

Role of membrane contact sites in protein import into mitochondria

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ABSTRACT: Mitochondria import more than 1,000 different proteins from the cytosol. The proteins are synthesized as precursors on cytosolic ribosomes and are translocated by protein transport machineries of the mitochondrial membranes. Five main pathways for protein import into mitochondria have been identified. Most pathways use the translocase of the outer mitochondrial membrane (TOM) as the entry gate into mitochondria. Depending on specific signals contained in the precursors, the proteins are subsequently transferred to different intramitochondrial translocases. In this article, we discuss the connection between protein import and mitochondrial membrane architecture. Mitochondria possess two membranes. It is a long-standing question how contact sites between outer and inner membranes are formed and which role the contact sites play in the translocation of precursor proteins. A major translocation contact site is formed between the TOM complex and the presequence translocase of the inner membrane (TIM23 complex), promoting transfer of presequence-carrying preproteins to the mitochondrial inner membrane and matrix. Recent findings led to the identification of contact sites that involve the mitochondrial contact site and cristae organizing system (MICOS) of the inner membrane. MICOS plays a dual role. It is crucial for maintaining the inner membrane cristae architecture and forms contact sites to the outer membrane that promote translocation of precursor proteins into the intermembrane space and outer membrane of mitochondria.

This article is dedicated to Karl Decker from the Institut für Biochemie und Molekularbiologie (Universität Freiburg) on the occasion of his 90th birthday.

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The view is emerging that the mitochondrial protein translocases do not function as independent units, but are embedded in a network of interactions with machineries that control mitochondrial activity and architecture.

Keywords: contact site; membrane architecture; MICOS; mitochondria; protein sorting; protein translocase

Introduction

Mitochondria consist of four compartments: outer membrane, intermembrane space, inner membrane, and matrix (Fig. 1). Mitochondria are known best for their central role in cellular energy metabolism, resulting in the synthesis of ATP by oxidative phosphorylation. However, mitochondria play many more crucial functions in eukaryotic cells, including the metabolism of amino acids and lipids, the biosynthesis of heme and iron-sulfur clusters, the involvement in cellular stress responses and a central role in programmed cell death (apoptosis).^{1–10}

Proteomic studies revealed that mitochondria contain more than 1,000 different proteins.^{11–16} Although mitochondria carry a complete genetic system in the matrix, only ~1% of mitochondrial proteins are encoded by the mitochondrial genome. Most mitochondrial-encoded proteins are strongly hydrophobic subunits of the oxidative phosphorylation machinery of the inner membrane. They are synthesized on mitochondrial ribosomes and are typically exported into the inner membrane by the OXA (cytochrome oxidase activity) translocase [Fig. 1, pathway (vi)].^{17,18}

The vast majority of mitochondrial proteins are encoded by nuclear genes and synthesized on cytosolic ribosomes. The precursor proteins carry targeting signals that direct the precursors to receptors on the mitochondrial surface, followed by translocation into mitochondria. Experimental systems to study mitochondrial protein import mostly use precursor proteins that can be imported in a post-translational manner. However, it has been shown that cytosolic ribosomes associate with mitochondria and various precursor proteins can initiate translocation into mitochondria while part of the precursor is still being synthesized on ribosomes (cotranslational import).^{19–27} It is likely that both post-translational and cotranslational mechanisms are important for the biogenesis of mitochondrial proteins.

From electron micrographic pictures of mitochondria, sites of close contact between outer and inner mitochondrial membranes have been known for decades.^{19,28–34} The molecular nature and function of membrane contact sites for mitochondrial activity and biogenesis are the subject of ongoing research. In the current view, mitochondria do not possess one defined, permanent contact site structure but contain multiple dynamic contact sites that involve a variety of protein complexes from outer

and inner membranes. Here, we first provide an overview on the five main protein import pathways into mitochondria and then discuss how preprotein translocases are involved in forming different types of contact sites. Recent findings indicate that contact sites are involved in at least three protein import pathways and stimulate the import of different classes of precursor proteins by promoting an efficient cooperation of translocases.

Five Mitochondrial Protein Import Pathways—An Overview

Depending on their intramitochondrial destination, mitochondrial precursor proteins contain distinct types of targeting and intramitochondrial sorting signals. Currently, at least five major classes of precursor proteins can be distinguished that use different pathways of translocation into mitochondria (Fig. 1). The protein translocase of the outer membrane (TOM) is the major mitochondrial entry site used by the majority of import pathways.

- i. The classical presequence pathway is used by ~60% of all mitochondrial precursor proteins.³⁵ The preproteins are synthesized with amino-terminal presequences that form positively charged amphipathic α -helices.³⁶ Upon recognition by Tom receptors, the preproteins are translocated through an outer membrane channel formed by Tom40 and are transferred to the presequence translocase of the inner membrane (TIM23 complex) [Fig. 1, pathway (i)].^{37–39} Hydrophilic preproteins are imported into the matrix with the help of the presequence translocase-associated motor (PAM).^{40–44} Preproteins destined for the inner membrane contain a hydrophobic sorting signal behind the positively charged matrix targeting signal. These preproteins are laterally released into the lipid phase of the inner membrane.^{45,46} For both, matrix-translocated and inner membrane-sorted preproteins, the positively charged matrix targeting signal is proteolytically removed by the mitochondrial processing peptidase (MPP).^{47–49}
- ii. The carrier pathway imports the precursors of noncleavable multispinning inner membrane proteins. The precursors contain internal targeting signals that are not removed during import but remain part of the mature mitochondrial protein.⁵⁰ The hydrophobic metabolite carriers of

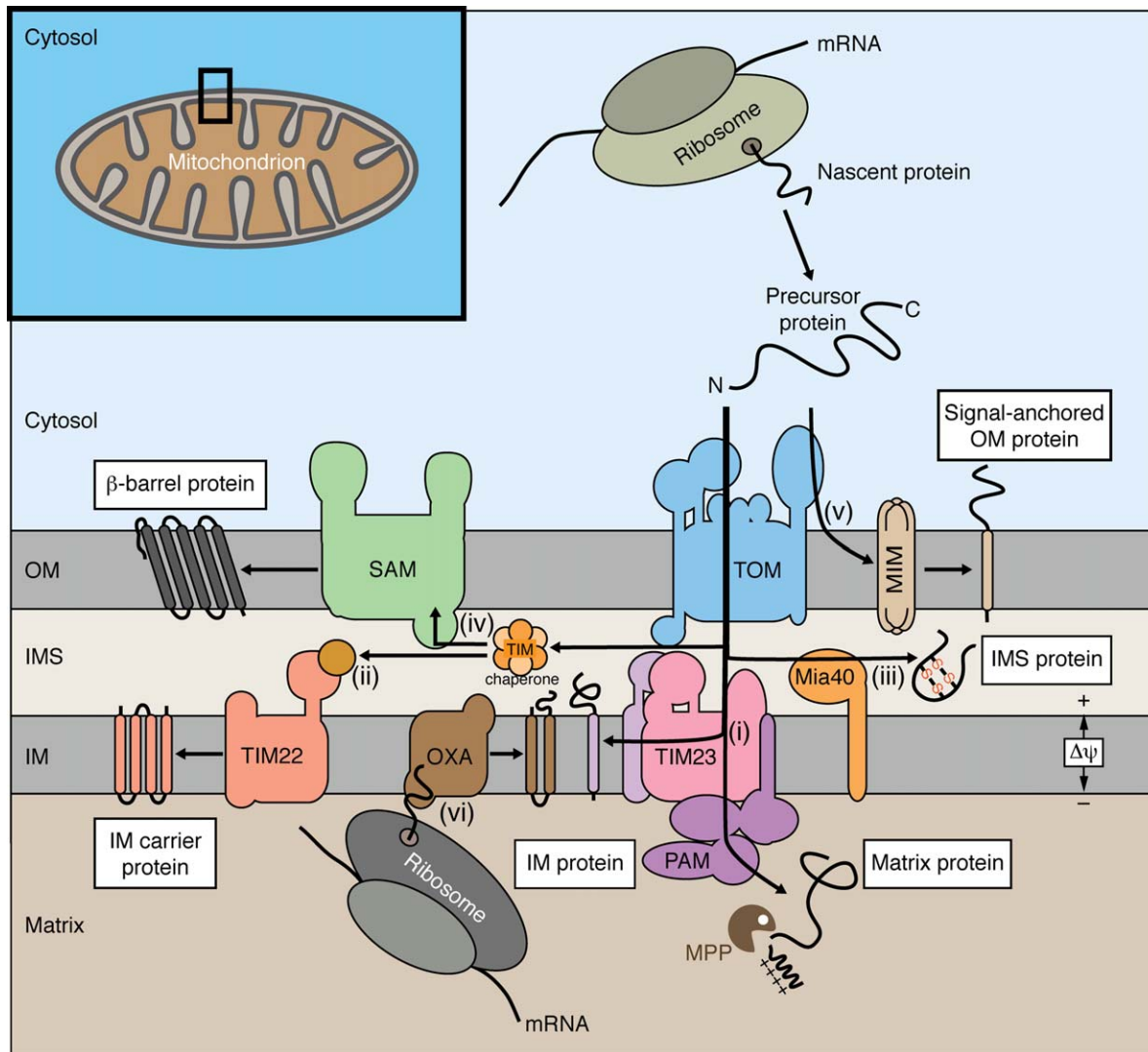


Figure 1. Biogenesis of mitochondrial proteins. Most mitochondrial proteins are synthesized on cytosolic ribosomes and imported into mitochondria by the translocase of the outer membrane (TOM). (i) Presequence-carrying preproteins are transferred from TOM to the presequence translocase of the inner membrane (TIM23). Hydrophilic preproteins are transported into the matrix with the help of the presequence translocase-associated motor (PAM). Preproteins with hydrophobic sorting signal are arrested in the TIM23 complex and are laterally released into the inner membrane (IM). The presequences are proteolytically removed by the mitochondrial processing peptidase (MPP). (ii) The precursors of multispanning inner membrane proteins like the carrier proteins are imported via TOM, the small TIM chaperones of the intermembrane space (IMS) and the carrier translocase of the inner membrane (TIM22). (iii) IMS proteins with cysteine motifs are imported via TOM and the mitochondrial IMS import and assembly (MIA) machinery. (iv) The precursors of outer membrane (OM) β -barrel proteins use TOM, small TIM chaperones and the sorting and assembly machinery (SAM) for insertion into the outer membrane. (v) For α -helical outer membrane proteins, different pathways have been described. Shown is the import of a precursor protein via the mitochondrial import (MIM) complex. (vi) A small number of hydrophobic proteins are encoded by mtDNA and synthesized in the matrix. These proteins are typically exported into the inner membrane by the cytochrome oxidase activity (OXA) translocase. $\Delta\psi$, membrane potential across the inner mitochondrial membrane (drives protein import via TIM23 and TIM22).

the inner membrane are the major substrates of this pathway. The precursors are initially translocated by the TOM complex, yet do not use the TIM23 complex, but bind to soluble TIM chaperones of the intermembrane space that are formed by small Tim proteins [Fig. 1, pathway (ii)].⁵¹ The small TIM chaperones guide the hydrophobic precursors to the carrier translocase of the inner membrane (TIM22 complex) that mediates

membrane insertion of the proteins in a membrane potential ($\Delta\psi$)-driven manner.⁵²

iii. It had been assumed that the mitochondrial intermembrane space is a reducing environment and thus proteins are in a reduced state. The identification of an oxidative protein import and folding system in the intermembrane space changed this view and led to the discovery of a large number of oxidized, disulfide-containing

intermembrane space proteins.^{53–60} We termed the system the mitochondrial intermembrane space import and assembly (MIA) machinery. The MIA machinery accepts preproteins after their translocation through TOM, recognizes a cysteine-containing targeting signal and inserts disulfide bonds into the proteins [Fig. 1, pathway (iii)].^{61–66}

- iv. The mitochondrial outer membrane contains two types of transmembrane proteins, proteins with α -helical transmembrane segments and β -barrel proteins. The precursors of β -barrel proteins are initially recognized and translocated by the TOM complex. The precursors are transferred to the intermembrane space side and bind to the small TIM chaperones [Fig. 1, pathway (iv)]. Insertion of the proteins into the outer membrane is mediated by the sorting and assembly machinery (SAM).^{67–70}
- v. The biogenesis of α -helical outer membrane proteins involves several distinct pathways and is only understood in part. The mitochondrial import (MIM) complex promotes insertion of some α -helical proteins into the outer membrane, in particular proteins with amino-terminal membrane anchor (termed signal-anchored proteins) as well as multispansing outer membrane proteins [Fig. 1, pathway (v)].^{71–75} Tom receptors can be involved in the recognition of multispansing proteins before their insertion by the MIM complex. As discussed below, a special signal-anchored protein uses the presequence pathway, followed by MIM-dependent outer membrane insertion.^{76,77} Various pathways have been discussed for proteins with carboxy-terminal membrane anchor (tail-anchored proteins), including a lipid-mediated insertion into the outer membrane,^{78,79} and an involvement of the SAM complex for some of the proteins.^{80,81}

In this article, we will discuss that physical contacts between protein complexes of mitochondrial outer and inner membranes promote the translocation of precursor proteins not only via the presequence pathway, but also via the MIA and β -barrel pathways.

TOM-TIM Contact Sites and the Presequence Pathway

The first evidence for import of mitochondrial proteins via contact sites was obtained by the accumulation of translocation intermediates of cleavable preproteins across both mitochondrial membranes.⁸² The two-membrane spanning preproteins were present in a hydrophilic membrane environment, suggesting that they were accumulated in hydrophilic transport channels.⁸³ The copurification of TOM and TIM23 complexes together with an accumulated preprotein directly demonstrated the existence of translocation contact sites.^{42,84–94} TOM and TIM23

complexes transiently interact in the absence of preproteins,^{95–97} yet their interaction is strongly stabilized by a preprotein in transit [Fig. 2(A)]. The channels formed by the TOM and TIM23 translocases are so narrow that folded proteins cannot pass^{98–102} and thus preproteins have to be translocated in an unfolded conformation.^{29,41,103–113}

Several Tom and Tim proteins participate in the formation of TOM-TIM23 translocation contact sites: the intermembrane space domain of the receptor Tom22, Tom40, and Tom7 of the outer membrane translocase,^{88,96,114–116} as well as Tim50, Tim23, and Tim21 of the inner membrane translocase^{85,86,89,95,97,117,118} [Fig. 2(A)]. Tim50 plays a central role in the TOM-TIM23 reaction cycle. In the absence of preproteins, the intermembrane space domain of Tim50 keeps the translocation channel formed by Tim23 in a closed state.¹¹⁹ Presequence-carrying preproteins are initially recognized by the receptors Tom20 and Tom22 on the cytosolic side of the outer membrane.^{120–124} Upon passing through the Tom40 channel of the outer membrane, the presequences bind to the intermembrane space domain of Tom22.^{125–128} Tom7 may be involved at this import stage. Tim50 promotes the efficient binding of preproteins to Tom22 at the *trans* side of the TOM complex,^{88,95,117,118} underscoring the close cooperation between Tom and Tim proteins. Tim50 then functions as inner membrane receptor for preproteins and in cooperation with the intermembrane space domain of Tim23 transfers the preproteins to the Tim23 channel.^{85,95,100,117,129–134} Tim21 plays a regulatory role in preprotein transfer through the intermembrane space.^{88,132} The membrane potential ($\Delta\psi$) across the inner membrane activates the Tim23 channel and exerts an electrophoretic effect on the positively charged presequences.^{100,135}

The TIM23 complex functions in a dynamic manner and distinct TIM23 forms have been identified [Fig. 2(A)].^{88,91,92,136} In an early stage of translocation, the TIM23 core complex consisting of Tim50, Tim23, and Tim17 is associated with Tim21 and the recently identified subunit Mgr2.^{137,138} The motor subunit Pam17 plays a regulatory role. Pam17 replaces Tim21 at the translocase and initiates assembly of further motor subunits to the TIM23 complex.^{90,91,132,139,140} Tim44 functions as membrane docking site for the central motor component, the matrix heat shock protein 70 (mtHsp70).^{139,141–145} Two further membrane-bound cochaperones, the J-protein Pam18 (also termed Tim14) and its partner Pam16 (Tim16) regulate the ATP-driven activity of mtHsp70.^{42–44,87,146–153} The nucleotide release factor Mge1 promotes the release of ADP from mtHsp70 to initiate a new round of the mtHsp70 reaction cycle.^{154–157} The mtHsp70 motor drives the ATP-dependent import of preproteins into the matrix by a combination of pulling and trapping of the

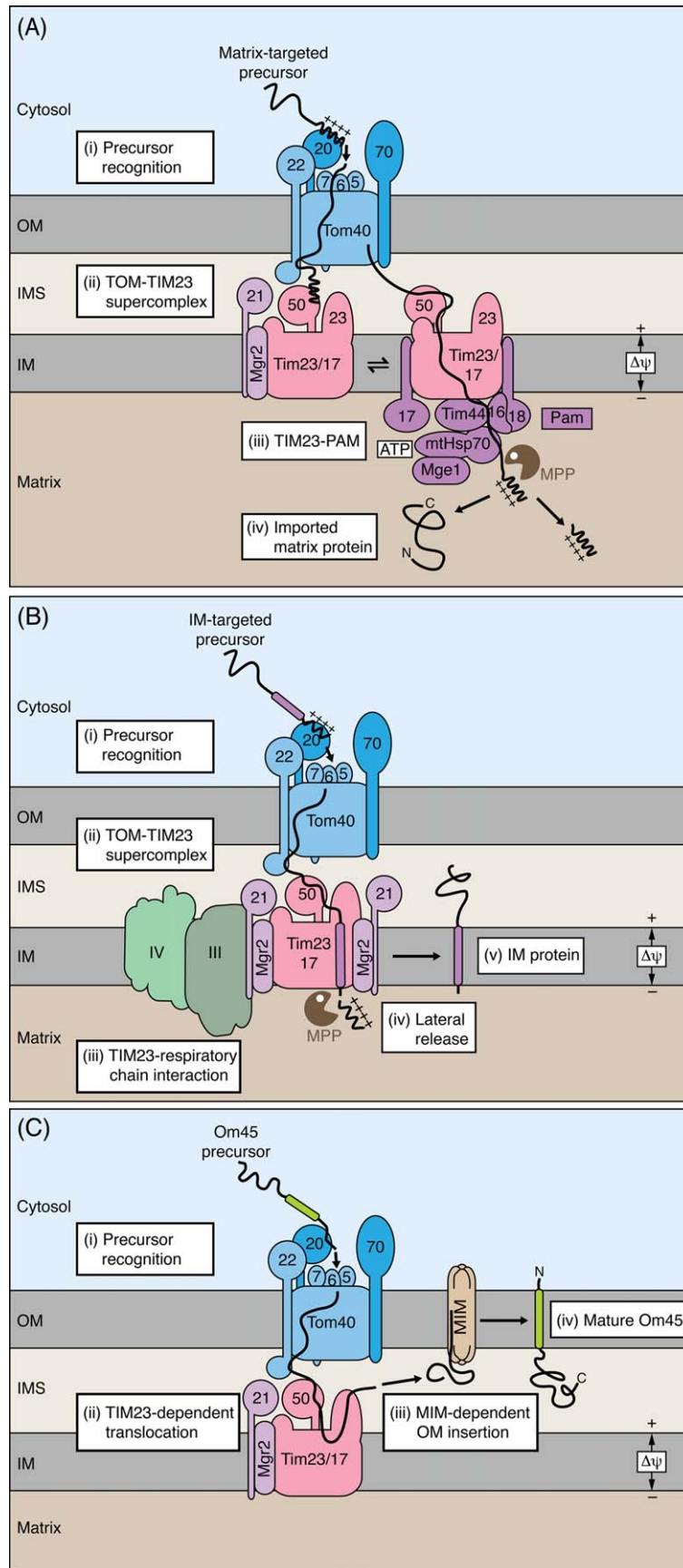


Figure 2.

precursor polypeptide and thus promotes unfolding of carboxy-terminal precursor domains on the mitochondrial surface.^{40,41,93,158–160}

During preprotein import, both forms of the TIM23 complex, TIM23-Tim21 and TIM23-PAM, cooperate with TOM to form TOM-TIM23-preprotein supercomplexes.⁹² TOM-TIM23-Tim21 is found in translocation contact sites at early import stages and TOM-TIM23-PAM preferentially at later stages. The translocation of presequence-carrying preproteins across the mitochondrial outer and inner membranes thus involves a dynamic interaction between TOM, different TIM23 states and the motor PAM and is driven by presequence binding, $\Delta\psi$ and ATP. The presequences sequentially interact with binding sites at multiple steps of their translocation pathway, including receptors on the cytosolic side, the channel protein Tom40, the intermembrane space domain of Tom22, Tim50, Tim23, and chaperones.^{40,122,123,128,130,132,161–166} During or after translocation into the matrix, the preproteins are cleaved by MPP to remove the positively charged presequences.

Imaging studies revealed that mitochondrial import sites are not equally distributed across the membranes, but cluster in microdomains,^{167,168} suggesting that several TOM-TIM23-preprotein supercomplexes are organized into larger translocation contact sites. Electron cryotomography shows that the contact sites are connected via protein–protein interactions, not via direct contact of the lipid phases of the membranes.¹⁶⁸ The high resolution structures of various hydrophilic import components and domains have been determined and provided important information on the recognition of presequences, function of cochaperones and processing of preproteins.^{48,114,116,123,131,149,169–175} However, high resolution structures of the membrane domains (channels) of TOM and TIM complexes are lacking so far. This is a major reason that despite a wealth of knowledge on individual reaction steps, the exact mechanisms of preprotein translocation and the complete TOM-TIM23-PAM reaction cycle have not been elucidated yet.

Lateral Escape from the Presequence Pathway

TOM-TIM23 supercomplexes efficiently guide preproteins from the outer membrane to the inner membrane. However, they do not form completely sealed structures, but preproteins in transit are partially exposed to the intermembrane space.¹⁷⁶

At the level of the inner membrane, the import of cleavable preproteins branches into the matrix route and the inner membrane sorting route.^{45,46} Hydrophilic preproteins are fully translocated into the matrix, whereas preproteins with a hydrophobic sorting signal behind the positively charged matrix targeting signal are typically arrested in the TIM23 complex and are laterally released into the inner membrane (stop transfer pathway).⁴⁵ The TIM23-Tim21 complex functions as insertase that can insert preproteins into the inner membrane without the motor PAM¹³⁶ [Fig. 2(B)] (whereas an association with PAM is strictly required for preprotein translocation into the matrix). The small hydrophobic TIM23 subunit Mgr2 acts as lateral gatekeeper of the translocase. Mgr2 is not required for the translocation of preproteins into the matrix, but plays a regulatory role by delaying the release of preproteins into the lipid phase of the inner membrane.⁹⁴ Mgr2 recognizes the inner membrane-sorting signal and permits its lateral exit, whereas preproteins without a functional sorting signal are prevented from entering the inner membrane.

A direct connection between the TIM23 complex and the respiratory chain complexes III (cytochrome *bc*₁ complex) and IV (cytochrome *c* oxidase) stimulates the insertion of preproteins into the inner membrane [Fig. 2(B)]. Tim21 provides the link between preprotein translocase and respiratory supercomplexes III-IV.^{137,177–179} The exact mechanism of how the TIM23-respiratory chain connection promotes preprotein import has not been identified. TIM23 complexes in the direct vicinity of proton-pumping respiratory complexes likely experience a higher proton-motive force and thus preprotein import is stimulated.¹⁸⁰ The Tim21-respiratory chain connection may also play a role in the assembly of respiratory chain complexes. Human Tim21 was

Figure 2. Translocation contact sites as core of the presequence pathway of mitochondria. (A) Matrix-targeted precursor proteins typically carry cleavable amino-terminal presequences. The presequences are recognized by receptors of the translocase of the outer membrane (TOM). Upon translocation through the Tom40 channel, the preproteins are transferred to the presequence translocase of the inner membrane (TIM23 complex). Several Tom and Tim proteins cooperate in the formation of dynamic TOM-TIM23 translocation contact sites. Two forms of the TIM23 complex are in dynamic exchange with each other. Both TIM23 forms are involved in the formation of TOM-TIM23 supercomplexes. In an early stage of translocation, Tim21 is associated with TIM23, whereas in later stages, the presequence translocase-associated motor (PAM) is associated with TIM23. The mitochondrial processing peptidase (MPP) removes the presequences. OM, outer membrane; IMS, intermembrane space; IM, inner membrane. (B) Cleavable preproteins inserted into the inner membrane (IM) carry a hydrophobic sorting signal behind the positively charged matrix targeting signal. Insertion of these preproteins into the inner membrane can be mediated by the Tim21-containing TIM23 complex without PAM. Mgr2 functions as lateral gatekeeper of the TIM23 complex and controls the proper sorting of preproteins into the inner membrane. The respiratory chain complexes III and IV interact with the Tim21-containing TIM23 complex and stimulate the $\Delta\psi$ -dependent preprotein insertion. (C) The outer membrane protein Om45 uses an unusual biogenesis pathway that involves TOM, TIM23 and the mitochondrial import (MIM) complex.

found to dynamically interact with the TIM23 complex and with assembly intermediates of the respiratory chain, termed MITRAC (mitochondrial translation regulation assembly intermediate of cytochrome oxidase).¹⁸¹ Human Tim21 thus helps to transfer preproteins from the presequence translocase to the respiratory chain. Additionally, a recent study showed that the mitochondrial ADP/ATP carrier associates with the TIM23 complex and may support preprotein import when the respiratory activity is low.¹⁸² When oxidative phosphorylation is inhibited, mitochondria do not export ATP, but can import glycolysis-generated ATP in exchange for ADP, thus generating a membrane potential and providing ATP to drive the TIM23-PAM machinery.

It should be added that not all preproteins with hydrophobic segments are arrested in the inner membrane. Some hydrophobic proteins or protein segments are transferred to the matrix via TIM23-PAM and are exported into the inner membrane by the OXA machinery that has been conserved from bacteria (YidC) to mitochondria. This pathway has been termed the conservative sorting pathway.^{183–188} A special case of conservative sorting represents the biogenesis of the Rieske iron-sulfur protein of respiratory complex III. After initial translocation of the precursor into the matrix and proteolytic processing, the folded iron-sulfur domain is exported to the intermembrane space by the AAA-ATPase Bcs1.¹⁸⁹ Currently, many more inner membrane proteins sorted by a stop transfer mechanism are known than proteins using conservative sorting. Further work will be required to define the substrates of the conservative sorting pathway.

Recent studies surprisingly showed that TOM-TIM23 contact sites can even be used for protein translocation into the outer membrane. The protein Om45 is anchored in the outer membrane by an amino-terminal anchor sequence and exposes a large domain into the intermembrane space.^{76,77,190,191} The precursor of Om45 initially embarks on the presequence pathway using TOM and TIM23 complexes and the $\Delta\psi$ across the inner membrane [Fig. 2(C)]. However, the precursor is not translocated into the matrix but escapes from the presequence route and is inserted into the outer membrane.^{76,77} The outer membrane MIM complex, which has been known to insert various α -helical outer membrane proteins arriving from the cytosolic side, is involved in this final stage of Om45 biogenesis.⁷⁶ MIM may mediate membrane insertion of Om45 from the intermembrane space side or promote the assembly of Om45 in the outer membrane. Thus, three translocases, TOM, TIM23 and MIM, cooperate to form a new intramitochondrial sorting pathway, revealing a high versatility of how contact sites are used for protein import into mitochondria.

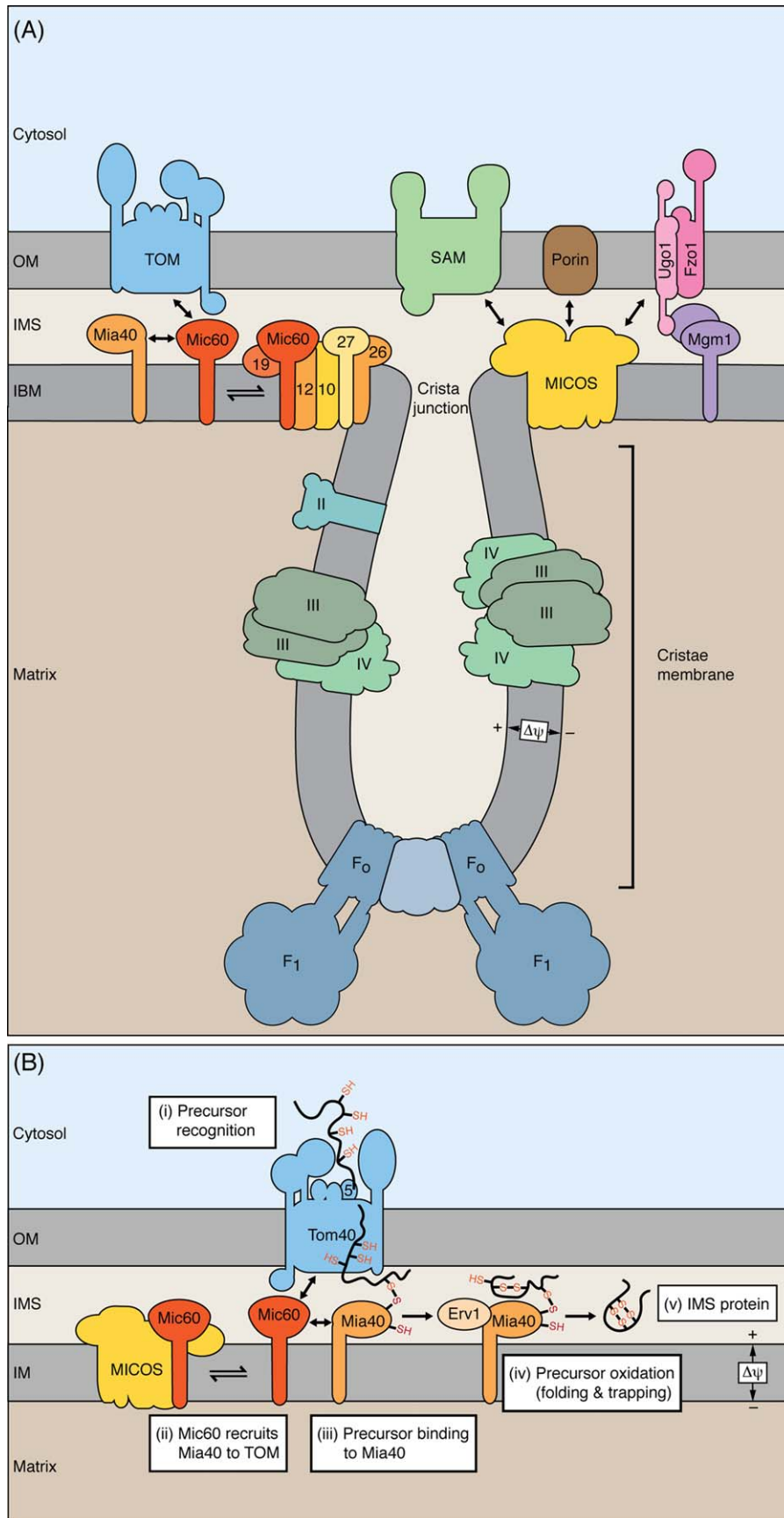
In summary, the presequence translocase shows a remarkable flexibility. It dynamically interacts with TOM, PAM, ADP/ATP carrier, and respiratory chain complexes and thus TOM-TIM23 translocation contact sites undergo a multi-step reaction cycle and control different intramitochondrial sorting routes.

Mitochondrial Contact Site and Cristae Organizing System

The mitochondrial inner membrane displays a characteristic architecture with extended membrane invaginations, termed cristae. Two domains of the inner membrane can be distinguished: the inner boundary membrane that is closely opposed to the outer membrane and the cristae membranes [Fig. 3(A)].¹⁹² The two membrane domains differ in their protein distribution: preprotein translocases and components of the fusion machinery are predominantly localized in the inner boundary membrane, whereas the F_1F_0 -ATP synthase and respiratory chain complexes are mainly found in the cristae membranes.^{193–201} Both membrane domains are connected through tubular openings, termed crista junctions, which probably limit diffusion of molecules and help to create a microenvironment in the intracristal space enhancing the capacity of the oxidative phosphorylation system of mitochondria.^{34,198,202–205}

A large hetero-oligomeric protein complex of the inner mitochondrial membrane is enriched at crista junctions.^{206,207} The complex and its evolutionary conserved components originally received several different names,^{26,206–215} yet recently the nomenclature was unified and the complex is termed MICOS for mitochondrial contact site and cristae organizing system.²¹⁶ MICOS is composed of six subunits that all expose domains to the intermembrane space [Fig. 3(A)]. The two core components, Mic10 and Mic60 (mitofilin/Fcj1), and three further subunits, Mic12, Mic26, and Mic27, are integral inner membrane proteins. The sixth subunit, Mic19, is a peripheral membrane protein. In addition, the inner membrane protein Aim24 is required for the integrity of MICOS, but is not a structural component of the mature complex.²¹⁷ Cells lacking structural MICOS subunits show a strongly altered morphology of the inner membrane.^{26,206–208,213,218–220} Most crista junctions are lost and the cristae membranes form large internal membrane stacks that are detached from the inner boundary membrane. MICOS is thus required for maintaining the connections between inner boundary membrane and cristae membranes.

MICOS plays a dual role. In addition to maintaining crista junctions, multiple interactions of MICOS with protein complexes of the outer membrane were observed. MICOS was found to interact with TOM and SAM complexes, with the abundant outer membrane channel protein porin (VDAC) and with a component of the mitochondrial fusion machinery, the



outer membrane protein Ugo1.^{26,207–209,221–223} MICOS is considered as an important element in the formation of outer–inner membrane contact sites.²²⁴ Super-resolution microscopy indicated that MICOS complexes are regularly distributed in the inner membrane, forming MICOS arrays.²²⁵ MICOS is thus involved in a network of interactions that are important for maintaining inner membrane architecture and formation of contact sites.

MICOS and Protein Import into Intermembrane Space and Outer Membrane

Cells lacking the largest MICOS subunit, Mic60, were found to import cysteine-rich intermembrane space proteins with reduced efficiency.^{26,226} Mutant mitochondria lacking other MICOS subunits imported these proteins like wild-type mitochondria. How does Mic60 influence import via the MIA pathway, yet none of the other MICOS subunits does? We propose that Mic60 can function in two modes, a MICOS-dependent one and a MICOS-independent one [Fig. 3(B)].

In the MICOS-dependent mode, Mic60 is of central importance for inner membrane architecture. In the MICOS-independent mode, Mic60 forms contact sites with the TOM complex and transiently interacts with Mia40, the core component of the oxidative protein import and folding machinery of the intermembrane space [Fig. 3(B)]. The oxidoreductase Mia40 functions as receptor for precursors upon their passage through the TOM channel.^{53–55,227,228} Since Mia40 interacts with the cysteine-containing targeting signals of the precursors as soon as they emerge from the TOM channel,^{229–232} a close spatial proximity of Mia40 to the TOM complex promotes efficient protein import via the MIA pathway.²⁶ However, a direct interaction between Mia40 and TOM has not been observed, but Mic60 performs an adapter-like function. By interacting with both TOM and Mia40, Mic60 positions Mia40 close to the exit of the TOM channel. In cooperation with the sulfhydryl oxidase Erv1, Mia40 oxidizes the precursors, leading to formation of disulfides and folding of the mature proteins.^{56–58,233–236} Thus, a close proximity to the TOM complex is important for Mia40 to capture precursor substrates directly at the entry gate.

Mitochondria deficient in Mic60 are not only impaired in the import of intermembrane space proteins, but also in the import of β -barrel proteins of the outer membrane. β -barrel precursors engage both TOM and SAM complexes on their biogenesis pathway (Fig. 4, left half). The precursor proteins are initially translocated through the TOM channel to the intermembrane space, bind to small TIM chaperones that shield hydrophobic regions of the precursors, and are inserted into the outer membrane by the SAM complex. TOM and SAM complexes transiently interact to form a supercomplex that promotes an efficient transfer of β -barrel precursors.²³⁷ In addition to the Mic60-TOM interaction, MICOS also interacts with the SAM complex (Fig. 4),^{207,209,221–223} and thus both interactions may contribute to an efficient biogenesis of β -barrel precursors. However, by dissecting the translocation of β -barrel precursors into distinct stages, Bohnert *et al.*²³⁸ indicated that the early step of precursor translocation through the TOM complex is promoted by Mic60, whereas the subsequent precursor interaction with SAM is not influenced by MICOS. Thus, the Mic60-TOM interaction is critical for stimulating β -barrel import. The molecular mechanism of β -barrel biogenesis stimulation by Mic60 remains to be elucidated.

Interestingly, for MIA substrates as well as for β -barrel precursors, only Mic60 is needed to stimulate their biogenesis, not any other MICOS subunit. Since the proper architecture of mitochondrial cristae depends on an intact MICOS complex, it is evident that the promotion of protein import by Mic60 does not depend on the morphology of the inner membrane. For example, in mitochondria lacking Mic10, most crista junctions are lost and large internal membrane stacks are formed like in mitochondria lacking Mic60.^{26,207,208,213} However, *mic10* Δ mitochondria possess fully active MIA and β -barrel pathways in contrast to *mic60* Δ mitochondria.^{26,238}

We propose that the MICOS complex and in particular Mic60 represent a multifunctional molecular hub in the mitochondrial intermembrane space. Mic60 is crucial for maintaining mitochondrial inner membrane architecture and promotes biogenesis of proteins of the intermembrane space and outer membrane by forming contact sites with TOM. At

Figure 3. Mitochondrial contact site and cristae organizing system and protein import into intermembrane space. (A) The mitochondrial inner membrane consists of the inner boundary membrane (IBM), cristae membranes and the connecting crista junctions. Protein translocases are enriched in the IBM, whereas respiratory complexes and the F_1F_0 -ATP synthase are enriched in cristae membranes. The MICOS complex is enriched at crista junctions and exposes protein domains into the intermembrane space (IMS). MICOS is crucial for maintenance of the cristae structure and forms multiple contact sites with outer membrane (OM) protein complexes. Fzo1, Ugo1, and Mgm1, components of the mitochondrial membrane fusion machinery. (B) The precursors of intermembrane space proteins with cysteine motifs are translocated through the Tom40 channel and are recognized by the oxidoreductase Mia40 that functions as receptor in the intermembrane space. Mic60 interacts with TOM and Mia40 and thus positions Mia40 close to the Tom40 channel. In cooperation with the sulfhydryl oxidase Erv1, Mia40 oxidizes the imported proteins, leading to disulfide formation and folding of the proteins.

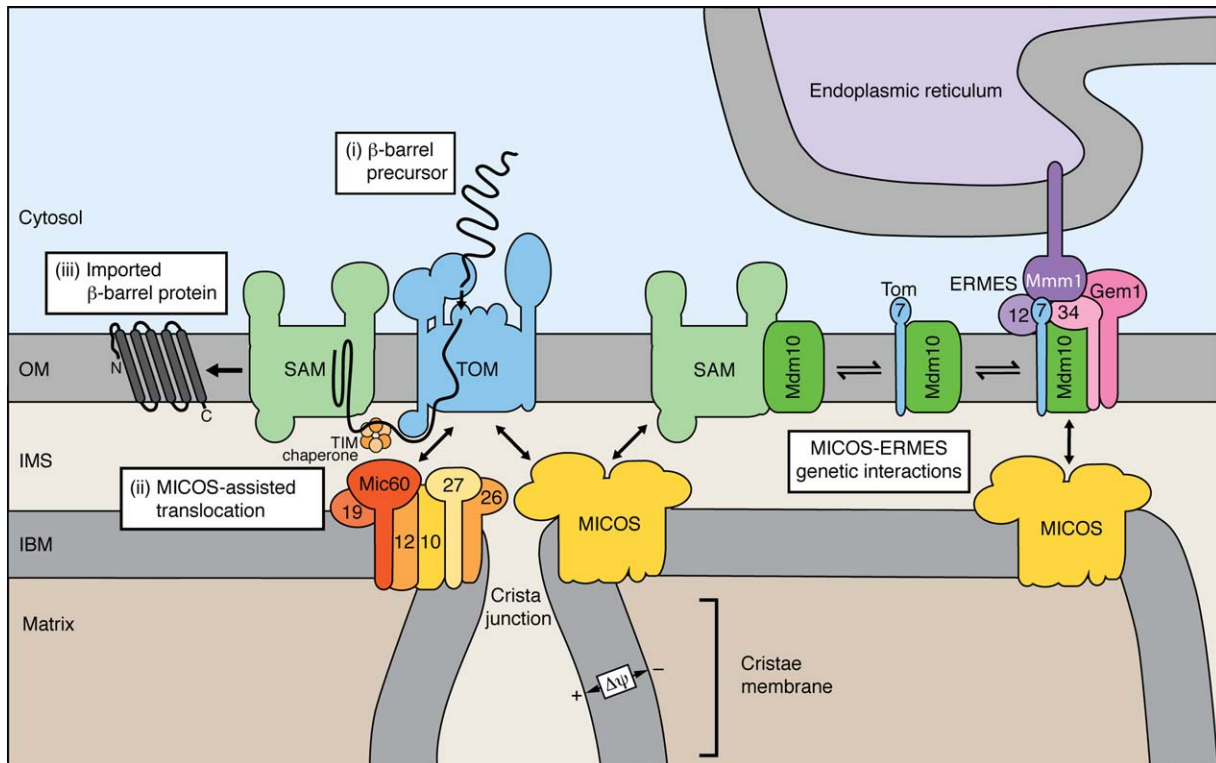


Figure 4. Biogenesis of mitochondrial outer membrane proteins and ER-mitochondria contact sites. Left half, β -barrel proteins of the outer membrane (OM) are imported into mitochondria via TOM, the small TIM chaperones of the intermembrane space (IMS) and the sorting and assembly machinery (SAM). MICOS interacts with TOM and SAM and stimulates β -barrel biogenesis. The transient interaction of Mic60 with TOM promotes translocation of β -barrel precursors to the intermembrane space. Right half, the SAM complex is in dynamic exchange with the ER-mitochondria encounter structure (ERMES) via the protein Mdm10. Tom7 binds to the SAM-free form of Mdm10 that can associate with ERMES. ERMES is composed of Mdm10, Mdm34, the adaptor protein Mdm12, the regulatory protein Gem1 and the ER localized protein Mmm1. MICOS and ERMES form genetic interactions.

present, two possibilities are conceivable to explain the distinct functional modes of Mic60 in wild-type mitochondria. (i) Mic60 molecules may be present in two distinct physical pools, a MICOS-integrated form and a form separated from MICOS, and thus perform MICOS-dependent and MICOS-independent functions (see model in Fig. 3). (ii) Alternatively, Mic60 may be part of MICOS in both functional modes (see model in Fig. 4). However, only the morphology function depends on the presence and activity of the other MICOS subunits. Mutant mitochondria show that Mic60 can interact with TOM and stimulate protein import also when other MICOS subunits are missing and MICOS is dissociated.^{26,238} Further studies will be required to define the molecular organization and functional mechanisms of Mic60.

Mitochondrial Organizing Network

We propose that translocation contact sites and MICOS are important parts of a large network of protein complexes that form an organizing center for mitochondrial biogenesis and activity. Mitochondrial outer and inner membranes are connected at sites

that apparently involve numerous transient interactions. Probably none of the individual contact sites is sufficient to maintain the close connection of the inner boundary membrane with the outer membrane, but multiple contact sites are required.

The TOM complex is one of the important players in mitochondrial contact sites since it interacts with the TIM23 complex and the MICOS complex. Imaging studies show that TOM-TIM23 supercomplexes are clustered in the vicinity of crista junctions but not directly at the junctions,¹⁶⁸ whereas MICOS complexes are enriched at crista junctions.^{206,207} Thus, the TOM-TIM23 and TOM-MICOS supercomplexes are in spatial vicinity, but form distinct contact sites that are part of a larger organizing network. It is open if these two membrane-spanning supercomplexes directly interact with each other. In addition, the TOM complex forms a transient supercomplex with the SAM complex in the outer membrane,²³⁷ and TOM is crucial for the import of carrier proteins, which are transferred to the small TIM chaperones but to our current knowledge do not use translocation contact sites.^{239,240} TOM complexes are thus engaged in several different

pathways and interact with different partner complexes. Since TOM complexes are more abundant than the other mitochondrial protein translocases,^{84,241} they may exist in separate pools dedicated to different protein import pathways. Alternatively, a large dynamic pool of TOM complexes may be used to recruit TOM complexes to different partner complexes depending on the activity of the import pathways. Since each of the TOM-containing supercomplexes is of dynamic nature, we prefer the second scenario that TOM complexes engaged in formation of different supercomplexes are in exchange with each other.

The SAM complexes can interact with TOM as well as MICOS. In addition, SAM is in dynamic exchange with the endoplasmic reticulum (ER)-mitochondria encounter structure (ERMES), a protein complex that stably connects ER and the mitochondrial outer membrane.^{242–244} Though no evidence for a direct physical contact between SAM and ERMES has been reported so far, both complexes share a subunit. The mitochondrial distribution and morphology protein Mdm10 is a subunit of both complexes (Fig. 4, right half). In the SAM complex, Mdm10 promotes assembly of the TOM complex,^{81,245,246} whereas its exact function in the ERMES complex is unknown.²⁴⁷ Mdm10 can shuttle between SAM and ERMES. Interestingly, Tom7, a small subunit of the TOM complex, has a dual localization as well. Tom7 is not only found in the TOM complex, but also forms a complex with Mdm10. Since Tom7 binds to the SAM-free form of Mdm10, Tom7 promotes segregation of Mdm10 from the SAM complex and may favor the assembly of Mdm10 with the ERMES complex.^{248–250}

Further ERMES subunits that are not in exchange with SAM were also found to be involved in the assembly of β -barrel proteins.²⁵¹ This finding links outer membrane biogenesis to contact sites between mitochondria and ER. Such interorganellar contact sites have gained a lot of interest in recent years since many vital cellular functions ranging from calcium homeostasis to phospholipid synthesis and mitochondrial fission have been associated with these tethering structures.^{242,244,252–258} In addition to yeast ERMES, further direct ER-mitochondria connections have been described, including the ER membrane protein complex (EMC) in yeast²⁵⁹ and mitofusin 2 in mammals.^{260,261} Two recent studies identified a molecular tether between vacuoles and mitochondria, termed vCLAMP, that may have complementary functions to ERMES in phospholipid metabolism.^{262,263}

Future studies will have to unravel the mechanisms of molecular crosstalk between interorganellar contact sites as well as their structural and functional relationship with the mediators of mitochondrial biogenesis and membrane architecture, like

protein translocases and MICOS. The importance of such wide-ranging connections is highlighted by the fact that MICOS not only interacts with TOM and SAM, but also shows strong genetic interactions with ERMES components (Fig. 4).²⁰⁸ Thus, multiple interactions involving TOM, SAM, MICOS, and ERMES point to a large ER-mitochondria organizing network, termed ERMIONE, which connects three membranes and functions as organizing center for mitochondrial architecture and biogenesis.²²⁴ The network likely involves further components such as mitochondrial fusion proteins, lipid synthesizing enzymes, the prohibitin ring complexes and the proteins Mdm31 and Mdm32 of the inner membrane that expose domains to the intermembrane space.^{211,217,256,264–274} ERMES, MICOS, and Mdm31/32 are also involved in the organization of mtDNA into nucleoids.^{220,247,265,275–277} Thus, ERMIONE likely spans from the ER lumen to the mitochondrial matrix and is also involved in the proper maintenance of mtDNA.

It will be a major task of future research to define the molecular functions of this mitochondrial organizing network. We propose that ERMIONE forms a central platform for coordinating mitochondrial biogenesis, architecture and activity, including import and assembly of proteins, biosynthesis and transfer of lipids, interorganellar and intraorganellar contact sites and mtDNA organization. Elucidation of the mechanisms that regulate these machineries will be an important topic. It had been assumed previously that mitochondrial protein import works in a constitutive, non-regulated manner. Recent studies, however, revealed that the TOM complex is extensively regulated by at least four cytosolic protein kinases that depending on the cellular growth conditions, metabolic state and cell cycle phases, stimulate or inhibit TOM biogenesis and activity and thus control the main mitochondrial entry gate.^{9,278–281} The efficiency of protein translocation into and across the inner membrane depends on the magnitude of the membrane potential and thus the protein import machinery can serve as a sensitive indicator for mitochondrial fitness and dysfunction. A reduced protein import efficiency has been found to trigger a mitochondrial stress response or the degradation of dysfunctional mitochondria by autophagy.^{282,283} Translocation contact sites are thus not only crucial for the import of proteins into mitochondria but also for the regulation of mitochondria and their integration into cellular metabolism and signaling.

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