOM and SAM. The precursors of β-barrel proteins are initially transported across the outer membrane by the TOM complex and are inserted into the outer membrane from the intermembrane-space side by the SAM complex[15] (FIG. 4a). The channel-forming core of the SAM complex, Sam50, is homologous to BamA (also known as Omp85) of the bacterial β-barrel assembly machinery (BAM)[16–18]. The basic mechanism of β-barrel insertion into the outer membrane has thus been conserved from Gram-negative bacteria to mitochondria, although the other components of SAM and BAM are not

related to each other[76]. The β-barrel precursors are not directly transferred from the TOM complex to the SAM complex, but the intermembrane space chaperones Tim9–Tim10 and Tim8–Tim13 participate in the transport of the precursors[77,78]. A sorting signal that corresponds to the most C-terminal β-strand of the precursor directs insertion into the SAM complex and binds Sam35, a partner protein of Sam50 (REF. 79). Sam37, the third subunit of the core SAM complex, promotes the release of precursors into the lipid phase of the outer membrane[80]. The exact mechanism of precursor insertion and release is not yet known. It is unknown whether the precursor proteins are translocated through a channel formed by monomeric Sam50 or whether a channel is located between several Sam50 molecules. A third possibility is that precursor proteins may be inserted at the SAM–lipid interface.

The insertion of typical β-barrel proteins such as the main outer membrane protein porin is mediated by this core SAM complex (FIG. 4a). A more complicated pathway is used by the precursor of Tom40. This central TOM subunit not only has to be inserted into the outer membrane but also has to assemble with the other TOM subunits to the oligomeric TOM complex. The SAM complex provides a modular assembly platform for the TOM machinery. A fraction of SAM complexes contain a fourth subunit, mitochondrial distribution and morphology 10 (Mdm10), which promotes the association of Tom40 with α-helical TOM proteins such as the precursor of Tom22 (REFS 81–84). Interestingly, Mdm10 is not only located in the SAM complex but is also a subunit of the MDM complex and thus connects protein assembly with mitochondrial distribution and morphology, as discussed below.

Outer membrane insertion of α-helical proteins. The outer membrane contains different types of α-helical proteins that can be distinguished by the location of their transmembrane segments in N-terminal, middle or C-terminal protein regions (FIG. 4b). The hydrophobic segments typically function as targeting signals of the proteins. The receptors Tom20 and Tom70 are representatives of N-terminally anchored proteins. They use a further outer membrane protein, mitochondrial import 1 (Mim1), for membrane insertion[85–87]. Mim1 was identified in a genome-wide screen in yeast[88] and transiently interacts with the SAM complex[86]. The insertion pathway for proteins with internal signals has only been analysed for a few proteins. The precursor of Tom22 uses TOM receptors for targeting to mitochondria and then the SAM complex for membrane insertion[89] (FIG. 4b). Precursors with multiple transmembrane segments were shown to use Tom70 and intermembrane-space components but not other TOM components for insertion into the outer membrane[90]. Tom70 and the intermembrane-space components may carry out chaperoning functions for the hydrophobic precursors. An insertase (proteinaceous machinery that mediates membrane insertion of the precursors) has not been identified so far. Different pathways have been suggested for proteins with a C-terminal membrane anchor (C-tail). The precursors of small TOM proteins were found to depend on Mim1 for membrane insertion[82].

Porin
A pore-forming protein of the mitochondrial outer membrane that is permeable for many metabolites. It is the most abundant outer membrane protein and is also called voltage-dependent anion channel (VDAC).
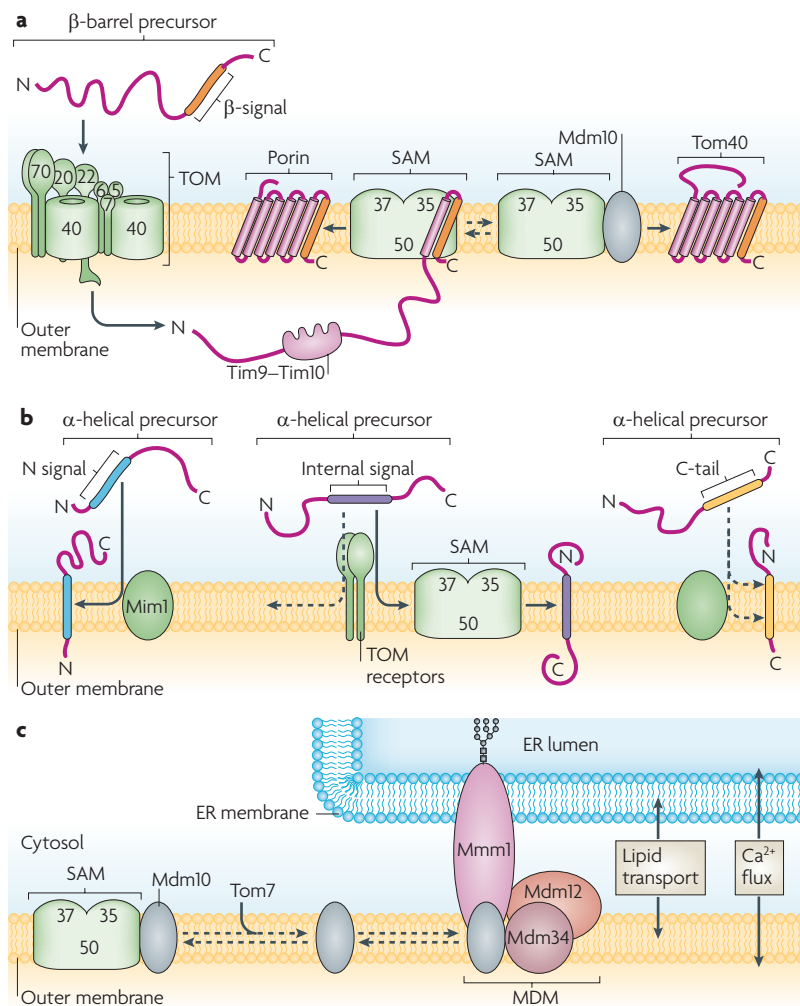
Figure 4 | **Protein sorting to the outer mitochondrial membrane and connection to the ER. a** | β-barrel proteins are imported by the translocase of the outer membrane (TOM) complex and bind to the translocase of the inner membrane 9 (Tim9)–Tim10 chaperone on the intermembrane-space side of the outer membrane. The β-sorting signal initiates the insertion of the β-barrel precursors into the sorting and assembly machinery (SAM) complex, which comprises Sam35, Sam37 and Sam50. β-barrel proteins such as porin (a voltage-dependent anion channel) are then inserted into the lipid phase. The β-barrel precursor of Tom40 assembles with α-helical TOM proteins, which involves a larger SAM complex that includes mitochondrial distribution and morphology 10 (Mdm10). **b** | α-helical outer membrane proteins are imported by different routes that have only been partly characterized. The membrane anchor sequences typically function as targeting signals. Precursors with amino-terminal signals can use mitochondrial import 1 (Mim1) for membrane insertion. Other precursors can use TOM receptors, the SAM complex or unknown mechanisms. **c** | Mdm10 is located in two different complexes: the SAM complex and the MDM complex, which comprises Mdm34, Mdm12 and maintenance of mitochondrial morphology 1 (Mmm1). Mmm1 is anchored in the endoplasmic reticulum (ER) membrane. The ER–mitochondrion junction formed by the MDM complex may be involved in the transport of lipids and Ca^{2+}. The small TOM subunit, Tom7, favours the dissociation of Mdm10 from the SAM complex and thus negatively regulates the assembly of the TOM complex[96].

Thus, our current knowledge on the sorting and insertion of α-helical outer membrane proteins is only fragmentary. It is clear that the proteins do not follow the classical route through the Tom40 import channel, in contrast to most mitochondrial proteins, and so the mechanisms and components of α-helical insertion pathways await further analysis.

Outer membrane assembly and ER–mitochondrion junctions. Mdm10 was identified in a genetic screen for components that affect mitochondrial distribution and morphology[93]. It is present in at least two different protein complexes: the SAM complex and the MDM complex[81,83,84,94–96] (FIG. 4c). The identification of Mdm10 as a subunit of SAM unexpectedly connected the fields of protein assembly and mitochondrial morphology, which had been considered as independent fields before.

A further surprise came when the MDM complex was characterized. In addition to Mdm10, the complex includes other so-called morphology components: maintenance of mitochondrial morphology 1 (Mmm1), Mdm12 and Mdm34 (also known as Mmm2) (REFS 95,97,98). Mmm1 was found to be inserted into the membrane of the ER and to be glycosylated[99] (FIG. 4b). Thus, the MDM complex connects part of the ER membrane with the mitochondrial outer membrane and was also termed the ER–mitochondrion encounter structure (ERMES). Junctions between the ER and mitochondria have been known for many years[100], but their molecular identity remained unknown. An important role of the MDM complex is in the formation of ER–mitochondria junctions. A study in mammalian cells indicated that another protein, mitofusin, is also involved in this process[101]. The current evidence suggests that ER–mitochondrion junctions are important for the transfer of lipids and Ca^{2+} between both organelles.

The exact molecular function of the MDM complex is not known. It may function as an organizing centre that provides a platform for the connection of mitochondria with the ER and the transfer of ions, lipids and possibly other molecules[102]. The dual localization of Mdm10 in MDM and SAM complexes suggests that the protein assembly machinery is connected to the organizing centre, and indeed an involvement of the MDM complex in protein assembly has been reported[84,94]. A close functional connection between SAM and MDM complexes is also suggested from the analysis of mitochondrial morphology in yeast cells. Whereas wild-type mitochondria form a tubular network, mutant cells of MDM components contain condensed mitochondria (so-called giant mitochondria)[93] and SAM mutants show similar changes in mitochondrial shape[81,94,96]. These morphological alterations may result from defects in outer membrane assembly and defects in ER–mitochondrion junctions.

Perspectives

The large-scale identification of the mitochondrial proteome and its systematic analysis by biochemical and genetic approaches provided invaluable information for the identification of new mitochondrial import components and pathways, including the oxidative folding

Mitofusin
A mitochondrial outer membrane protein required for fusion of mitochondria and maintenance of mitochondrial morphology.

It was reported that other C-tail proteins do not require any of the known outer membrane machineries, but the lipid composition of the membrane is important for their efficient insertion[91,92]. These proteins may use an unknown insertase or be directly inserted into the lipid phase of the outer membrane.

Cardiolipin
A large, dimeric phospholipid that is a characteristic of mitochondria and consists of two phosphatidyl moieties linked by glycerol.

Liposome
An artificial lipid vesicle that is typically formed by a phospholipid bilayer (membrane).

pathway of the intermembrane space and the β-barrel pathway of the outer membrane. Protein translocases do not function as independent units but are integrated into dynamic networks. The TIM23 complex, which interacts with complexes in three different mitochondrial compartments (the TOM complex, the respiratory chain and the matrix motor), serves as a paradigm. The dynamic association of Mdm10 with different outer membrane complexes indicates that the translocases are also connected to machineries that function in ER–mitochondrion junctions, lipid transport and maintenance of mitochondrial morphology.

Seeing the rapid increase in the number of identified mitochondrial proteins (BOX 1), the question of whether, and how, it may be possible to achieve a complete coverage of the mitochondrial proteome arises. As not all mitochondrial proteins are expressed under the same growth conditions or in all organs of multicellular organisms, proteomic analyses of cells from different growth phases and media will be required for single-cell organisms, whereas the analysis of multiple organs and different developmental stages will be required for animal or plant mitochondria. It is likely that the search for new mitochondrial import components is not yet finished as the mitochondrial proteome still contains many proteins with unknown function (BOX 2) and several pathways of protein import are only understood in part, such as the insertion of α-helical precursors into the outer membrane. Additionally, the mitochondrial inner membrane may contain further insertases. Although the presequence pathway and the carrier pathway strictly require a $\Delta\psi$, $\Delta\psi$-independent import has been observed for a few precursor proteins[103,104]. These proteins may be imported by an alternative machinery that can operate in the absence (or at low levels) of a $\Delta\psi$.

There are different views about whether and how protein synthesis at ribosomes and translocation into mitochondria are coupled. As the *in vitro* import assays with isolated mitochondria work efficiently in a post-translational manner (that is, with fully synthesized precursor proteins), it has been assumed that a coupling of ribosomes to mitochondria is not involved. However, most precursor proteins were not analysed in the *in vitro* assays and several proteins tested could not be imported[105]. The presence of ribosomes on the mitochondrial surface has been known for many years[106]. More recently, mRNAs for mitochondrial proteins were found to be enriched at mitochondrion-associated ribosomes[107,108] and the corresponding precursor proteins

were found to accumulate at the outer membrane[109]. Future studies have to consider the possibility that co-translational targeting of mitochondrial proteins may be much more important than anticipated.

The role of the lipid environment of the membranes has been underestimated for a long time. In addition to the studies on outer membrane protein insertion that indicate the importance of the lipid composition[92,102], the analysis of the mitochondrial signature lipid, the dimeric phospholipid cardiolipin, provided important information. The efficient integration of purified TIM23 complexes into liposomes requires cardiolipin[38], and the lack of cardiolipin influences the organization of the TIM23–PAM complex[110–112]. Whereas cardiolipin is enriched in the mitochondrial inner membrane, a small but significant fraction of cardiolipin was found in the outer membrane and shown to influence the activity of the TOM and SAM complexes[113]. Lipids may affect protein biogenesis by influencing the activity of translocase complexes but may also directly participate in the process of membrane insertion.

The structural analysis of mitochondrial import components is an important field of research. In addition to single-particle electron microscopic analysis of purified translocase complexes[9,114,115], high resolution structures have been reported for receptor domains, mitochondrial chaperones and the processing peptidase MPP[6,10,29,37,42,46,116]. To understand the molecular mechanisms of membrane translocation, it will be crucial to obtain high resolution structures of the membrane-integrated translocation channels, including that of preproteins in transit.

As recent studies revealed that mitochondria are involved in numerous human diseases[117], a systematic analysis of the mitochondrial proteome will serve as a basis to define their molecular mechanisms. Little is known about the regulation of the mitochondrial protein import machinery. The mitochondrial proteome led to the identification of several protein kinases, phosphatases and GTPases[2,3]. A first analysis of the mitochondrial phosphoproteome suggested that many more proteins than assumed are phosphorylated[118,119]. We expect that a systematic analysis of the phosphorylation of mitochondrial proteins and the assignment of the responsible kinases and phosphatases, as well as the analysis of GTPases, will provide a wealth of information of how mitochondria are embedded into cellular signalling networks and will help us understand the molecular basis of mitochondrial diseases.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

UniProtKB: http://www.uniprot.org
Erv1 | Mdm10 | Mdm12 | Mdm34 | Mia40 | Mim1 | Mge1 | Mmm1 | Pam16 | Pam17 | Pam18 | Sam35 | Sam37 | Sam50 | Tim8 | Tim9 | Tim10 | Tim12 | Tim13 | Tim17 | Tim18 | Tim21 | Tim22 | Tim23 | Tim44 | Tim50 | Tim54 | Tom7 | Tom20 | Tom22 | Tom40 | Tom70

FURTHER INFORMATION

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