VERSATILITY OF THE MITOCHONDRIAL PROTEIN IMPORT MACHINERY

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The vast majority of mitochondrial proteins are synthesized in the cytosol and are imported into mitochondria by protein machineries located in the mitochondrial membranes. It has become clear that hydrophilic as well as hydrophobic preproteins use a common translocase in the outer mitochondrial membrane, but diverge to two distinct translocases in the inner membrane. The translocases are dynamic, high-molecular-weight complexes that have to provide specific means for the recognition of preproteins, channel formation and generation of import-driving forces.

TRANSLOCASE
A system catalysing the transfer of a substrate across a barrier.

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The division of eukaryotic cells into numerous membrane-bounded compartments — the cell organelles — poses a fundamental problem for the correct localization of proteins. With the exception of a few proteins that are synthesized within mitochondria or chloroplasts, all proteins are synthesized on cytosolic ribosomes. About half of the proteins in an eukaryotic cell, however, do not stay in the cytosol upon synthesis, but are imported into organelles such as the nucleus, mitochondria, chloroplasts, peroxisomes and the endoplasmic reticulum. The correct sorting of these proteins is essential for the life of any cell. This article focuses on the translocation of proteins into mitochondria, which contain about 15–20% of all cellular proteins and must import about 1,000 different proteins. Three main questions have to be answered. How are the newly synthesized proteins directed to the correct organelle? How can fully synthesized polypeptide chains be translocated across biological membranes that form efficient barriers for macromolecules? And how are the proteins sorted to their correct location (subcompartment) within mitochondria? The basic concept includes the idea that these proteins carry signal sequences that are either in cleavable extensions or within the mature protein[1]. The signal sequences are decoded by protein machines of the target organelles that direct them to their correct destination. Each organelle has its own specific apparatus for the recognition and translocation of preproteins. Studies over the past years have revealed that these protein transport machineries are dynamic, multisubunit systems with a much higher complexity and versatility than expected.

Protein translocation into mitochondria is complex because these organelles are divided into four subcompartments: the outer membrane, the intermembrane space, the inner membrane and the matrix. Hydrophilic preproteins destined for the matrix must cross two membranes as well as the intermembrane space before reaching their final destination. The precursors of hydrophobic proteins that have to be inserted into the inner membrane must cross the outer membrane without getting stuck in it and must additionally pass through the aqueous intermembrane space. Moreover, all components of the mitochondrial import machinery are themselves synthesized on cytosolic polysomes — their preproteins must be correctly sorted and inserted into the membranes by pre-existing TRANSLOCASE components. What are the molecular mechanisms that allow such versatility and accuracy of targeting?

Principles of mitochondrial protein import

Mitochondria are not only the site of oxidative phosphorylation, carrying the complexes of the respiratory chain in the inner membrane, but they also house the

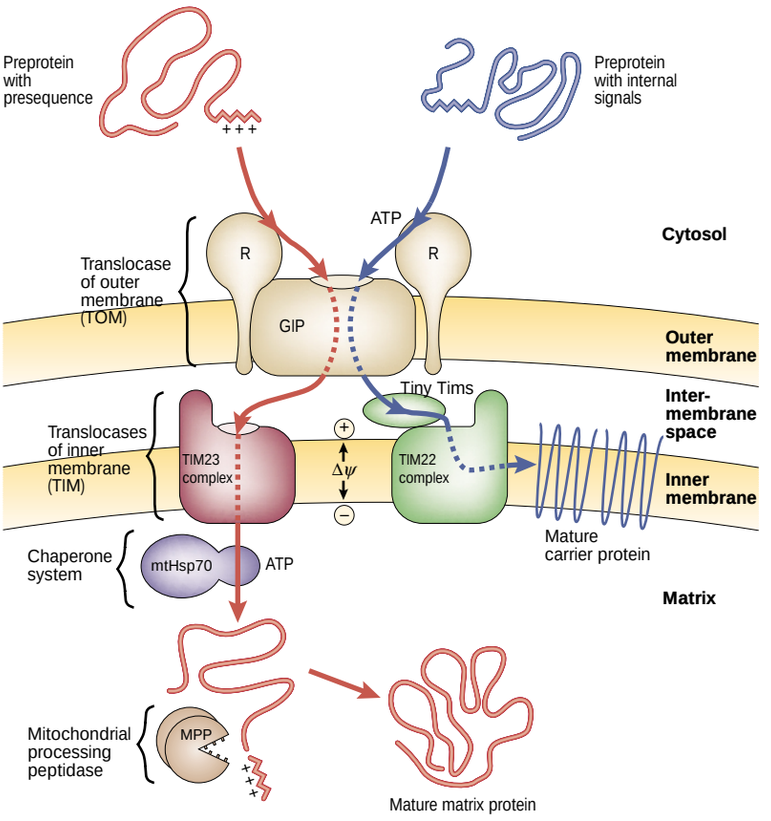


Figure 1 | **The two main pathways of protein import into mitochondria.** Preproteins with an amino-terminal presequence (red) as well as preproteins with internal targeting signals (blue) are recognized by receptors (R) and translocated by the general import pore (GIP) of the translocase of the outer membrane (TOM). Preproteins with a presequence are translocated across the inner membrane by the TIM23 complex. This requires the membrane potential ($\Delta\psi$) and the ATP-dependent action of mitochondrial heat-shock protein 70 (mtHsp70). The presequences are cleaved off by the mitochondrial processing peptidase (MPP) in the matrix. Preproteins with internal signals are guided by tiny Tim proteins across the intermembrane space to the TIM22 complex of the inner membrane and inserted into the membrane in a $\Delta\psi$-dependent step.

MOLECULAR CHAPERONES
Proteins that assist the correct folding, assembly or disassembly of other proteins *in vivo*, but are not components of the final functional structures.

TOM
(Translocase of the outer mitochondrial membrane). A protein complex that recognizes nuclear-encoded mitochondrial preproteins and mediates their translocation across the membrane.

GIP
(General import pore). Pore of the mitochondrial outer membrane translocase that translocates virtually all nuclear-encoded mitochondrial proteins.

citric acid (Krebs) cycle in the matrix and are involved in important steps of the urea cycle, haem biosynthesis and fatty-acid metabolism. These functions require efficient transfer of many metabolites to and from the cytosol. The most abundant protein in the outer mitochondrial membrane is porin, which forms a large pore for various metabolites. The mitochondrial inner membrane must maintain an electrochemical gradient and therefore metabolite transport across this membrane has to be tightly controlled to prevent leakage of ions. The inner membrane contains a large family of carrier proteins that are responsible for the specific translocation of metabolites.

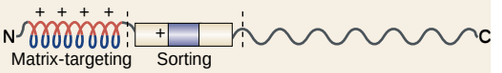
Mitochondrial preproteins are preferentially, although not exclusively, imported post-translationally[2–4]. Most soluble proteins of the matrix and some proteins of the inner membrane and intermembrane space are synthesized with an amino-terminal extension of about 10–80 amino-acid residues, termed a presequence, which is proteolytically removed upon import[5,6]. Other preproteins, however, are synthesized without cleavable extensions and contain targeting signals in the mature protein[7]. This group includes all outer membrane proteins, some intermembrane space proteins and many inner membrane proteins. Although these proteins are often quite hydrophobic, they can be transported through the aqueous cytosol; the preproteins are probably in a different folding state than the mature proteins and are protected from aggregation by MOLECULAR CHAPERONES[8–10]. Both types of signal sequence are recognized by receptors that are associated with the translocase of the outer mitochondrial membrane, TOM[11–14] (FIG. 1). The preproteins cannot be translocated across the mitochondrial membranes in a folded conformation; they must be at least partially unfolded to fit through the import channels[15,16].

After crossing the outer membrane through the general import pore (GIP) of TOM[11,17,18], imported preproteins are directed to one of two translocases of the inner membrane, or TIM (FIG. 1). All presequence-carrying preproteins, including the numerous matrix proteins, are directed to the TIM23 complex, which forms a channel across the membrane[19–21]. The membrane potential ($\Delta\psi$) across the inner membrane is required for transfer of the presequence across the membrane[22]. The mitochondrial HEAT-SHOCK PROTEIN 70 (mtHsp70) forms the central part of an ATP-dependent molecular motor that drives import of the preprotein into the matrix[23–26]. The mitochondrial processing peptidase (MPP) then cleaves off the presequence[27], and the protein folds into its active form — a process that can be assisted by folding helpers, such as Hsp70, the CHAPERONIN Hsp60 or peptidyl-prolyl *cis/trans* isomerases[23,28–30]. Hydrophobic inner membrane proteins with internal targeting signals, such as the metabolite carriers, are directed with the help of so-called tiny Tim proteins to a second TIM translocase, termed the TIM22 complex, which inserts them into the inner membrane in a $\Delta\psi$-dependent manner[31–34].

Mitochondrial targeting signals

The classic mitochondrial-targeting signal is an amino-terminal cleavable presequence, which functions as a matrix-targeting signal. When attached to non-mitochondrial proteins, presequences can specifically direct the passenger protein across both mitochondrial membranes into the matrix[5,6]. Presequences contain many positively charged, hydrophobic and hydroxylated amino-acid residues[35]. A characteristic property of presequences is their high tendency to form an amphipathic α-helix that presents one positively charged surface and one hydrophobic surface[14,36] (TABLE 1). It has previously been assumed that the positive charges of the presequences are crucial for the recognition by import receptors and that the amphipathic property favours insertion into the outer membrane. Recent studies have indicated, however, that the different surfaces of a presequence might be sequentially recognized by distinct receptor proteins of the TOM machinery[13]: the hydrophobic side by Tom20 and the positively charged side by Tom22. The first high-resolution structure of a preprotein receptor together with a signal sequence indeed showed that the hydrophobic residues of a presequence were crucial for

Table 1 | Mitochondrial targeting and sorting signals

Signals	Preproteins	Import machinery	Location of signals in preproteins
Presequences			
At amino terminus of preproteins Cleavable upon import Enriched in positively charged, hydroxylated and hydrophobic amino-acid residues Amphipathic α-helix	Matrix proteins	TOM complex TIM23 complex	
Variations on presequences for sorting to mitochondrial subcompartments			
Non-cleavable 'presequence' plus hydrophobic anchor	Some outer membrane proteins	TOM complex	
Presequence plus hydrophobic anchor	Some inner membrane proteins	TOM complex TIM23 complex	
Bipartite presequence with dual targeting and sorting information: matrix-targeting sequence followed by more hydrophobic sorting signal (prokaryotic type)	Some proteins of inner membrane or intermembrane space	TOM complex TIM23 complex	
Presequence-like signal (positively charged) at internal position, often preceded by hydrophobic segment	Some inner membrane proteins	TOM complex TIM23 complex	
Multiple internal targeting signals			
Multiple internal signals in non-cleaved preprotein Charged and uncharged signals (unknown recognition motif) Signals can function independently but cooperate for efficient targeting and translocation	Metabolite carriers of inner membrane	TOM complex TIM22 complex	

TIM, translocases of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane; blue, hydrophobic. Cleavage sites are indicated by dashed lines.

interaction with the binding groove of Tom20 (REF. 14). Moreover, the positive net charge of presequences is also important for the membrane potential $\Delta\psi$ across the inner membrane to exert its electrophoretic effect on presequences[22].

Whereas the presequences of most matrix proteins are cleaved off upon import, a few matrix proteins, for example, chaperonin 10 (Hsp10), are synthesized with an amino-terminal presequence that remains a permanent part of the protein[37]. Moreover, one matrix protein, the DNA helicase Hmi1, has a presequence-like targeting signal at its carboxyl terminus. In contrast to the usual amino- to carboxy-terminal translocation of presequence-carrying preproteins, this preprotein seems to be translocated in the reverse orientation, showing that the mitochondrial import system is highly flexible[38].

Presequence-like signals are also used for several preproteins that are destined for the three other mitochondrial subcompartments — the outer membrane, the intermembrane space and the inner membrane (TABLE 1). In these cases, the positively charged sequences are complemented by more hydrophobic sorting signals that lead to a specific arrest of the preproteins in the outer or inner membranes, respectively (stop-transfer signal)[39–41]. These sorting signals are not simply arrested in the lipid phase of a membrane owing to their hydrophobic nature, but seem to be specifically recognized by components of the TOM complex or TIM23 complex before they are released into the lipid membrane[42,43]. However, it is unknown which TOM or TIM components are directly responsible for recognizing the sorting signals. First, some outer membrane proteins contain a non-cleavable presequence, followed by a long hydrophobic stretch. These preproteins are directed to mitochondria by the presequence and get stuck in the outer membrane through the hydrophobic stretch[40]. Second, some inner membrane proteins carry a cleavable presequence and a hydrophobic membrane anchor sequence in the mature part[44]. Third, a bipartite presequence is used to sort some proteins to the inner membrane (for example, cytochrome c_1) or the intermembrane space (for example, cytochrome b_2)[39]. In this case, a positively charged matrix-targeting sequence is followed by a sorting sequence related to the signals found in bacterial and eukaryotic secretory proteins, including a hydrophobic stretch preceded by a few positively charged residues. These sorting sequences are cleaved off at the outer surface of the inner membrane by the heterodimeric inner membrane peptidase (Imp1–Imp2)[45,46]. Last, some inner membrane proteins contain an internal targeting signal that consists of a transmembrane segment and a positively charged segment directly after it. This internal signal is thought to form a hairpin–loop structure in the inner membrane[47]. It is a continuing debate as to whether, apart from the stop-transfer mechanism described above, some of the sorting signals might be imported into the matrix and then redirected into the inner membrane, thereby resembling prokaryotic protein export ('conservative sorting')[47,48].

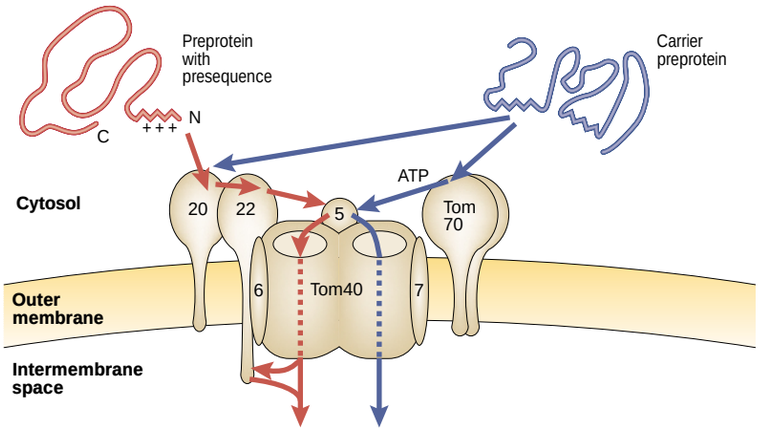
Metabolite carriers of the inner membrane do not

TIM
(Translocases of the inner mitochondrial membrane). Protein complexes in the inner membrane and intermembrane space of mitochondria that mediate transport of preproteins into and across the membrane.

HEAT-SHOCK PROTEINS
Heat-shock proteins are synthesized in larger amounts when cells have been exposed to a temperature that is higher than normal. Many heat-shock proteins function as molecular chaperones and are crucial for cellular functions also under non-stress conditions.

CHAPERONINS
A subclass of molecular chaperones, including the chaperonin 60 family and the chaperonin 10 family.

Box 1 | The translocase of the outer mitochondrial membrane



Initial receptors

Tom20

- Mainly recognizes presequences (hydrophobic surface) and some hydrophobic preproteins

Tom70

- Mainly recognizes hydrophobic preproteins with internal targeting signals

General import pore (GIP) complex

Tom22

- Receptor for presequences (positively charged surface)
- Docking point for initial receptors (Tom20, Tom70)
- Organizer of GIP complex
- *Trans*-binding site for presequences

Tom5

- Preprotein transfer into Tom40 pore
- Receptor-like properties

Tom40

- Import channels (oligomeric complex)

Tom6

- Assembly of Tom22 with Tom40

Tom7

- Favours dissociation of translocase and exchange of GIP complex with late-assembly intermediate

contain a cleavable presequence, but have about three to six internal targeting segments, distributed throughout the entire length of the preprotein[7,16,49] (TABLE 1). Contrary to cleavable presequences, these internal signals do not necessarily contain charged amino-acid residues; however, a consensus sequence has not yet been found. Each targeting segment of about ten amino acids can function by itself and is recognized by a mitochondrial surface receptor, albeit with low efficiency. The signals seem to function in a cooperative manner to recruit several receptors to one preprotein[16,50]. Other membrane proteins, such as the components of the inner membrane translocases also contain several targeting and sorting signals, including hydrophobic segments and positively charged loops[51–53].

Translocase of the outer membrane

The translocase of the outer membrane machinery consists of three receptor proteins, Tom20, Tom22 and Tom70, the channel forming protein Tom40, and three small Tom proteins (Tom5, Tom6 and Tom7)[11,17,18,54–56] (BOX 1). Tom22, Tom40 and the three small Tom proteins form the stable TOM core complex of ~400 kDa, termed the GIP COMPLEX.

Preproteins with a presequence are first recognized by Tom20 and subsequently by Tom22 (REFS 12–14). Hydrophobic preproteins with several internal signals, such as the carrier proteins, are recognized by the dimeric Tom70 and, to a smaller extent, also by Tom20. Several Tom70 dimers simultaneously bind to one carrier preprotein, probably to prevent aggregation of the hydrophobic preproteins[16,50]. The cytosolic domain of Tom22 not only functions as the receptor for preproteins, but it also acts as the docking point of the GIP complex to which the peripheral receptors Tom20 and Tom70 can associate. We propose that preproteins are initially bound by Tom20 or Tom70 and are subsequently donated to Tom22 through a direct contact between the cytosolic domains of the receptors[55].

Tom5 is required for the transfer of preproteins from Tom22 to the Tom40 channel. In fact, Tom5 can be seen as a receptor at the third level[57], acting after Tom20 and Tom22. Some preproteins destined for the intermembrane space, such as the precursors of the tiny Tim proteins, do not require any of the three receptors with a large cytosolic domain, but depend only on Tom5 and Tom40 for their passage across the outer membrane[58].

Only Tom40 is strictly required to form the channel across the outer membrane, as shown by reconstitution of expressed Tom40 into liposomes[59]. Tom40, the only Tom protein that is essential for cell viability under all growth conditions[60], is present in about six copies per GIP complex, but the exact stoichiometry of the complex has not yet been unravelled. The current model proposes that a single channel is formed by two Tom40 molecules and that a complete GIP complex contains two to three channels[17,18,54,55]. Each channel has a pore diameter of ~22 Å, a size that is sufficient for the passage of up to two α-helical segments[18,59,61]. At the exit of the channel, presequence-containing proteins bind to the intermembrane space domain of Tom22 (REFS 12,62) before they are sorted to the TIM23 inner membrane translocase[19,20].

Interestingly, Tom40 itself also provides a specific binding site for presequences directly at the channel[59]. A presequence-containing preprotein is thus successively recognized by at least five binding sites on its trip across the outer membrane — Tom20, the cytosolic domain of Tom22, Tom5, Tom40 and the intermembrane space domain of Tom22 (BOX 1). As several of these Tom proteins contain negatively charged patches, it has been suggested that the positively charged presequences are recognized by ionic forces (acid chain hypothesis)[57,63]. However, other non-covalent forces, including hydrophobic interactions, are also crucial for the interactions between presequence-containing preproteins and Tom proteins, as shown for Tom20 and Tom40 (REFS 13,14,56). We propose that the Tom proteins (and some Tim proteins) provide a chain of binding sites, including various types of non-covalent interaction, to

GIP COMPLEX
Stable core complex of the preprotein translocase of the outer mitochondrial membrane, consisting of the channel and several associated proteins.

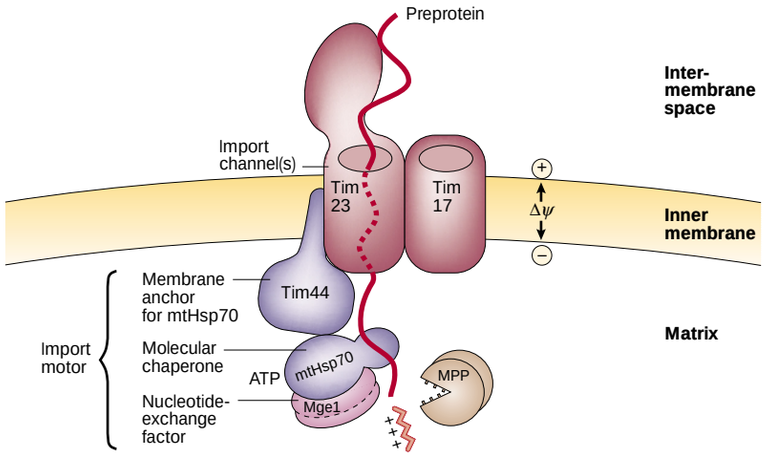


Figure 2 | **The translocase of the inner mitochondrial membrane for presequence-carrying preproteins.** The presequence translocase (TIM23 complex) consists of the transmembrane proteins Tim23 and Tim17 and the peripherally attached import motor. Tim23–Tim17 form a channel (or channels) for preproteins with amino-terminal presequences. The membrane potential ($\Delta\psi$) exerts an electrophoretic effect on the positively charged presequences and might activate Tim23. The import motor consists of the ATP-driven mitochondrial heat-shock protein 70 (mtHsp70), its membrane anchor protein Tim44, and the homodimeric co-chaperone mitochondrial GrpE (Mge1) that promotes the reaction cycle of mtHsp70 by supporting nucleotide exchange. Tim23 and Tim44 are probably also present as homodimers[20,100] (not shown). The heterodimeric mitochondrial processing peptidase (MPP) removes the presequences.

guide presequence-containing preproteins into mitochondria (binding chain hypothesis).

Tom6 and Tom7 modulate the dynamics of the TOM machinery in a partially antagonistic manner. Tom6 promotes the assembly of Tom22 with Tom40 (REF. 17). The membrane anchor sequence of Tom22 is required to maintain a stable association between the individual Tom40 channels. In yeast mutants lacking either Tom6 or Tom22, the GIP complex is destabilized and dissociates into 100-kDa basic units, each containing a Tom40 dimer[17,55]. Tom7 supports a dissociation of the translocase and thereby allows a lateral release of preproteins[64]. Mutant mitochondria lacking Tom7 are only slightly impaired in the translocation of presequence-containing preproteins across the outer membrane, but are more strongly inhibited in the sorting of proteins such as porin into the outer membrane[17,64,65]. The precursor of porin is targeted through the receptors Tom20 and Tom22 to the Tom40 channel. Tom7 probably supports an opening of the translocase to release the protein into the lipid phase of the membrane[65].

The inner-membrane presequence translocase

After guidance across the outer membrane by the chain of Tom proteins, preproteins with presequences interact with the TIM23 complex of the inner membrane[19,20] (FIG. 2). In contrast to the TOM channel, the TIM channels need a tight regulation of opening and an adaptation to the polypeptide chain in transit[61] to prevent a pronounced leakage of ions. Otherwise, the electrochemical gradient across the inner membrane and thus the mitochondrial capacity for oxidative phosphorylation would break down.

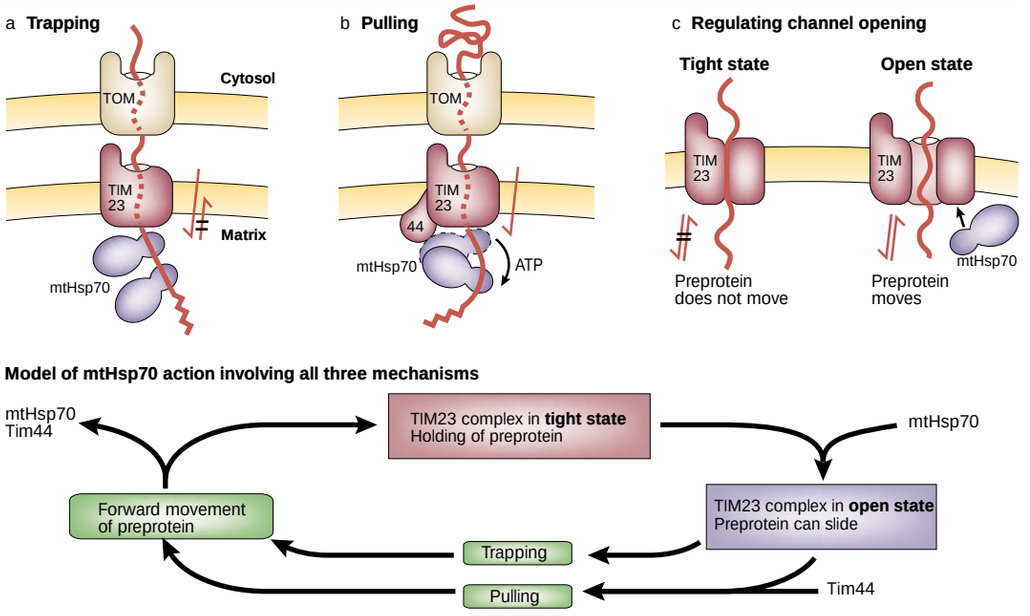
The translocases of the inner and outer membranes form independent structural and functional units. If the outer membrane is ruptured, preproteins can be directly translocated across the inner membrane, without a need for the TOM machinery[66]. Only the presence of a preprotein in transit, simultaneously arrested in both translocases in a two-membrane-spanning fashion, maintains a stable contact between the TOM complex and the TIM23 complex[19,67]. A TOM–TIM23–preprotein supercomplex of ~600 kDa has been identified[19]. It cannot be excluded, however, that loose contacts exist between the TOM complex and the TIM23 complex *in vivo* in the absence of preproteins, for example through hypothetical adaptor proteins. These contacts could be disrupted by the current methods of isolation and the opening of mitochondria and could therefore have eluded detection so far.

The core of the TIM23 translocase is formed by a 90-kDa complex, consisting of two essential proteins, Tim23 and Tim17 (FIG. 2). Both proteins contain a membrane-integrated domain with several (probably four) transmembrane segments[68,69]. Tim23 additionally exposes an amino-terminal domain into the intermembrane space. Recently, it has been reported that the amino terminus of Tim23 extends into the outer membrane and links both mitochondrial membranes; however, no contact with the TOM machinery has been found and so the relevance for protein import is not yet clear[70]. Although the membrane domains of Tim23 and Tim17 are homologous, they cannot substitute for each other; each protein is essential for cell viability. The intermembrane space domain of Tim23 is thought to function as the binding site for presequences[20,63], and the membrane domains of Tim23 and Tim17 probably form channels for the translocation of preproteins.

Protein translocation across the inner membrane requires two driving forces: a membrane potential ($\Delta\psi$) across the inner membrane and an ATP-dependent import motor. The translocation of the presequence into and across the inner membrane strictly depends on the presence of $\Delta\psi$, which is negative on the matrix side. Two roles have been assigned to $\Delta\psi$[20,22,71] — an electrophoretic effect on the positively charged presequences and the activation of Tim23. The electrophoretic effect seems to provide the most important contribution to protein import.

The ATP-dependent import motor, consisting of mtHsp70, the translocase subunit Tim44 and the co-chaperone Mge1, has the most important role in translocating the mature portion of the preprotein into the matrix[23–26,72]. All three components of the import motor are essential for viability. mtHsp70 binds extended segments of preproteins as they emerge on the matrix side of the TIM channel, and is powered by the binding and hydrolysis of ATP. Tim44 functions as a membrane anchor for the ATPase domain of mtHsp70 directly at the exit site of the import channel[73], whereas the peptide-binding domain of mtHsp70 interacts with the preprotein in transit. Mge1 is a nucleotide-exchange factor, homologous to bacterial GrpE, and promotes the reaction cycle of mtHsp70. Two models for the action of

Box 2 | Proposed roles of mitochondrial Hsp70 in protein import



a Trapping (Brownian ratchet or holding)[25,26,72,75,100]

- mtHsp70 binds to preprotein segments emerging on the matrix side of the import channel
- Prevents backsliding of preprotein
- Import is favoured

b Pulling

- mtHsp70 is simultaneously bound to membrane anchor (Tim44) and preprotein in transit
- ATP-induced conformational change of mtHsp70 generates a pulling force on the preprotein
- Supports unfolding (labilization) of preprotein domains on the cytosolic side and helps to overcome preprotein–pore interactions[23,25,72,74,76]

c Regulatory effect on import channel

- mtHsp70 might bind to the TIM channel and favour opening of the channel
- In the absence of mtHsp70 binding, the TIM channel is tight and the preprotein cannot move back or forth[19,78]

mtHsp70 are debated at present[72,74–76] (BOX 2). In the first model, mtHsp70 passively traps the polypeptide chain when it emerges on the matrix side, and this confers directionality onto the Brownian movement of the chain in the translocase. In the other model, Tim44-bound mtHsp70 undergoes conformational changes and pulls the chain through the translocon. Recent studies indicate that, in fact, both mechanisms might cooperate in protein import[25,72,77]. For loosely folded preproteins, a trapping mechanism could be sufficient, but preproteins with tightly folded domains might require an additional pulling action of mtHsp70. Folded protein domains undergo spontaneous fluctuations that can lead to complete or partial unfolding. Passive holding by mtHsp70 will be sufficient for import of preproteins when their spontaneous unfolding is complete. Conformational changes of mtHsp70 will help to harness smaller fluctuations (partial unfolding) of folded domains and provide additional energy to overcome the activation barrier required for complete unfolding.

Surprisingly, the TOM–TIM–preprotein supercomplex isolated from mitochondria is very stable, but contains neither mtHsp70 nor Tim44, indicating that a preprotein can be stably kept in the import channels without being trapped by mtHsp70. The TIM23 channel is apparently not just a passive pore for diffusion of preproteins, but actively interacts with and holds the preprotein[19]. So a pulling force, generated by mtHsp70, is probably needed to overcome preprotein–pore interactions[19,76].

Moreover, mtHsp70 probably also functions in protein import by regulating the opening of the TIM23 channel (BOX 2). In the absence of mtHsp70, the channel is tight and the preprotein does not move back or forth[19]. In the presence of mtHsp70, channel opening is promoted, possibly by a Tim44-independent influence of mtHsp70 on the channel properties[78]. Then the preprotein is free to move, and the direct binding of mtHsp70 to the preprotein will promote a forward movement by pulling and trapping (BOX 2).

Import of hydrophobic carrier proteins

Whereas the TIM23 complex is preferentially used by presequence-carrying hydrophilic matrix proteins and inner membrane proteins with a limited number of transmembrane segments, inner membrane proteins

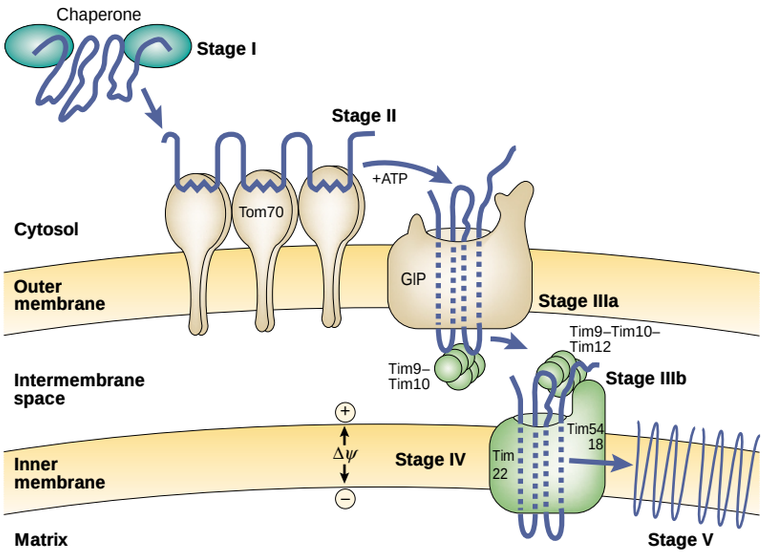


Figure 3 | **Import of a hydrophobic carrier protein into the inner mitochondrial membrane.** Stage I: the preprotein is transported through the cytosol in a complex with molecular chaperones. Stage II: several molecules of the receptor Tom70 bind to the preprotein. Stage IIIa: translocation through the general import pore (GIP) of the outer membrane in a loop formation and interaction with Tim9–Tim10 of the intermembrane space. Stage IIIb: the preprotein binds to the surface of the inner membrane through association of Tim9–Tim10 with Tim12. Stage IV: insertion of the preprotein into the TIM22 complex of the inner membrane requires a membrane potential ($\Delta\psi$). Stage V: the carrier protein assembles into a functional homodimer in the inner membrane.

with several hydrophobic segments, such as metabolite carriers, are typically imported by a second inner membrane translocase, the TIM22 complex[31,32,79,80] (FIG. 3). In fact, most components of the import machinery are different for the two types of preprotein, except for the outer membrane GIP complex. The import pathway of the carrier proteins follows a new principle, distinct from that of preproteins that carry presequences[9,16,49,81,82]. Whereas presequences direct the targeting and translocation of polypeptides as linear chains, carrier proteins contain several signals dispersed throughout the primary structure and seem to be transported in partially folded forms[7,16,49].

The cytosolic transport form of a carrier preprotein (FIG. 3, stage I) is protected from aggregation by molecular chaperones, such as cytosolic Hsp70 and the mitochondrial import stimulating factor (MSF)[10,63]. Tom70 functions as the main receptor for these preproteins. The multiple targeting signals that are present in a carrier recruit several Tom70 dimers to one preprotein[7,16,50]. Carrier preproteins consist of three related domains of ~100 amino-acid residues, termed modules[7,49]. Each module can bind to Tom70 and, by cooperation with the other two modules, seems to induce an oligomerization of three Tom70 dimers[7,16,50] (FIG. 3, stage II). The release of a carrier preprotein from Tom70 requires ATP[81]. We propose that Tom70 not only acts as a receptor, but also has chaperone-like properties[16], protecting hydrophobic carrier preproteins (FIG. 3, stage II) from aggregation during the transfer from the cytosolic transport complex (FIG. 3, stage I) to the import pore (FIG. 3, stage IIIa).

Carrier preproteins do not cross the outer membrane as a linear chain, as preproteins that contain presequences do; instead, an internal loop is translocated first through the Tom40 channel (FIG. 3, stage IIIa). This has been directly shown by blocking both termini of a carrier preprotein with stably folded domains that remain on the cytosolic side, while an internal segment contacts translocase components of the intermembrane space[16]. Efficient accumulation of a carrier preprotein in the channel requires the presence of more than one import signal. We propose that the translocation of carrier proteins in a loop formation allows the cooperation of import signals that would be separated from each other in a linear mode of translocation.

After leaving the outer membrane translocase, carrier proteins interact with the Tim9–Tim10 complex, which probably protects these hydrophobic proteins from aggregation in the aqueous intermembrane space. Tim9 and Tim10 are homologous proteins that form a hetero-oligomeric complex, probably a hexamer, in the intermembrane space. Like most Tim proteins, these tiny Tims are essential for cell viability, emphasizing the importance of the carrier import pathway[33,34,83,84]. The tiny Tim proteins form a family with five members: Tim8, Tim9, Tim10, Tim12 and Tim13 (REFS 33,34,58,83–85). Tim8 and Tim13 are also assembled in a hetero-oligomeric complex in the intermembrane space, but they are not essential for cell viability. Tim8 and Tim13 seem to be involved in the import of some special preproteins; they trap the precursor of Tim23 in the intermembrane space, and influence the interaction of the Tim9–Tim10 complex with the inner membrane[52,53,85,86]. The first human disease caused by a defect in the mitochondrial protein import machinery — the deafness dystonia (Mohr–Tranebjaerg) syndrome — is caused by a mutation in the human homologue of Tim8 (REF. 85).

Tim12 is an essential protein located at the outer surface of the inner mitochondrial membrane. It is part of the TIM22 complex (~300 kDa), which also contains three integral membrane proteins, Tim22, Tim54 and Tim18. Tim12 represents the functional link between the soluble TIM complexes of the intermembrane space and the membrane-integrated components of the TIM22 complex[33,34,86] (FIG. 3, stage IIIb). The insertion of preproteins into the TIM22 complex (FIG. 3, stage IV) strictly depends on the presence of a membrane potential. Tim22 is homologous to Tim23 and Tim17 of the presequence translocase[31], indicating that it might form a channel. Tim54 exposes a large domain to the intermembrane space[32], and might bind preproteins or other translocase components. Tim18 might be involved in the assembly and stability of the TIM22 complex[79,80]. The current model for membrane insertion of carrier proteins suggests that the preprotein forms several loops, possibly one loop per module, during the translocation into the TIM22 complex (FIG. 3, stage IV). Finally, the carrier is released through an unknown mechanism into the lipid phase of the inner membrane and assembles into a functional homodimer (FIG. 3, stage V).

Variations on import pathways

In addition to presequence-containing preproteins and carrier preproteins, mitochondria import many other preproteins using variations of the two principal pathways (FIG. 4a). Outer membrane proteins, as well as some proteins destined for the intermembrane space or the inner membrane, initially follow the presequence pathway (FIG. 4a, pathway A) and then diverge into the corresponding subcompartment, directed by sorting signals in the preproteins[39–41,87] (TABLE 1). A special sorting mechanism has been observed for yeast mitochondrial NADH-cytochrome b_5 reductase, which ends up in two mitochondrial subcompartments, although only a single type of preprotein with a bipartite amino-terminal presequence (a positively charged matrix-targeting sequence followed by a hydrophobic sorting sequence) is synthesized[40]. During insertion into the outer membrane, about one-third of the preproteins become firmly anchored in the membrane by the hydrophobic sorting segment and remain uncleaved (see TABLE 1, first variation). However, the translocation arrest is incomplete and the remaining molecules pass through the outer membrane, are proteolytically processed and sorted to the intermembrane space. This pathway is comparable to that of other preproteins, such as cytochrome b_2, that carry a bipartite presequence (see TABLE 1, third variation).

On the carrier-import pathway (FIG. 4a, pathway B), some preproteins can bypass the receptor Tom70 and use Tom20, albeit with lower efficiency[88]. The Tim8–Tim13 complex probably functions in parallel to the Tim9–Tim10 complex in the import of some inner membrane proteins[52,53,86]. Moreover, some preproteins bound to the Tim9–Tim10 complex might be delivered to the TIM23 complex rather than the TIM22 complex[86]. Connections are also conceivable at the level of the TIM23 complex and TIM22 complex, for example, by transient contacts between the translocases.

Further variations of import pathways were revealed by analysing the biogenesis of the Tim proteins of the carrier import pathway[58] (FIG. 4b). First, the precursors of the tiny Tim proteins follow one of the simplest pathways, bypassing the surface receptors and using only the GIP complex to reach the intermembrane space (FIG. 4b, pathway C). Crossing over between the presequence pathway and the carrier pathway happens during import of the precursors of Tim22 and Tim54. Whereas the precursor of Tim22 first binds to Tom20 like presequence-containing proteins, after passing the GIP, it diverges into the carrier import route through the TIM22 complex (FIG. 4b, pathway D). Conversely, the precursor of Tim54 is recognized by Tom70 and then joins the pathway for presequence-containing preproteins through the TIM23 complex. Tim54 subsequently diverges from the presequence pathway and is released into the inner membrane (FIG. 4b, pathway E). Tim54 carries internal targeting signals and a non-cleaved segment at its amino terminus that is enriched in positively charged residues. This segment does not function as a bona fide presequence as it does not form an amphipathic α-helix, so it is not recognized by Tom20. It is the internal targeting signals of Tim54 that are recognized by Tom70. The amino-terminal segment, on the other hand, is needed for translocation through the TIM23 complex, probably by an electrophoretic effect of the membrane potential on the positively charged segment.

Some preproteins enter the import channels in a co-translational manner, while a portion of the protein is still being synthesized on ribosomes[89,90]. An interesting case is the preprotein of fumarase, a protein found both in mitochondria and in the cytosol, despite the fact that

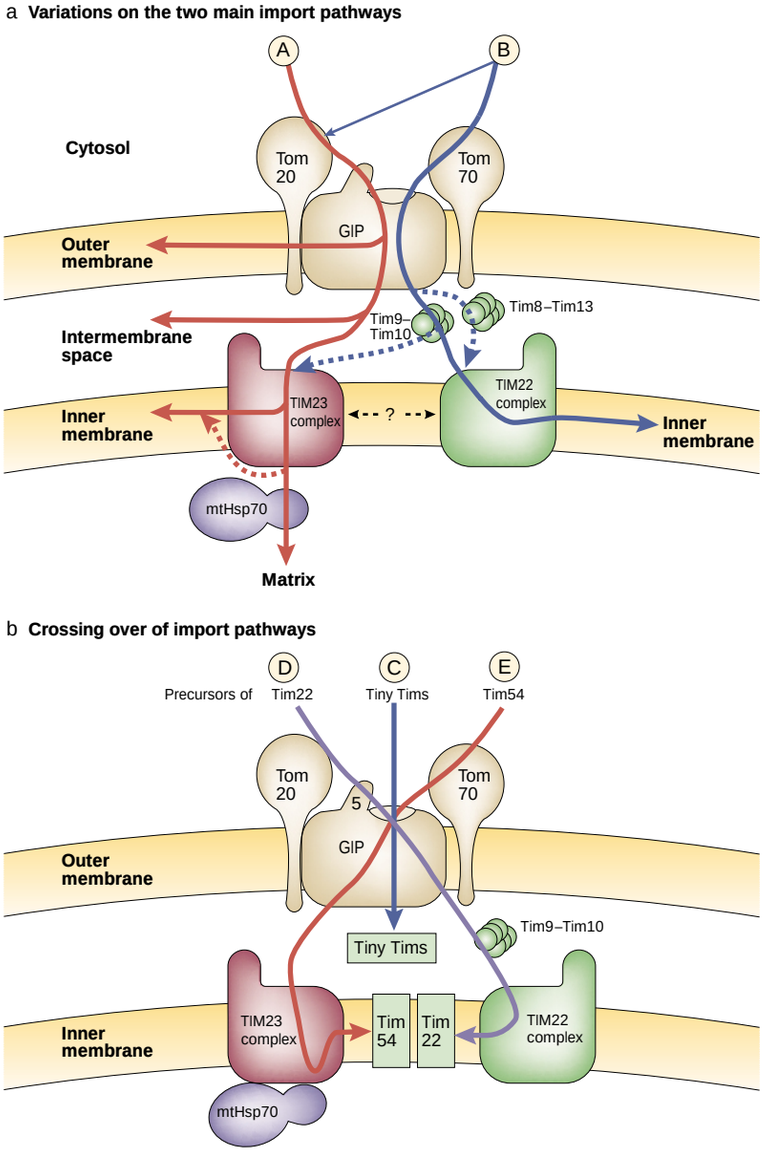


Figure 4 | **Variations on mitochondrial import pathways. a** | Variations on the two main import pathways. Pathway A is used by presequence-carrying preproteins destined for the matrix and various preproteins destined for the outer membrane, intermembrane space or inner membrane. Sorting signals in the latter preproteins direct their divergence from the main pathway. Pathway B is used by the precursors of inner membrane carriers and additional inner membrane proteins with multiple internal signals. **b** | Crossing over of import pathways (adapted from REF. 58). Pathway C for the precursors of the tiny Tim proteins of the intermembrane space is simple, and does not require surface receptors, but only Tom5 and Tom40 of the GIP. The pathways D (precursor of Tim22) and E (precursor of Tim54) combine distinct portions of the two main import pathways A and B, leading to a crossing over at the level of the GIP.

only a single preprotein with presequence is synthesized. The preprotein is partially imported into mitochondria in a co-translational manner and the presequence is removed by MPP in the matrix. Most of the processed mature-sized proteins are subsequently released into the cytosol, probably by a retrograde movement through the translocation channels, and only a minor fraction is fully imported, representing the mitochondrial form[91].

In conclusion, the original model of one principal import pathway into mitochondria that is used by the various preproteins must be radically changed. Mitochondrial preproteins do not carry a consensus targeting sequence, but have various targeting and sorting signals that are probably more characterized by physicochemical properties than by defined amino acids. Different types of import signal can be found in many combinations in preproteins. We propose that the mitochondrial translocases, the receptors, channels and transfer components, have a high flexibility for interaction with preproteins, as shown by the relatively low affinity of individual Tom or Tim proteins for preproteins[13,63]. Together with the observed dynamic interactions between translocase components[19,55,67,84,86], this favours the generation of a network of import pathways (FIG. 4). How, then, can a cell ensure the specificity of mitochondrial protein import? A preprotein cannot be imported by simply interacting with only one import component. Typically, preproteins must be recognized at numerous levels of their import pathway and only when they have the targeting characteristics required at each level will they finally end up at the correct location. This view of cooperation of multiple low-affinity binding sites (binding chain hypothesis) is supported by the observation that the entire TOM complex interacts with a preprotein with significantly higher affinity[92] than individual Tom proteins do[13,63].

Perspectives

Mitochondrial protein import will hold many more surprises and questions for us. As all Tom and Tim proteins are encoded by nuclear genes, their preproteins have to be imported as well. It is vital for any cell that the precursors of the import components are correctly targeted and assembled. Indeed, mistargeting one of the import receptors to another organelle would lead to the mistargeting of many other preproteins. We are only beginning to understand the biogenesis of the TOM and TIM machineries. The current evidence indicates that none of the Tom receptors can import its own preprotein, but requires other Tom proteins for specific import[93]. This provides a safety system to avoid mistargeting of an individual receptor molecule, which would cause a subsequent mistargeting of many receptor molecules to the wrong organelle. New Tom and Tim proteins seem to be continuously integrated into pre-existing complexes[17,19,94,95]. A possible model is that the dynamic translocase complexes are in rapid exchange with subcomplexes, into which late-assembly intermediates can be integrated. Indeed, it has recently been suggested that the mature GIP complex of 400 kDa is in exchange with a 100-kDa subcomplex that functions as an assembly site for newly imported TOM subunits[95], including the essential preprotein of the channel Tom40.

An important question concerns the poorly understood mechanism that allows mitochondria to export proteins from the matrix to the inner membrane, including components of the respiratory chain that are encoded and synthesized in the matrix. The mitochondrial inner membrane protein Oxa1 (cytochrome oxidase assembly 1) has been identified as a component of the export machinery that directs proteins from the matrix into the inner membrane[96,97]. Except for the inner membrane peptidase, which is related to the bacterial leader peptidase[45,46], Oxa1 is the first mitochondrial transport component with homology to a bacterial export component (YidC)[98]. Oxa1 might therefore represent a machinery for conservative, that is, prokaryotic-like sorting of some mitochondrial proteins that are encoded by either the mitochondrial or the nuclear genome. Recently, Pnt1 has been identified as a possible additional component of an export machinery for mitochondrially encoded proteins[99], but the composition and mode of action of the export machinery are not yet known.

A pressing issue is to unravel the structural organization of the receptors and channels of the mitochondrial import machinery. How are the targeting and sorting signals decoded by receptors and downstream import components? The relevance of co-translational translocation *in vivo* and the roles of cytosolic cofactors for co-translational as well as post-translational translocation are not understood. How can the inner membrane translocase channels be kept impermeable to protons during the translocation of entire polypeptide chains? How is the energy input converted into directional protein translocation at the molecular level? Which components are responsible for protein sorting to the mitochondrial subcompartments? It is conceivable that the mitochondrial inner membrane contains a third import translocase complex[86] and thus, like bacteria and thylakoids (of chloroplasts), might have several translocation and sorting machineries. Finally, we would like to know how the flow of preproteins through the different import pathways is coordinated and regulated.

Links

DATABASE LINKS porins | mtHsp70 | MPP | Hsp60 | Tom20 | Tom22 | Hsp10 | Hmi1 | cytochrome c_1 | cytochrome b_2 | Imp1 | Imp2 | Tom70 | Tom40 | Tom5 | Tom6 | Tom7 | Tim23 | Tim17 | Tim44 | Mge1 | MSF | Tim9 | Tim10 | Tim8 | Tim12 | Tim13 | Tim22 | Tim54 | Tim18 | NADH-cytochrome b_5 reductase | fumarase | deafness dystonia syndrome | Oxa1 | Pnt1

FURTHER INFORMATION Pfanner lab

ENCYCLOPEDIA OF LIFE SCIENCES Protein translocation across membranes | Mitochondria: structure and role in respiration

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