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# Mitochondrial protein organization: from biogenesis to networks and function

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## Abstract

Mitochondria are essential for the viability of eukaryotic cells, perform crucial functions in bioenergetics, metabolism and signaling, and have been linked to numerous diseases. Recent functional and proteomic studies revealed a remarkable complexity of mitochondrial protein organization. Protein machineries with diverse functions such as protein translocation, respiration, metabolite transport, membrane architecture and quality control interact with each other in dynamic networks. Here we discuss that the mitochondrial protein import machinery forms a housekeeping system that plays a central role in organizing the mitochondrial protein networks. The preprotein translocases not only deliver newly synthesized proteins to their proper intramitochondrial destination, but are also directly involved in establishing dynamic networks. Translocases form building blocks that cooperate with numerous mitochondrial protein complexes. Understanding mitochondrial protein organization requires an integrative view of organelle biogenesis and protein network formation.

## Introduction

Mitochondria, the double membrane-bounded powerhouses, are a hallmark of eukaryotic cells. Derived from an α-proteobacteria-related ancestor, mitochondria have retained the high capacity and system of bacteria to synthesize ATP via oxidative phosphorylation, but are also deeply integrated into the metabolism and signaling pathways of their eukaryotic host cells.

Mitochondria consist of two membranes and two aqueous compartments (FIG. 1). The mitochondrial inner membrane possesses a several-fold larger surface than the outer membrane, resulting in an invagination of so-called cristae membranes that harbor the oxidative phosphorylation system, including the respiratory complexes I to IV and the $F_1F_O$-

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ATP synthase for production of ATP. A small set of proteins are encoded by the mitochondrial genome, including 13 proteins in humans and eight proteins in the model organism baker's yeast. The mitochondrially encoded proteins include highly hydrophobic proteins that form core parts of the oxidative phosphorylation complexes of the mitochondrial inner membrane. Approximately 99% of mitochondrial proteins are encoded by nuclear genes, including originally prokaryotic genes that were transferred to the nucleus as well as genes coding for novel mitochondrial proteins developed by eukaryotic cells. Nuclear encoded mitochondrial proteins are equipped with specific targeting signals that direct the newly synthesized proteins from the cytosol to mitochondrial surface receptors and subsequently into the proper mitochondrial subcompartments[1–4].

Whereas research in previous decades mainly focused on mitochondrial bioenergetics, studies in the past 10-15 years revealed an unexpected complexity and versatility of mitochondrial activities, integrating mitochondrial energetics with protein biogenesis, metabolic pathways, cellular signaling, stress response and apoptosis. It is becoming more and more evident that mitochondrial protein machineries do not function as independent units, but protein complexes of diverse function are physically and functionally connected. It is currently discussed how these complex networks are formed and maintained and what are the ordering principles behind them.

In this Review, we first discuss recent proteomic studies that showed a large spectrum of mitochondrial proteins and functions. Quantitative proteomics revealed for the first time absolute numbers for the abundance of all important mitochondrial machineries under different growth conditions, unveiling the protein import machinery as a housekeeping system under respiratory and non-respiratory conditions. We then address how preprotein translocases build core parts of dynamic protein networks that link organelle biogenesis, energy metabolism, membrane morphology and dynamics. Based on this integrative view, we discuss the connection of the mitochondrial protein import machinery to mitochondrial stress response, quality control and diseases.

## Multifunctional mitochondria

Systematic analyses of the mitochondrial proteome provided a comprehensive overview not only of the mitochondrial protein complement, but also of the huge variety of functions performed by mitochondria, summarized in FIG. 1. We present an overview of the various functions of mitochondria and then discuss how absolute quantification of the mitochondrial proteome considerably shaped our view of mitochondrial activities.

### Numerous functions of mitochondria

The typical textbook knowledge about mitochondria comprises the respiratory complexes and the $F_1F_O$-ATP synthase in the inner membrane cristae, transporters and channels for metabolites and ions in both mitochondrial membranes, and numerous metabolic pathways mainly localized to the matrix and inner membrane[5] (FIG. 1). Major metabolic pathways concern the energy metabolism, such as the tricarboxylic acid cycle (TCA) also known as citric acid cycle or Krebs cycle, and the metabolism of amino acids, lipids and nucleotides. Electrons derived from oxidation of metabolites are fed into the respiratory chain that

generates an electrochemical gradient by pumping protons from the matrix to the intermembrane space side. The proton gradient is then used to drive ATP synthesis by the $F_1F_O$-ATP synthase, as well as the import of precursor proteins and the transfer of some metabolites across the inner membrane.

Mitochondria perform two tasks that are essential for cell viability under all growth conditions. (i) They contain a complete system for the biosynthesis of iron-sulfur-clusters inside the organelle and provide a not yet identified essential compound for the cytosolic biosynthesis of iron-sulfur-clusters[6]. (ii) Mitochondrial import and maturation of precursor proteins are essential processes and thus a considerable number of protein import, processing and folding components are essential for cell viability[1–4].

The mitochondrial matrix contains a complete genetic system, including the mitochondrial genome, numerous factors for maintaining, regulating and expressing the genome, and the mitochondrial ribosomes that differ in size and composition from cytosolic ribosomes[7]. Proteins encoded by the mitochondrial genome are typically inserted into the inner membrane in a co-translational mechanism by coupling translating ribosomes to the membrane integrated insertase, termed oxidase assembly (OXA)[1,2,4,8].

Several machineries have been identified that control mitochondrial membrane architecture and dynamics. This includes protein factors that mediate fusion or fission of the mitochondrial membranes, in particular dynamin-related GTPases located at the outer and inner membranes, and membrane-shaping components. Mitochondria typically form a dynamic network in most cell types that is continuously remodeled by fusion and fission of the organelles[9–12]. The mitochondrial contact site and cristae organizing system (MICOS) is critical for maintaining the characteristic shape of inner membrane cristae[13–15]. MICOS and several further protein complexes are involved in forming contact sites between the mitochondrial outer and inner membranes to promote the transfer of proteins, lipids and metabolites[13,14,16,17].

An intensively studied area includes mitochondrial signaling processes, regulation and quality control. Numerous cytosolic signaling cascades target mitochondria under physiological and pathophysiological conditions. The metabolic activity of the organelle serves as a measure for mitochondrial fitness and quality[18], and elaborate pathways for mitochondrial stress responses, selective degradation of damaged mitochondria by mitophagy (autophagy)[19] and programmed cell death (apoptosis) via mitochondria have been identified[20,21]. Mitochondria contain a set of internal proteases that are also involved in quality control and turnover of mitochondrial proteins[22]. In addition, mitochondria are a major site of cellular production of reactive oxygen species and contain numerous redox pathways[23].

## Quantitative analysis of the mitochondrial proteome

The large number of mitochondrial activities is overwhelming and has led to differential views of mitochondrial organization. Three stages of understanding of mitochondrial functions can be seen. (i) The original research focus on metabolism and ATP production established energetics and metabolism as hallmarks of mitochondria. (ii) The initiation of

systematic proteomic studies on mitochondria about 15 years ago led to the identification of numerous new mitochondrial proteins. We currently estimate that mitochondria contain more than 1,000 (yeast) to 1,500 different proteins (humans)[24–42]. The functional classification of the mitochondrial proteome, based on the number of different proteins, suggested an unexpected perspective[30,36]. Only up to 15% of the different mitochondrial proteins were directly involved in energy metabolism, including the energy metabolizing pathways and all structural subunits of the oxidative phosphorylation system. However, 20-25% of the mitochondrial proteome were required to maintain, regulate and express the mitochondrial genome that codes for only ~1% of mitochondrial proteins. In addition, numerous components involved in signaling, regulation and membrane dynamics were identified. Thus, regulatory proteins emerged as important factors in mitochondria, suggesting a considerable revision of the textbook view of mitochondria as cellular powerhouses. (iii) The recent systematic quantification of the majority of the mitochondrial proteome yielded the absolute copy numbers of mitochondrial proteins per one cell[30,43–47]. As detailed in BOX 1, proteins involved in energy metabolism form by far the most abundant protein classes in respiring yeast mitochondria. The ~15% different mitochondrial proteins with a direct role in energy metabolism and respiration mentioned above represent more than half of the mitochondrial protein mass under respiratory conditions, reinforcing the original view of mitochondria as cellular powerhouses. Taking the various metabolic processes, oxidative phosphorylation and metabolite carriers/channels together, ~75% of the protein mass of respiring mitochondria are dedicated to metabolism and bioenergetics[30].

Thus, both seemingly controversial views, mitochondria as powerhouses vs. mitochondria as organelles with a huge number of different functions, have to be combined to understand the cellular significance of mitochondria. Based on absolute protein mass, metabolism and bioenergetics indeed represent the quantitatively major tasks of mitochondria and the term powerhouses is justified. However, many additional functions are of central importance for mitochondrial fitness, cellular growth and development. A striking example is the system for biosynthesis of iron-sulfur clusters that comprises less than 1% of the protein mass of respiring mitochondria, but is essential for the viability of eukaryotic cells[6]. Similarly, the machineries for mitochondrial membrane morphology and dynamics represent less than 1% of the mitochondrial protein mass[30], although they play crucial roles in mitochondrial architecture, fusion and fission and thus are critical for maintaining the mitochondrial network in cells and for remodeling mitochondria under different growth conditions[9–12]. Mitochondria can thus be seen as super powerhouses that in addition to their predominant metabolic and energetic functions, are deeply integrated into cellular signaling, biosynthesis and dynamics by performing a multitude of functions. We discuss below that these functions are not independent tasks, but the various machineries and proteins are physically connected in large, dynamic networks.

## Plasticity of the mitochondrial proteome

The mitochondrial content of a cell can vary considerably under different growth conditions and between different organisms and tissues[25,28,30–32,48]. The systematic analysis of yeast mitochondria revealed that the total mitochondrial protein mass represented ~9% of the cellular protein mass under fermentable conditions, where less activity of mitochondria is

required. Under respiratory growth conditions, the mitochondrial protein mass is more than doubled to ~20% of the cellular protein mass[30]. As outlined in BOX 1, the changes from fermentation to respiration are quite different for mitochondrial proteins belonging to different functional classes. The strongest increase toward respiration is observed for proteins directly involved in energy metabolism and respiration with a more than three-fold increase of the absolute protein copy numbers of these proteins per cell. Proteins functioning in signaling, redox processes and membrane dynamics are increased about two-fold, like the overall increase of mitochondrial mass[30].

Remarkably, the protein classes responsible for the two mitochondrial processes, which are essential for cell viability in all cell types and growth conditions, protein biogenesis & folding and biosynthesis of iron-sulfur clusters, are only moderately altered in their overall copy numbers upon shift of yeast cells from fermentation to respiration[30]. Thus, these systems are very well equipped already under non-respiratory conditions and are only slightly increased in their abundance during respiration. It seems that these systems are so central to mitochondrial and cellular behavior that their protein complement is largely present under all conditions, although their activity has to be strongly increased under respiratory conditions. The protein import machinery has to import more than double the amount of proteins during respiration and thus it is evident that the machinery is working at considerably lower yield under fermentation. We conclude that the protein import machinery and the system for biosynthesis of iron-sulfur clusters are central housekeeping systems of yeast mitochondria and are not or only moderately regulated by their copy numbers. Indeed, studies on the phosphorylation of the main protein entry gate, the translocase of the outer membrane (TOM) complex, revealed that at least four different cytosolic signaling systems regulate the TOM activity by phosphorylation, leading to a sophisticated pattern of stimulatory and inhibitory effects depending on the kinase and TOM subunits involved[49–52]. The advantage of a stable presence of housekeeping systems and their regulation by reversible modification such as phosphorylation is the rapid response to changing conditions. When an increased capacity of the protein import machinery would require an increased gene expression, translation and import, the system would be rather slow in adapting to different requirements. Similarly, inhibitory effects are faster when directly executed by protein modification than by the slower copy number reduction. For example, upon fermentable growth, where less translocation of metabolites into and out of mitochondria is needed, the activity of the TOM receptor of 70 kDa (Tom70), which is critical for targeting of metabolite carriers, is inhibited by phosphorylation, leading to an immediate decrease of carrier import into mitochondria[52]. Therefore, the presence of essential housekeeping systems with largely stable protein copy numbers and the regulation of these systems by post-translational modification will provide a high flexibility of mitochondrial biogenesis and plasticity.

## Protein assembly and functional networks

The analysis of protein import from the cytosol into mitochondria was originally based on the assumption that one central pathway is responsible for translocating the 1,000-1,500 different proteins to their mitochondrial destination, however, the characterization of precursor proteins carrying different targeting signals revealed a higher complexity. We first

present that mitochondria use at least five major protein import pathways, each one directed by a different type of targeting signal. Then we discuss the next level of complexity as preprotein translocases do not operate as isolated units, but are connected to numerous mitochondrial protein complexes that belong to different, at first glance unrelated functional categories.

## Five major import pathways of precursor proteins into mitochondria

The presequence pathway is the best known mitochondrial protein import pathway responsible for the transport of ~60% of all mitochondrial proteins[53]. The precursor proteins carry amino-terminal targeting signals, termed presequences, which form positively charged amphipathic α-helices. These presequences are typically recognized by the TOM receptors of ~20 and ~22 kDa (Tom20 and Tom22)[54,55] on the mitochondrial surface and direct translocation through the main protein translocation channel of the outer membrane of ~40 kDa (Tom40)[2,4,56,57] (FIG. 2). Upon translocation across the outer membrane, the preproteins are engaged by the presequence translocase of the inner membrane (TIM23 complex) that directs their transfer across the inner membrane[58–61]. The membrane potential ($\Delta\psi$) across the inner membrane (negative on the inside) activates the Tim[23] channel and drives the positively charged presequences toward the matrix[62–65]. The presequence translocase-associated motor (PAM) contains the mitochondrial heat shock protein 70 (mtHsp70) as central ATP-driven chaperone[66]. Together with five co-chaperones, PAM promotes translocation of the entire polypeptide chain into the matrix[67–72]. The presequences are removed by the mitochondrial processing peptidase (MPP)[2,4,73]. Additional processing enzymes in the matrix are involved in quality control functions as mitochondrial proteins can be degraded according to the N-end rule pathway, depending on the presence of stabilizing or destabilizing amino acid residues at the amino-termini of proteins[53,74]. The intermediate cleaving peptidase of 55 kDa (Icp55) and the octapeptidyl peptidase (Oct1) remove destabilizing amino acid residues, which can be located at the amino-termini of imported proteins after cleavage by MPP, and thus generate stable amino-termini that are less prone to degradation by matrix proteases[53,75,76]. With the help of mtHsp70 and further chaperones such as Hsp60-Hsp10[77,78], the proteins are then folded to their active form.

Presequence-carrying precursors that become integrated into the inner membrane follow two distinct routes (FIG. 2). A number of presequence-carrying preproteins possess a hydrophobic sorting signal behind the matrix targeting signal. This sorting signal arrests translocation in the TIM23 complex and the lateral gatekeeper Mgr2 permits a release of the protein into the inner membrane (stop transfer pathway)[79]. Other inner membrane proteins are first transported into the matrix and are translocated into the inner membrane by the OXA insertase that has been conserved from bacteria to mitochondria and is also used by mitochondrially synthesized proteins (conservative sorting)[8,80–83].

Most other protein import pathways also use the TOM channel for preprotein translocation across the outer membrane[84,85] (FIG. 2), although the dependence on the three TOM receptors Tom20, Tom22 and Tom70 and the mode of delivery from the cytosol to TOM can differ[86–88]. The carrier pathway is dedicated to the import of hydrophobic multi-spanning




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inner membrane proteins, which do not possess amino-terminal presequences, but distinct types of internal targeting signals that are not fully defined, yet include hydrophobic elements. Cytosolic chaperones of the Hsp90 and Hsp70 classes deliver the hydrophobic precursor proteins to the receptor Tom70[89]. The precursors are released from the chaperones, translocated through the Tom40 channel in a loop formation[90,91] and engaged by small TIM chaperones of the intermembrane space to prevent aggregation of the hydrophobic precursors[92–94] (FIG. 2). The small TIM chaperones guide the carrier precursors to the carrier translocase of the inner membrane (TIM22 complex) that operates in a $\Delta\psi$-dependent manner to drive membrane insertion of the multi-spanning proteins[95–99].

Many proteins of the mitochondrial intermembrane space contain characteristic cysteine motifs that become oxidized to form stabilizing disulfide bonds in the mature proteins. The mitochondrial intermembrane space import and assembly (MIA) system consists of two main components: the oxidoreductase with disulfide isomerase activity and intermembrane space receptor of ~40 kDa, Mia40[100,101], and the sulfhydryl oxidase essential for respiration and viability, Erv1[102] (FIG. 2). Upon translocation of the precursors through the Tom40 channel, Mia40 recognizes the precursors that contain a mitochondrial intermembrane space sorting signal, typically consisting of a hydrophobic element flanked by a cysteine residue[103–105]. Mia40 forms a transient disulfide bond with the precursor protein and then transfers disulfide bonds to the imported protein, leading to an oxidation and stabilization of the protein by intramolecular disulfide bonds[106,107]. Upon each transfer of a disulfide bond to an imported protein, cysteines of Mia40 become reduced and are re-oxidized by Erv1. In this disulfide relay, disulfide bonds are thus transferred from Erv1 to Mia40 to imported intermembrane space proteins.

The mitochondrial outer membrane contains different classes of membrane proteins: single-spanning and multi-spanning proteins with α-helical transmembrane segments; and β-barrel proteins. The precursors of β-barrel proteins are initially translocated by the TOM complex to the intermembrane space and interact with small TIM chaperones like the carrier precursors[108] (FIG. 2). Insertion of β-barrel precursors into the outer membrane is mediated by the sorting and assembly machinery (SAM)[109–111] in a step-wise process that involves translocation into the SAM channel and lateral release into the lipid phase of the membrane[112]. The carboxy-terminal β-strand of the precursor functions as β-signal that directs insertion via SAM[113]. The β-signal and the central channel-forming protein Sam50 have been conserved from bacteria to humans[114]. α-Helical outer membrane proteins follow distinct import routes that in most cases do not involve the Tom40 channel. The sorting signal is typically contained within the α-helical transmembrane segment(s) and flanking positively charged amino acid residues. Single-spanning proteins with amino-terminal membrane anchor (signal-anchored proteins) as well as multi-spanning outer membrane proteins can use the mitochondrial import (MIM) channel for membrane insertion, assisted by the receptor Tom70 at least in the case of multi-spanning proteins[115–120]. In case of single-spanning proteins with carboxy-terminal membrane anchor (tail-anchored proteins) and some multi-spanning proteins, the lipid composition of the membrane seems to be important, yet the exact molecular mechanism is unknown[121–124]. The views reach from a protein-independent insertion directly into the phospholipid membrane to MIM complex-

assisted insertion or to the possible involvement of an as yet unknown proteinaceous insertase of the outer membrane.

## Abundance and versatility of import machineries

The inset of FIG. 2 shows the absolute copy numbers of characteristic translocase components in a respiring yeast cell, and for comparison also the abundance of respiratory complexes, metabolite channels and carriers of the mitochondrial membranes[30]. The TOM complex is the most abundant translocase consistent with its role in feeding precursors into at least four distinct down-stream translocase systems. The abundance of the TIM23 presequence translocase fits to the major role of the presequence import pathway. Interestingly, the central motor component mtHsp70 is about ten times more abundant than Tim23 and other motor subunits such as Tim44. mtHsp70 plays a dual role: a fraction of mtHsp70 molecules act in the TIM23-associated motor PAM to drive preprotein import, whereas the major fraction of mtHsp70 is dedicated to protein folding in the mitochondrial matrix. Sam50 and Tim22 are present in quite low amounts, reflecting their more specialized role in protein sorting. A comparison with the abundance of their major substrates, however, underscores the importance and activity of the translocases. The outer membrane β-barrel metabolite channel porin (isoform Por1) is one of the most abundant mitochondrial proteins and also the inner membrane metabolite carriers are of high abundance.

The TOM complexes can form different types of dynamic supercomplexes: a TOM-SAM supercomplex for efficient transfer of β-barrel precursors and a two membrane-spanning TOM-TIM23-preprotein supercomplex[125–130]. TOM also interacts with the small TIM chaperones, and in mammals TIM29 of the TIM22 complex was found to associate with TOM[85,131,132]. The differential abundance of the translocases (FIG. 2, inset) indicates that indeed sufficient TOM complexes are available to form the different supercomplexes. It is currently discussed whether separate pools of TOM complexes exist for different import pathways or whether the TOM complexes are freely interchangeable in one large dynamic pool. The translocation of several intermembrane space precursor proteins across the outer membrane depends on Tom40 but does not require the TOM receptor domains[133,134] and competition experiments suggest that intermembrane space precursors and presequence-carrying precursors do not use the same TOM complexes[133], supporting the view of distinct forms of the main protein entry gate. We speculate that in addition to the full-size TOM complex, which contains Tom40 channels, all three receptors and three small Tom proteins[85], simplified forms of TOM complexes may exist. These simplified TOM complexes may just contain the Tom40 channel and possibly some of the small Tom subunits and may be dedicated to the import of e.g. intermembrane space precursors, which are recognized by the receptor Mia40 and do not depend on classical TOM receptors. The differential phosphorylation of TOM complexes by cytosolic signaling cascades also contributes to a heterogeneity of TOM complexes and thus favors the engagement in distinct import routes in a signal-controlled manner[49–52].

In metazoans, the presequence translocase exists in two forms with a differential tissue distribution, containing either the stably expressed housekeeping subunit TIM17B (skeletal muscle) or the stress-regulated subunit TIM17A (brain)[135]. Under stress conditions, the

levels of TIM17A are decreased by two means, decreased synthesis and increased degradation by the ATP-dependent AAA protease of the inner membrane that is exposed to the intermembrane space (*i*-AAA protease) (FIG. 3), promoting the induction of a mitochondrial unfolded protein response[136]. Thus, whereas the overall abundance of the mitochondrial protein import machinery of rapidly growing uni-cellular organisms like yeast is quite stable under different metabolic conditions, the abundance of tissue-specific isoforms can be regulated in metazoans, suggesting an additional regulatory level in multi-cellular organisms[135–137].

Elements of different import pathways can be combined for the creation of new import pathways. For example, the single-spanning outer membrane protein Om45 contains a large domain in the intermembrane space. The precursor is first transported by the presequence pathway through the TOM complex and interacts with the TIM23 complex, followed by escape into the intermembrane space and insertion into the outer membrane by a reverse action of the MIM complex[138,139]. Further examples of the versatility of the mitochondrial import machinery are cleavable carboxy-terminal targeting signals that are imported via the presequence pathway and are removed by matrix or intermembrane space peptidases, followed by differential sorting to intramitochondrial destinations[140–142]. Thus, the diversity and versatility of mitochondrial protein import pathways go substantially beyond the known five major pathways and we expect that the systematic analysis of the large number of substrates will reveal the existence of further import routes and possibly also of further translocases.

## Respiratory chain interactions link bioenergetics, biogenesis and quality control

The respiratory chain complexes of the mitochondrial inner membrane form the center of a large network that connects bioenergetics to biogenesis, regulation and turnover processes (FIG. 3). The respiratory complexes I (NADH:ubiquinone oxidoreductase), III (cytochrome *c* reductase) and IV (cytochrome *c* oxidase) assemble into large supercomplexes. The I-III-IV supercomplexes are also termed respirasomes[143–147]. The existence of respiratory supercomplexes is now generally accepted, however, different views exist about the functions of these large assemblies[144,146]. The supercomplexes may influence the assembly and stability of respiratory complexes, regulate the activity of the complexes and/or reduce the formation of reactive oxygen species (ROS). Various factors involved in the formation of respiratory supercomplexes have been reported (FIG. 3), yet it is currently discussed if they mainly function in the assembly of individual respiratory complexes or in the formation of supercomplexes[144,146,148–151].

Preprotein translocases are not only in crosstalk with each other, but form physical contacts with other mitochondrial machineries, including machineries involved in mitochondrial energy metabolism. The TIM23 presequence translocase forms a hub in the sorting of preproteins at the inner membrane and cooperates with a remarkable number of different protein complexes. In addition to the complexes directly involved in preprotein import (TOM and PAM), the TIM23 complex forms supercomplexes with the respiratory complexes III and IV as well as with the ADP/ATP carrier (FIG. 3; BOX 2). These interactions of the TIM23 complex support protein import under energy-limiting conditions[152–154]; and in

human mitochondria, TIM23-respiratory chain interactions have been shown to promote assembly of respiratory complexes[155–157] (BOX 2). The respiratory complexes as well as the ADP/ATP carrier are several-fold more abundant than the TIM23 complex[30] (FIG. 2, inset) and thus only a fraction of them are engaged in the interaction with TIM23. Respiratory complexes are preferentially located in cristae membranes, yet a smaller fraction is found in the inner boundary membrane, which is adjacent to the outer membrane[158], and can thus interact with the TIM23 complexes. The interaction is likely not permanent, but TIM23 and respiratory complexes form dynamic supercomplexes[152,153].

A further link between the mitochondrial respiratory chain and protein biogenesis was found by analyzing the effects of mitochondrially generated ROS. The mitochondria respiratory chain is a major source for ROS. Stress conditions and dysfunction of the respiratory chain can lead to increased ROS production with oxidative damage of proteins, DNA and membranes. ROS can also perform various signaling functions and thus mitochondrially produced ROS can signal the functional state of mitochondria[23,146,159]. ROS was found to target redox-sensitive cysteine residues (redox switches) of the cytosolic translation apparatus, including the ribosome and translation factors[159]. When mitochondrial dysfunction leads to increased ROS production, the efficiency of translation is decreased in a reversible manner (FIG. 3). The respiratory chain thus participates in controlling cytosolic protein synthesis to decrease the protein load under mitochondrial stress conditions[159].

Coenzyme Q (CoQ), also termed ubiquinone, is a central molecule for the function of the respiratory chain[160,161]. CoQ is a redox-active lipid that mediates electron transfer from respiratory complexes I and II to complex III (FIG. 3) and functions as cofactor of many further enzymes. Several enzymes of CoQ biosynthesis were only identified recently, using systematic mass spectrometry profiling at the proteomic, lipidomic and metabolomic level[30,75,160,161]. The CoQ biosynthetic complex located at the matrix side of the mitochondrial inner membrane contains numerous enzymes involved in CoQ biosynthesis as well as lipids and cofactors. All protein subunits of this dynamic CoQ biosynthetic complex are encoded by nuclear genes and have to be imported by the TOM and TIM machineries[160,161]. The precursor of the methyltransferase Coq5 is processed twice. The first processing by MPP generates an instable intermediate and thus a second processing by the octapeptidyl peptidase is required to generate the stable mature Coq5 enzyme (FIG. 3). Disturbance of the processing by Oct1 leads to CoQ deficiency and respiratory defects[75]. Import and specific processing of the Coq precursor proteins thus functionally link the mitochondrial protein import machinery and the CoQ biosynthetic complex.

The mitochondrial inner membrane carries two large ATP-dependent protease complexes, the *i*-AAA protease and the matrix-exposed *m*-AAA protease[22,162–164]. These proteases are main elements of a quality control system for protein turnover and processing in mitochondria. AAA proteases not only cleave/degrade inner membrane proteins such as some subunits of respiratory complexes and preprotein translocases, but are also involved in the quality control and turnover of selected matrix, intermembrane space and outer membrane proteins (FIG. 3). In fungi, the adapter proteins Mgr1 and Mgr3 associate with the *i*-AAA protease and promote substrate recognition by the protease[162,164]. A recent proteomic study of yeast mitochondria identified the respiratory chain interacting proteins

Rci37 and Rci50 and demonstrated that they also interacted with the AAA proteases, Rci37 with the *m*-AAA protease and Rci50 with the *i*-AAA protease[30], revealing specific connections between respiratory complexes III and IV and the inner membrane quality control system.

The functional network of the mitochondrial respiratory chain thus includes respiratory supercomplexes and machineries for protein biogenesis, cofactor biosynthesis, regulation and quality control.

## Mitochondrial membrane architecture and protein interactions

Contact sites between the mitochondrial outer and inner membranes and between the outer membrane and the endoplasmic reticulum (ER) are crucial elements of a large network that functions in protein and lipid biogenesis, membrane architecture and dynamics, metabolite/ion transport, mitochondrial dynamics and inheritance (FIG. 4). Preprotein translocases form central building blocks of this ER-mitochondria organizing network (ERMIONE)[165]. The TOM and SAM complexes of the outer membrane interact with the large MICOS morphology complex of the inner membrane[13–15,17,166–169]. MICOS is enriched at crista junctions[170,171], the tubular entry gates into the cristae lumen. The largest MICOS subunit, Mic60, plays an important role in the formation of outer-inner membranes contact sites. In addition, Mic60 transiently interacts with the receptor and oxidoreductase Mia40 of the intermembrane space assembly machinery[13]. MICOS thus helps to position the down-stream machineries MIA and SAM close to the main protein import channel TOM and promotes the efficient import of cysteine-rich precursors into the intermembrane space and of β-barrel precursors into the outer membrane[13,166].

The MICOS-SAM-TOM core of ERMIONE undergoes multiple interactions with further mitochondrial machineries. FIG. 4 gives an overview of this huge network. Most interactions of ERMIONE partners have been observed by biochemical means via direct physical associations or by genetic means, i.e. synthetic growth defects of double mutants. Analyses in vivo and in organello provided evidence for functional crosstalks. The molecular mechanisms governing this network and the regulation of mitochondrial functions by interaction of diverse machineries are currently the subject of intensive research. (i) In the inner membrane, Mic10, a core component of MICOS[172,173], and its partner protein Mic27 are in dynamic contact with the dimeric $F_1F_O$-ATP synthase that shapes cristae rims, leading to a crosstalk between the two major membrane-shaping machineries of the inner membrane, MICOS and $F_1F_O$-ATP synthase[147,174,175]. Assembly of the Mic10-containing subcomplex of MICOS is linked to respiratory complexes and the mitochondria-specific dimeric phospholipid cardiolipin[176]. In addition, MICOS is connected to the machineries for mitochondrial fusion, including the inner membrane fusion protein optic atrophy 1 (OPA1, termed mitochondrial genome maintenance 1 [Mgm1] in yeast)[147,177,178]. (ii) By mutant studies, MICOS has been functionally linked to nucleoid aggregation and inheritance of mtDNA[179,180]. The underlying molecular mechanisms will require further analysis. (iii) At the outer membrane, the SAM complex not only directly interacts with a fraction of TOM complexes in TOM-SAM supercomplexes, but is also in exchange with the ER-mitochondria encounter structure (ERMES) that links the mitochondrial outer membrane to the ER (FIG.

4). The outer membrane β-barrel protein mitochondrial distribution and morphology 10 (Mdm10) is a subunit of both SAM, where it functions in TOM biogenesis, and ERMES, where it contributes to lipid transfer and maintenance of mitochondrial morphology[181–184]. The shuttling of Mdm10 between SAM and ERMES is regulated by Tom7. The small protein Tom7 has a dual localization. It is mainly located in the TOM complex, but additional Tom7 molecules act as regulatory factors that promote Mdm10 transfer to ERMES[185–188]. Non-assembled Tom7 retards TOM assembly by shifting Mdm10 from the SAM-form to the ERMES-form, representing a direct regulatory mechanism when an excess of non-assembled TOM subunits accumulate in mitochondria. (iv) The major outer membrane metabolite channel porin, also termed voltage-dependent anion channel (VDAC), interacts with MICOS as well as the TOM complex[14,189], linking metabolite transport to ERMIONE. (v) Tom70 performs receptor functions in the TOM complex, but is also found in pools outside of the TOM complex and plays crucial roles in forming ER-mitochondria contact sites (FIG. 4). Tom70 and its isoform Tom71 interact with the lipid transfer protein anchored at membrane contact sites termed Lam6/Ltc1, which contains lipid binding sites and regulates contact sites between mitochondria, ER and further organelles[190,191]. Mammalian TOM70 also interacts with inositol trisphosphate (IP3) receptors of the ER to promote $Ca^{++}$ transfer from the ER to mitochondria[192]. The TOM complex with is central component Tom40 participates in the formation of vacuole-mitochondria contact sites, termed vacuole and mitochondria patch (vCLAMP), involving the vacuolar GTPase Ypt7 and the bridging protein Vps39/Vam6[190,193]. (vi) Moreover, Tom70/Tom71 have been linked to mitochondrial quality control and degradation systems. Vesicles can be released from the mitochondrial outer membrane that may direct selected cargo to degradation[194–196] and Tom70/71 were found to be required for the formation of at least some mitochondria-derived vesicles[197]. The outer membrane ATPase Msp1 promotes the extraction of mistargeted proteins[198,199]. Upon accumulation of non-imported precursor proteins, Msp1 is recruited to Tom70 via the peripheral membrane protein Cis1, leading to removal of non-imported proteins and their degradation by the proteasome[200]. (vii) Finally, as discussed in the following chapter, TOM and MICOS are involved in the accumulation of the PTEN-induced putative kinase 1 (PINK1) at the outer membrane, under conditions of mitochondrial dysfunction that lead to removal of damaged mitochondria by mitophagy[19,201–203].

The mitochondrial membranes thus contain at least two large protein networks, both containing TOM complexes: the TOM-TIM23-respiratory chain-AAA network coupling protein import to bioenergetics and quality control, and the MICOS-SAM-TOM-ER network (ERMIONE) that links protein biogenesis to membrane contact sites and membrane morphology. Whereas MICOS is enriched at crista junctions, TOM-TIM23-preprotein supercomplexes are preferentially found in a distance of ~30-60 nm away from crista junctions[127]. At present, it is open if these two large networks function independently of each other or if the exchange of TOM complexes and further components between the networks may provide a dynamic coordination between protein biogenesis, energetics, membrane morphology and quality control. Though substantial future work will be required for understanding ERMIONE on a molecular and functional level, the identification of these networks clearly demonstrates that mitochondrial machineries do not function as stand-alone units, but are intimately linked to each other.

## Protein import and pathophysiology

The efficiency of protein import into mitochondria is a sensitive indicator of the energetic state and the fitness of mitochondria. Various disorders of mitochondrial respiration and metabolism lead to a reduction of the inner membrane potential[204,205]. Since the membrane potential is crucial for protein translocation into and across the inner membrane, the import of preproteins is diminished[1–4]. Defects of protein homeostasis in the mitochondrial matrix by the accumulation of misfolded proteins also lead to a reduced protein import, likely by disturbing the mtHsp70 import motor[206]. An impaired activity of the mitochondrial protein import machinery under stress conditions or in mitochondrial diseases is a direct indicator of impaired mitochondrial functions and can induce several responses from rescuing stress responses to removal of damaged mitochondria by mitophagy (BOX 3).

A mild disturbance of mitochondrial protein import can trigger the activation of the mitochondrial unfolded protein response (UPRmt) by impairing mitochondrial import of the transcription factor ATFS-1/ATF5, leading to its transport into the nucleus[207,208], where it induces a mitochondrial stress response to rescue partially damaged mitochondria (BOX 3). The stress-regulated decrease of the levels of TIM17A also leads to decreased mitochondrial protein import and promotes the induction of an UPRmt[136]. In addition, accumulation of mitochondrial precursor proteins in the cytosol leads to an attenuation of cytosolic protein synthesis and activation of the proteasome to clear the mistargeted proteins from the cytosol[209–212]. This process is termed unfolded protein response activated by mistargeted mitochondrial proteins (UPRam) or mitochondrial precursor over-accumulation stress (mPOS).

Upon severe damage of mitochondrial protein import, the kinase PINK1 is not imported, processed and degraded, but associates with the TOM complex as full-length protein, initiating a cascade that leads to removal of damaged mitochondria by mitophagy[19,201–203] (BOX 3). Since mutations of PINK1 have been linked to Parkinson’s disease, it is discussed that an insufficient mitophagy may be one of the factors in the development of the disease[201]. Recently, PINK1 and MIC60 of the MICOS complex were found to interact transiently, suggesting a crosstalk between PINK1 accumulation and inner membrane cristae remodeling[213,214] (FIG. 4).

It has been suggested that mitochondria may function as guardian of cytosolic proteins (MAGIC) by importing and degrading misfolded cytosolic proteins[215]. This process will require further studies to define its relevance for cellular protein homeostasis (proteostasis) and its relation to stress responses initiated by a decreased mitochondrial protein import efficiency such as UPRmt, UPRam and PINK1/parkin.

Proteolytic processing of precursor proteins also plays a role in mitochondrial quality control (BOX 3). One mechanism is the removal of destabilizing amino-terminal amino acid residues by the processing enzymes Icp55 or octapeptidyl peptidase to stabilize imported proteins against proteolytic degradation[53,75,76] (FIG. 2). Another mechanism concerns the differential processing of imported proteins, yielding two or more isoforms with distinct amino-termini, exemplified with the inner membrane fusion protein OPA1. OPA1 is first

processed by MPP, yielding a long isoform. Further processing by inner membrane-proteases generates short isoforms, depending on stress conditions and the energetic state of the inner membrane. The balance between long and short isoforms that is important for membrane fusion and fission is thus modulated by stress and mitochondrial activity[216–218].

An impaired processing of preproteins has been linked to mitochondrial dysfunctions in Alzheimer's disease[219]. The matrix peptidasome degrades presequences and other peptides such as Alzheimer linked amyloid β-peptides. Upon accumulation of amyloid β-peptides in mitochondria, the degradation of presequences is slowed down competitively, leading to an inhibition of processing peptidases. As a consequence, proteins imported into mitochondria are retained in precursor or intermediate forms that cannot fold properly and are prone to rapid degradation. The accumulation of amyloid β-peptides thus causes numerous changes in mitochondrial protein composition, providing possible explanations for a wide variety of mitochondrial alterations observed in Alzheimer's disease.

Studies in recent years provided increasing evidence for the involvement of mitochondrial protein import and processing in the pathogenesis of human diseases. At present, different views exist if mitochondrial dysfunctions are directly or indirectly involved in the development of major neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease220. In BOX 4, we provide an overview of more rare diseases and disorders that have been linked to specific components of the mitochondrial machineries for protein import and maturation, suggesting an involvement in disease pathogenesis. The diseases mostly affect the nervous system and other tissues with a high energy demand such as heart, muscles and kidney. On a mechanistic level, defects in preprotein targeting, presequence pathway, processing and folding, MIA pathway and carrier pathway have been observed (BOX 4).

The elaborate networks between preprotein translocases and other mitochondrial machineries have been mostly studied under physiological conditions. We expect that these networks will play an important role in understanding the mechanistic basis of mitochondrial stress responses and pathogenesis of diseases, exemplified with the role of TOM and MICOS in the accumulation of PINK1 at the outer membrane and the subsequent removal of damaged mitochondria by mitophagy[213,221–223].

## Conclusions and perspectives

Here, we have discussed that mitochondrial preprotein translocases, respiratory complexes, metabolite transporters, proteases, morphology complexes and membrane contact sites do not function as independent machineries, but are physically and functionally connected in large dynamic networks. The protein translocases represent an essential housekeeping system of mitochondria. The translocases are not only responsible for importing ~1,000-1,500 different proteins, but also form stable building blocks of the mitochondrial protein networks.

The rapid progress in identifying connections between machineries of different functions[15,30,147,160,161,224,225] indicates that we have not reached a saturation in the

analysis of mitochondrial protein networks. In addition to the experimentally established connections described in this Review, interesting further network candidates include: scaffold protein complexes that locally organize the protein-lipid composition of the inner membrane, such as the prohibitin ring complexes and stomatin-like protein 2 that associates with protease complexes and regulates the processing of PINK1 and OPA1[15,226]; lipid biosynthesis and remodeling enzymes; and cytosolic machineries that are involved in transferring preproteins, lipids or metabolites to mitochondria. Whereas several contact sites between mitochondria and other cell organelles have been identified recently, we have only a limited understanding of the interplay between cytosolic proteins/protein complexes and the mitochondrial outer membrane. This includes the potential involvement of specialized pools of cytosolic ribosomes in protein delivery to mitochondria[227], the role of cytosolic chaperones, co-chaperones and potential targeting factors in cytosol-mitochondria crosstalks, and the emerging evidence that numerous mitochondrial proteins possess a dual function and localization[30].

Important questions concern the dynamics, regulation and turnover of the protein networks. It is likely that partner complexes in networks are turned over in different rates. Examples are the stress-regulated degradation of the TIM17A isoform of metazoan presequence translocases[136] and the selective degradation of the outer membrane proteins Tom22 and porin-associated Om45 by the *i*-AAA protease[164] (FIG. 3), whereas the other subunits of the complexes are turned over by different proteolytic machineries. The differential control of the networks by mitochondrial proteolytic systems, the cytosolic ubiquitin-proteasome system and mitophagy, as well as the role of lipids in establishing and maintaining the networks will become central topics of research.

The large number of distinct functions observed in mitochondrial protein networks may give the initial impression that collaborations of protein machineries have developed in a random manner. The mechanistic studies performed so far, however, indicate that the interactions are highly specialized and specifically regulated, such as between presequence translocase and respiratory supercomplexes, and between MICOS, TOM, SAM and ER-mitochondria contact sites. To date, the studies have been mainly performed in yeast and partially in human mitochondria that both belong to the same supergroup of eukaryotes, opisthokonts, which include fungal and metazoan kingdoms. Since the characterization of the mitochondrial protein import machinery in different supergroups yielded remarkable insight into core machineries and the high variability of transport complexes[1,228], a systematic analysis of mitochondrial protein networks in the five eukaryotic supergroups will represent a rich source for defining core principles and variable parts of mitochondrial organization.

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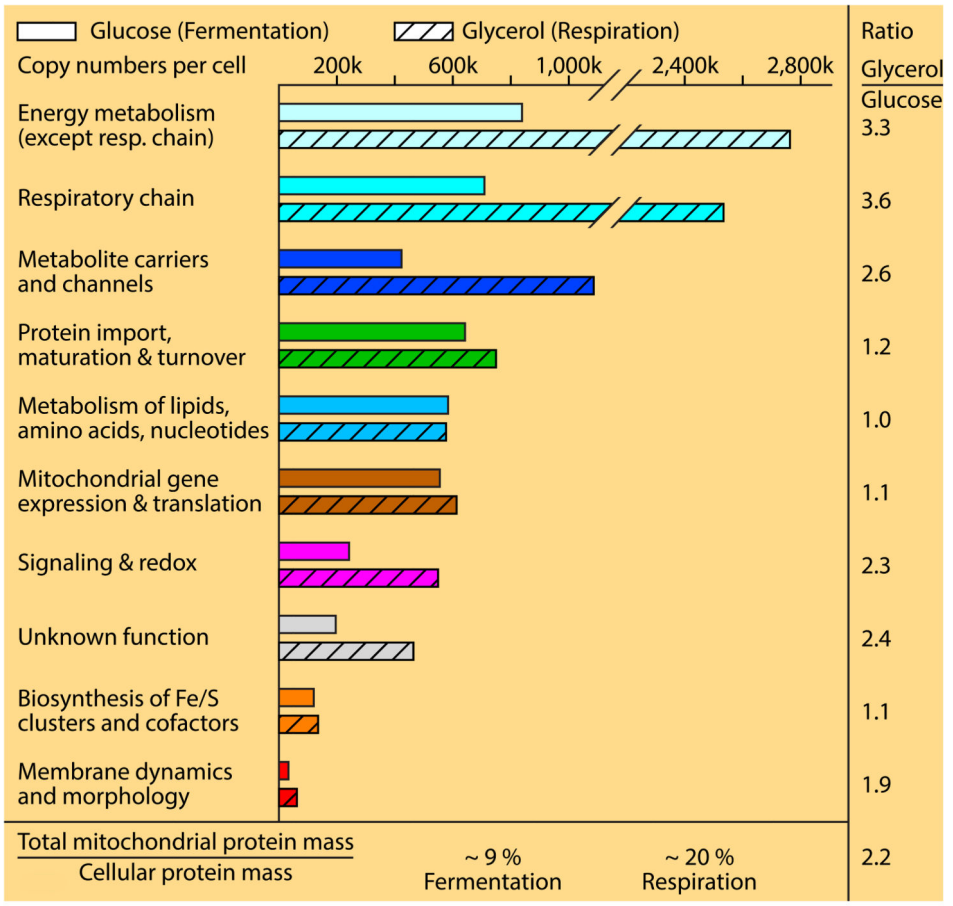
**Box 1**

### The mitochondrial proteome: from fermentation to respiration

The analysis of the mitochondrial proteome developed from the identification of individual proteins to the systematic determination of most mitochondrial proteins and the quantification of the relative abundance of mitochondrial proteins[24–42]. Recent studies determined the absolute copy numbers of proteins per cell[30,43–47], providing unprecedented insight into the composition and functional organization of mitochondria. Proteins involved in energy metabolism, including enzymes, respiratory chain complexes, $F_1F_O$-ATP synthase and metabolite carriers/channels, represent by far the most abundant classes of mitochondrial proteins[30]. The abundance of these proteins is strongly regulated by the growth conditions. When shifting baker's yeast from fermentation to respiratory conditions, their protein levels are increased about three-fold or more. The levels of proteins involved in signaling, redox processes, membrane dynamics and morphology are increased about two-fold from fermentation to respiration, comparable to the increase of the total mitochondrial protein mass. However, the two essential processes of mitochondria, protein import & maturation and biosynthesis of iron-sulfur clusters, are only mildly affected by different growth conditions; e.g., the absolute copy numbers per yeast cell of the proteins functioning in protein import, maturation and turnover are only changed from ~640,000 to 750,000 (i.e. a factor of 1.2) when cells are shifted from fermentation to respiration[30]. These essential machineries are thus identified as housekeeping systems that are present and active under all growth conditions. The protein classes involved in mitochondrial gene expression & translation and in the metabolism of lipids, amino acids and nucleotides are only mildly affected in their overall abundance by different growth conditions, however, differences can be observed for individual groups. For example, proteins involved in lipid metabolism are considerably more expressed under respiratory conditions, whereas proteins involved in amino acid metabolism are more strongly expressed under fermentable conditions[30].







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## Box 2

### Mitochondrial presequence translocase and respiratory chain assembly and function

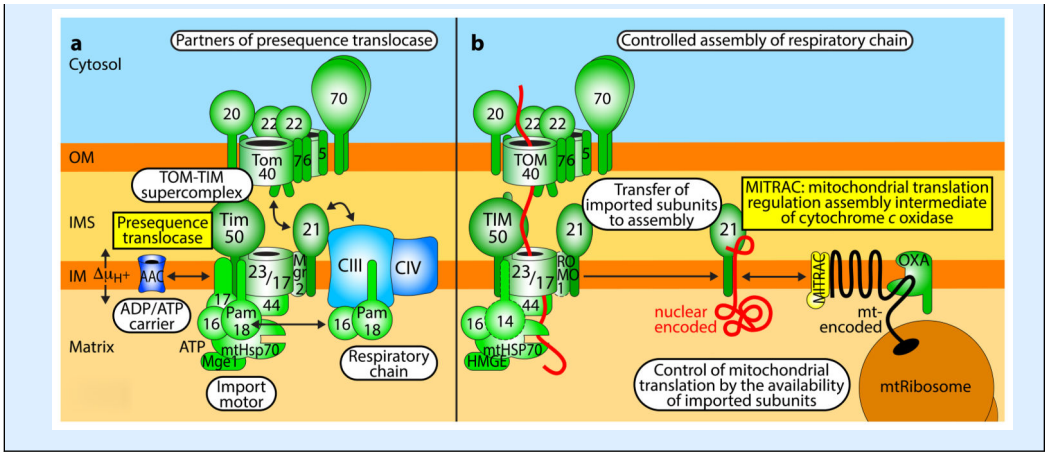
**Interaction network of presequence translocase**

The TIM23 complex of the inner membrane (IM) is a central junction in the presequence import pathway and interacts with several partner complexes in a dynamic manner: with the TOM complex during preprotein transfer from the outer membrane (OM) to the IM, forming a TOM-TIM-preprotein supercomplex; with the ATP-driven presequence translocase-associated motor (PAM); with the respiratory chain complexes III and IV that generate an electrochemical proton gradient ($\Delta\mu_H+$) driving preprotein insertion[152,153]; and with the ADP/ATP carrier that also supports preprotein translocation[154,229]. The translocase subunit Tim21 functions as a dynamic coupling factor that interacts with TOM and the respiratory supercomplex III-IV in an alternating manner. In fully active, respiring yeast mitochondria, the activity of the presequence translocase drives preprotein import efficiently. However, when the respiratory activity is decreased, coupling of the translocase to machineries involved in bioenergetics is beneficial to maintain the energy-dependent action of the translocase[152–154]. Preprotein translocases in the immediate vicinity of proton pumping respiratory complexes likely experience an increased proton motive force (localized proton gradients) and thus under energylimiting conditions preprotein insertion into the IM is still possible[152,153], ensuring that the biogenesis of respiratory complexes is ongoing e.g. under limited food supply.

**Coupling of presequence translocase to respiratory chain assembly**

The characterization of respiratory chain biogenesis in human mitochondria revealed a further level of cooperation between the protein import machinery and the respiratory chain, mediated by the mitochondrial translation regulation assembly intermediate of cytochrome *c* oxidase (MITRAC)[155–157]. MITRAC comprises several assembly intermediate complexes of the respiratory chain and plays a dual role. (i) It links the presequence translocase to respiratory chain assembly intermediates via the TIM21-mediated transfer of imported proteins from TIM23 to MITRAC[156]. (ii) MITRAC cooperates with the machineries for mitochondrial protein synthesis and insertion by adapting the efficiency of mitochondrial translation to the import of nuclear encoded partner proteins (translational plasticity). MITRAC assembly factors bind to partially synthesized/membrane inserted proteins such as the highly hydrophobic COX1 protein and delay their synthesis till the appropriate partner proteins have been imported, thus promoting a proper balance of nuclear and mitochondrially encoded subunits[157].

OXA, insertion machinery (oxidase assembly). The role of human TIM14/PAM18 and ROMO1/MGR2 in the TIM23 complex has not been defined so far (indicated by dashed borders).







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## Box 3

### Mitochondrial protein import and processing in cellular quality control

**Mitochondrial protein import machinery in stress response and quality control**

- Mitochondrial protein import machinery in stress response and quality control Mitochondrial unfolded protein response (UPRmt): The activating transcription factor associated with stress ATFS-1/ATF5 contains mitochondrial and nuclear localization signals. The factor is imported into healthy mitochondria and degraded. When mitochondrial import is impaired, the transcription factor accumulates in the cytosol, is translocated into the nucleus and induces expression of chaperones, proteases and further factors to promote recovery of impaired mitochondria[207,208].
- Unfolded protein response activated by mistargeted mitochondrial proteins (UPRam), also termed mitochondrial precursor over-accumulation stress (mPOS): Upon disturbance of mitochondrial protein import, precursor proteins accumulating in the cytosol trigger a stress response that reduces the efficiency of cytosolic protein synthesis and increases the activity of the proteasome, thus reducing the accumulation of mistargeted proteins in the cytosol[209,210].
- PINK1/parkin: The mitochondrial kinase PINK1 has been identified in familial cases of Parkinson's disease. In healthy mitochondria, PINK1 is imported by the presequence pathway and processed by MPP and the presenilin-associated rhomboid-like protease PARL, followed by release into the cytosol and degradation by the proteasome. When protein import or processing by the presequence pathway are disturbed, unprocessed PINK1 accumulates at the TOM complex[221–223] (FIG. 4), where it phosphorylates ubiquitin and the E3 ubiquitin ligase parkin, triggering the removal of damaged mitochondria by mitophagy.
- Mitochondria as guardian in cytosol (MAGIC): Some aggregation-prone or misfolded cytosolic proteins may be imported into mitochondria and degraded[215], suggesting a role of mitochondria in cytosolic proteostasis.

**Mitochondrial preprotein processing, turnover and membrane dynamics**

- After processing by MPP, the matrix peptidases Icp55 and octapeptidyl peptidase can remove destabilizing amino acid residues from the amino-termini of imported yeast mitochondrial proteins (FIG. 2), generating proteins with stabilizing amino-termini that are less susceptible to degradation by matrix proteases[53,75,76].
- The inner membrane fusion protein OPA1 is present in long and short isoforms. Upon import, MPP performs the first cleavage to generate the long isoform. A second processing by inner membrane-bound proteases such as AAA proteases (FIG. 3) and OMA1 in mammals generates short isoforms[216–218]. The efficiency of the second processing is influenced by

stress and energetic conditions (ATP, membrane potential), leading to different ratios between long and short isoforms that modulate mitochondrial fusion and fragmentation.



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**Box 4**

## Disorders and diseases linked to distinct steps of human mitochondrial protein import and maturation

- *Mitochondrial targeting signal:* mutations of targeting signals can impair import of individual proteins, causing pyruvate dehydrogenase E1α deficiency[230] or mitochondrial aspartyl-tRNA synthetase import defect linked to leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (LBSL)[231].
- *Mitochondrial targeting:* L-alanine:glyoxylate aminotransferase resides in peroxisomes in humans, however, mutations can generate a mitochondrial targeting signal, leading to mistargeting to mitochondria and primary hyperoxaluria type 1[232,233]. Similarly, in a form of renal Fanconi syndrome, a mutation generates a mitochondrial targeting signal in a peroxisomal protein involved in fatty acid oxidation, causing its mistargeting to mitochondria and disturbance of mitochondrial energy production in the proximal tubule[234].
- *Growth factor, augmenter of liver regeneration ERV1 homolog (GFER/ALR):* a mutation in the gene for the disulfide relay component ERV1/GFER/ALR causes impairment of FAD cofactor binding resulting in myopathy with cataract and combined respiratory chain deficiency[235,236].
- *TIMM50:* mutations of the presequence translocase receptor lead to epileptic encephalopathy, 3-methylglutaconic aciduria and variable complex V deficiency[237].
- *DnaJ domain containing protein DNAJC19:* mutations in the gene for the human PAM18/TIM14 homolog cause dilated cardiomyopathy with ataxia, anemia and testicular dysgenesis[238,239]. Since DNAJC19 is mainly associated with prohibitin complexes affecting cardiolipin metabolism, cardiolipin alteration is likely involved in disease pathogenesis[240]. DNAJC15/MCJ (methylation-controlled J-protein), a further human PAM18/TIM14 homolog[241], has been linked to tumorigenesis. DNAJC19 and DNAJC15 have been connected to distinct presequence translocase forms[135], yet their exact relevance for protein import needs further analysis (indicated by a dashed border).
- *Mitochondria-associated granulocyte macrophage colony stimulating factor-signaling molecule (MAGMAS):* a mutation in the gene for human PAM16/TIM16 is linked to a severe spondylodysplastic dysplasia[242].
- *Mitochondrial processing peptidase (MPP):* mutations in the genes for MPP subunits α (PMPCA) or β (PMPCB) cause defects in preprotein processing, linked to cerebellar ataxia[243] or early childhood neurodegeneration with cerebellar atrophy[244].

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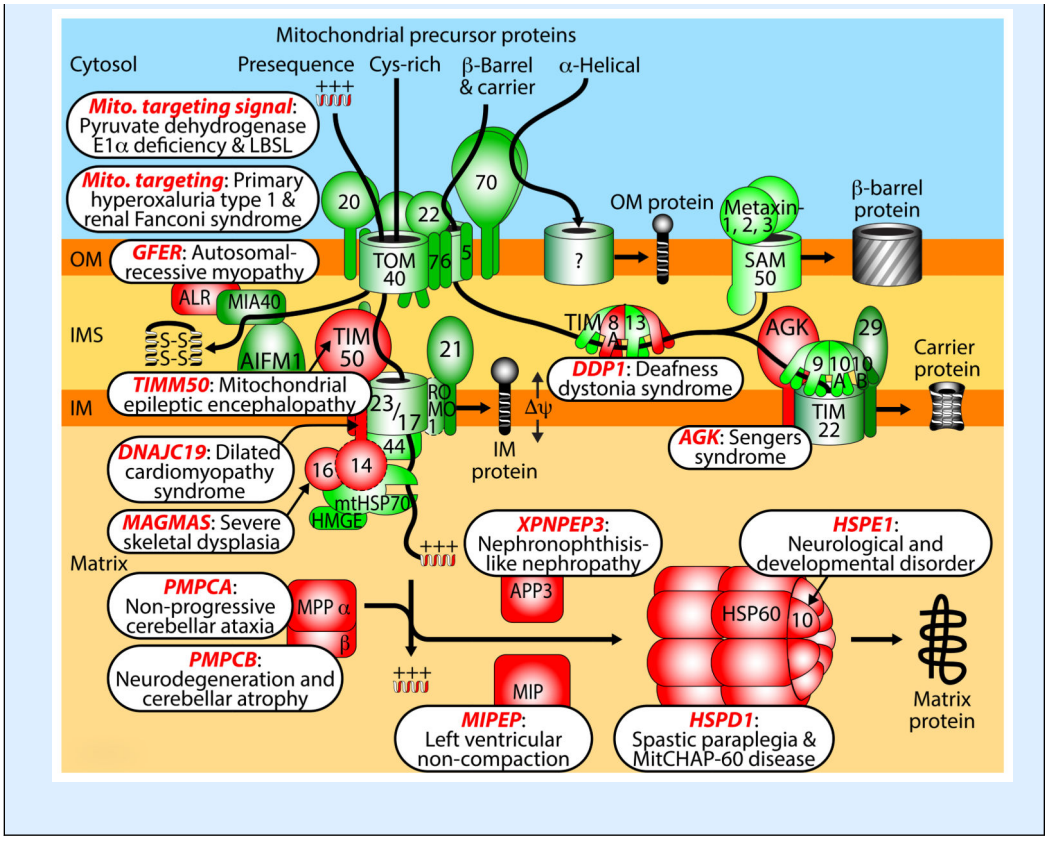
- *Mitochondrial intermediate peptidase (MIPEP):* mutations of octapeptidyl peptidase cause left ventricular non-compaction cardiomyopathy with hypotonia and developmental delay[245].
- *X-prolyl aminopeptidase 3 (XPNPEP3):* mutations of the human intermediate cleaving peptidase are linked to nephronophthisis-like cystic kidney disease[246,247].
- *Heat shock protein family D member 1 (HSPD1):* mutations of HSP60 lead to neurodegenerative disorders, spastic paraplegia and mitochondrial chaperonin-60 disease[248,249].
- *Heat shock protein family E member 1 (HSPE1):* a mutation of the co-chaperonin HSP10 is associated with infantile spasms and developmental delay[250].
- *Deafness/dystonia protein 1 (DDP1):* mutations of subunit TIMM8a of small TIM chaperones cause deafness dystonia syndrome, also termed Mohr-Tranebjaerg syndrome[251,252].
- *Acyl glycerol kinase (AGK):* mutations in the *AGK* gene lead to cataracts, cardiomyopathy and skeletal myopathy (Sengers syndrome)[253]. AGK plays a dual role as lipid kinase and as subunit of the human TIM22 carrier translocase, linking lipid metabolism and protein import to Sengers syndrome[254,255].



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## Glossary terms

**Oxidative phosphorylation**

Oxidation of metabolites liberates energy that is used to synthesize ATP, in mitochondria performed by the respiratory chain that generates a proton gradient across the inner membrane to drive the $F_1F_O$-ATP synthase.

**Insertase**

Membrane-bound machinery that facilitates the insertion of precursor proteins into the lipid phase of a membrane, such as the oxidase assembly (OXA) insertase of the mitochondrial inner membrane.

**MICOS**

The mitochondrial contact site and cristae organizing system is a large protein complex of the inner membrane with a dual role, formation of contact sites to the outer membrane and maintenance of the cristae architecture of the inner membrane.

**TOM complex**

The translocase of the outer membrane forms the major mitochondrial entry site for precursor proteins synthesized in the cytosol.

**SAM complex**

The sorting and assembly machinery inserts β-barrel proteins into the mitochondrial outer membrane and is also called topogenesis of outer membrane β-barrel proteins (TOB).

**Heat shock proteins 70**

Large family of ATP-dependent molecular chaperones of ~70 kDa that bind loosely folded proteins and prevent their misfolding or aggregation. The major mitochondrial heat shock protein 70 (mtHsp70) has a dual role in driving ATP-dependent protein import into the matrix and assisting in folding of imported proteins.

**Respirasomes**

Large supercomplexes in the mitochondrial inner membrane consisting of the complexes I, III and IV of the respiratory chain.

**ERMES**

The endoplasmic reticulum-mitochondria encounter structure is a multi-subunit protein complex that connects endoplasmic reticulum and the mitochondrial outer membrane. ERMES is likely involved in lipid transfer between the organelles and is required for maintaining the morphology of mitochondria.

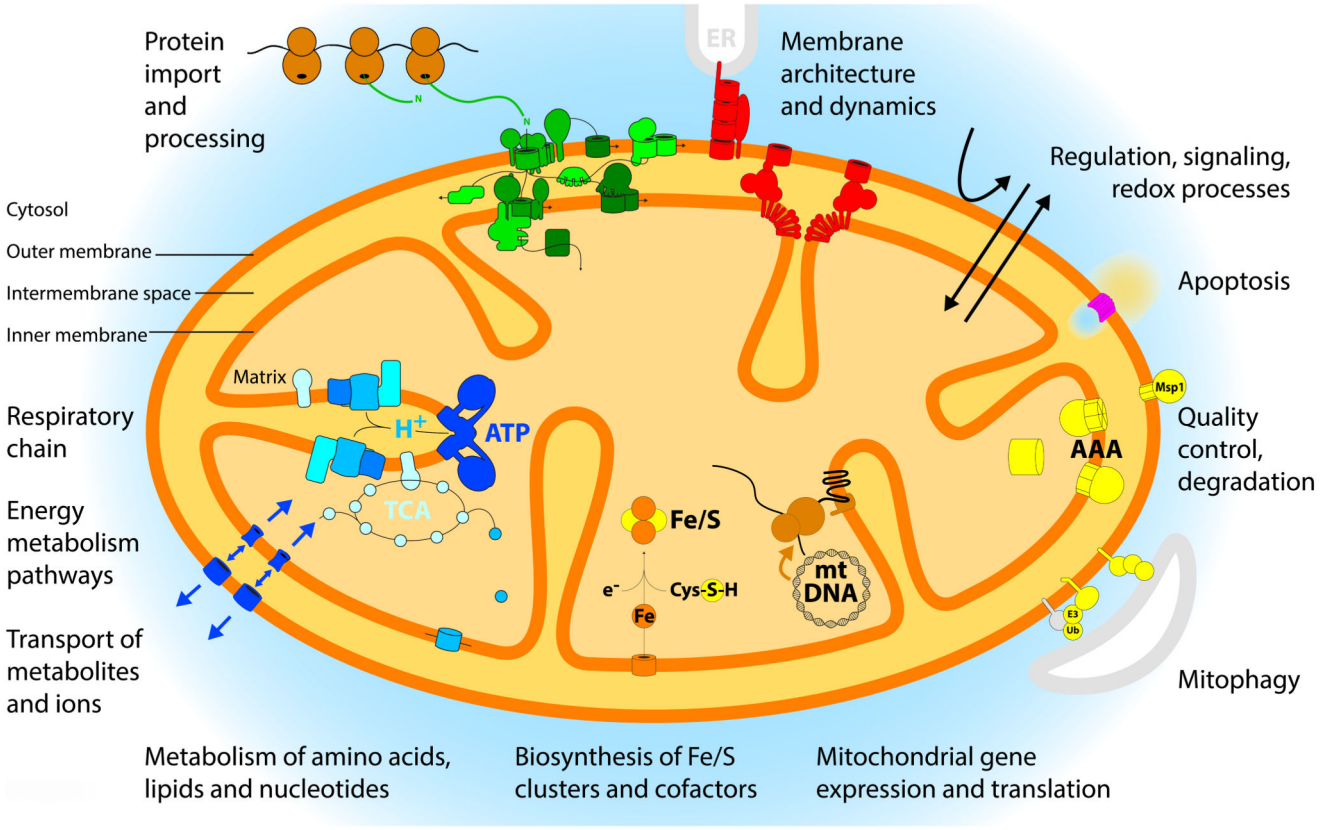


**Figure 1. Overview of mitochondria and their functions.**

Mitochondria consist of four compartments: outer membrane, intermembrane space, inner membrane and matrix. A large variety of functions have been assigned to mitochondrial proteins and protein complexes and are indicated in the figure: energy metabolism with respiration and synthesis of ATP; metabolism of amino acids, lipids and nucleotides; biosynthesis of iron-sulfur (Fe/S) clusters and cofactors; expression of the mitochondrial genome; quality control and degradation processes including mitophagy and apoptosis; signaling and redox processes; membrane architecture and dynamics; and the import and processing of precursor proteins that are synthesized on cytosolic ribosomes. AAA, ATP-dependent proteases of the inner membrane; E3, ubiquitin ligase; Msp1, mitochondrial sorting of proteins, extracts mistargeted proteins; TCA, tricarboxylic acid cycle; Ub, ubiquitin.




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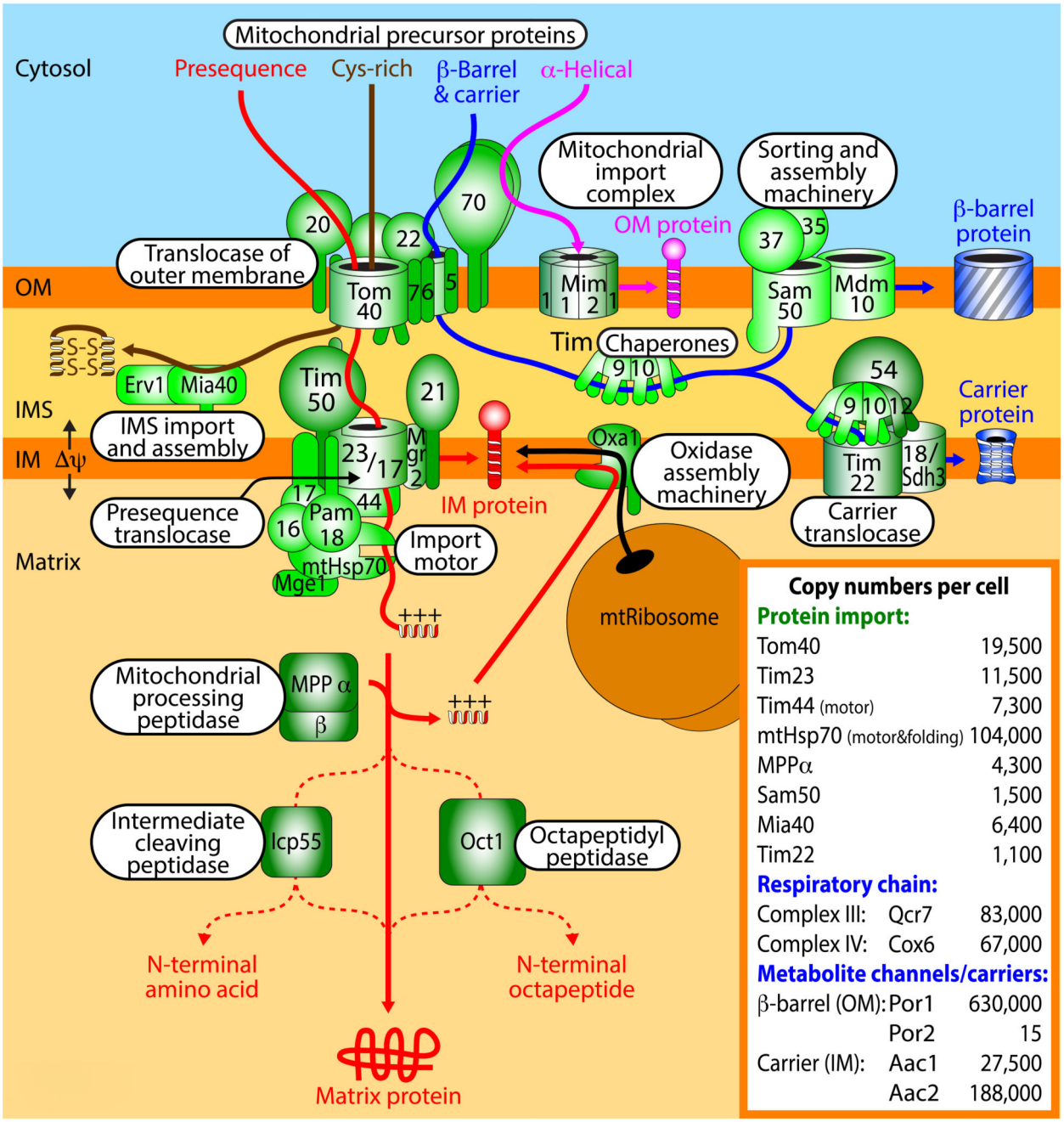


**Figure 2. Protein import pathways into mitochondria.**

Five major pathways of mitochondrial protein import have been identified. The protein import machineries have been well conserved from fungi (shown in this figure) to mammals (shown in BOX 4). First, the presequence pathway transports presequence-carrying cleavable preproteins through the translocase of the outer membrane (TOM) and the presequence translocase of the inner membrane (TIM23) with the presequence translocase-associated motor (PAM). The membrane potential Δψ across the inner membrane (IM) activates the TIM23 channel and drives translocation of the positively charged presequences

into the matrix. The presequences are removed by the mitochondrial processing peptidase (MPP) and additional proteolytic processing can occur by the intermediate cleaving peptidase (Icp55) or the octapeptidyl peptidase (Oct1). Cleavable IM proteins are either laterally released from the TIM23 complex or are transported via the matrix and inserted into the IM by the oxidase assembly (Oxa1) insertase. IM proteins synthesized on mitochondrial ribosomes are also inserted by Oxa1. Second, cysteine-rich proteins destined for the intermembrane space (IMS) are imported through the TOM complex and are recognized by the mitochondrial IMS import and assembly protein (Mia40) that functions as oxidoreductase to insert disulfide bonds into the imported proteins. The sulfhydryl oxidase Erv1 forms a disulfide relay with Mia40, transferring disulfides from Erv1 to Mia40 to imported proteins. Third, the precursors of non-cleavable IM proteins such as the carrier proteins are imported by the TOM complex, followed by transfer to the small TIM chaperones in the IMS and insertion into the IM by the TIM22 carrier translocase. Fourth, the precursors of outer membrane (OM) β-barrel proteins use the TOM complex and small TIM chaperones and are inserted into the OM by the sorting and assembly machinery (SAM). Fifth, many OM proteins with α-helical transmembrane segments are inserted into the membrane by the mitochondrial import (MIM) complex. α-helical OM proteins typically do not use the Tom40 channel, but Tom70 can be involved in their recognition. Inset, assessment of absolute copy numbers of mitochondrial proteins in a respiring yeast cell[30]. The porin isoform Por1 of the OM is one of the most abundant mitochondrial proteins, whereas the isoform Por2 is one of the least abundant proteins.

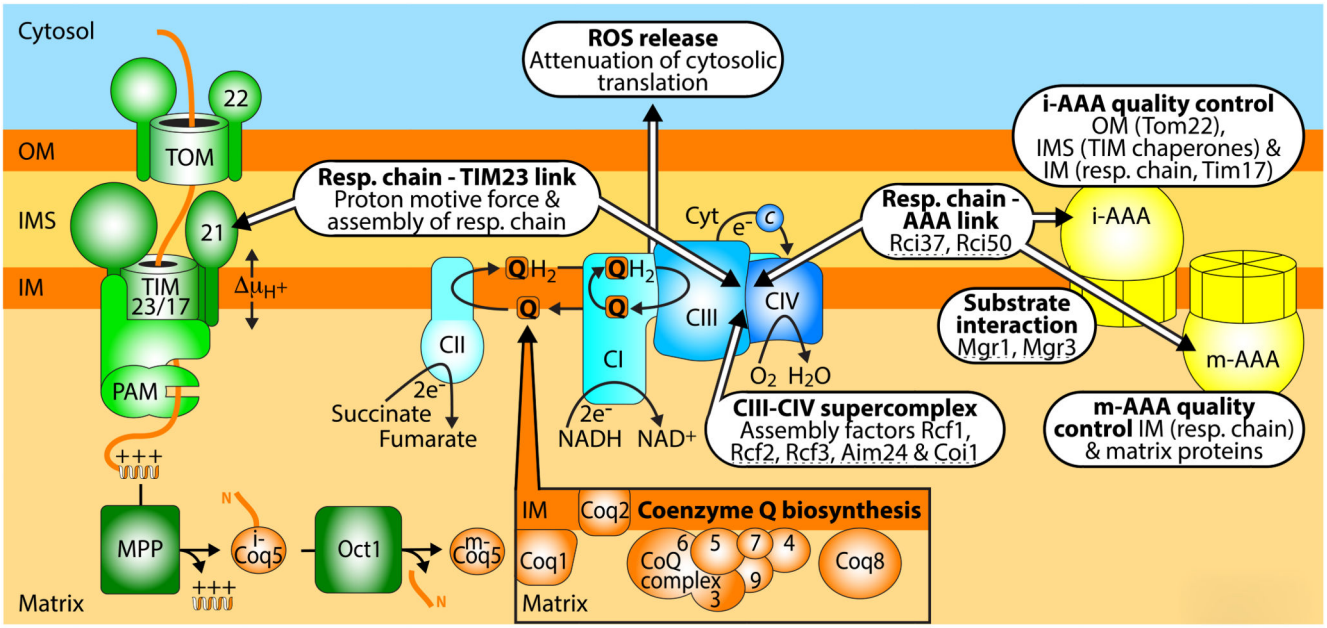


**Figure 3. Interaction network of respiratory complexes, biogenesis and quality control machineries.**

Supercomplexes of the mitochondrial respiratory chain are integrated into functional networks with the presequence translocase TIM23 (see BOX 2) and the AAA proteases of the inner membrane (IM). The ATP-dependent AAA proteases not only degrade several IM proteins, but also selected proteins of the matrix, intermembrane space (IMS) and outer membrane (OM), functioning as a quality control system of mitochondria. Several respiratory chain-AAA linker proteins, AAA-substrate adapter proteins, and assembly factors for respiratory supercomplexes were identified in fungi (indicated by dashed lines). The coenzyme Q (CoQ, Q) biosynthetic complex on the matrix side of the IM provides CoQ for the respiratory chain and further enzymes. The precursors of the CoQ complex are imported by the TOM and TIM machineries. Proteolytic processing in the matrix can involve two steps like for the precursor of Coq5. The mitochondrial respiratory chain is a main source for the generation of ROS that can exert harmful effects but also function in signaling. Targeting of the cytosolic translation machinery by ROS leads to a decreased protein synthesis, providing a link between the status of the respiratory chain and protein biogenesis.




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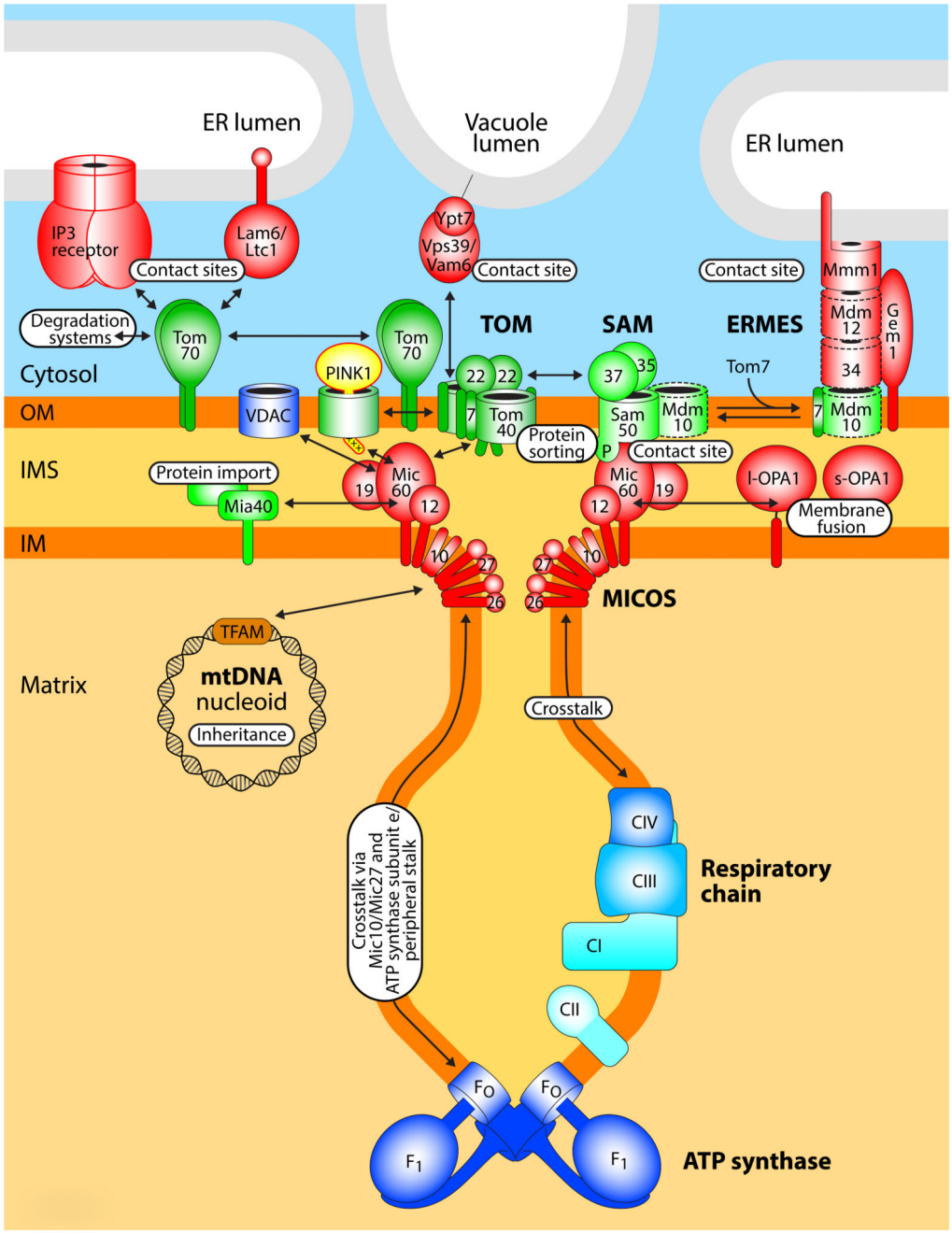


**Figure 4. Mitochondrial organizing network.**

The mitochondrial contact site and cristae organizing system (MICOS) of the inner membrane (IM) and the protein translocases TOM and SAM of the outer membrane (OM) form the core of a large ER-mitochondria organizing network (ERMIONE) that includes multiple dynamic interactions: to the ER-mitochondria encounter structure (ERMES); to further ER-mitochondria contact sites that involve the receptor Tom70 and IP3 receptors or the lipid transfer protein Lam6/Ltc1, as well as to vacuole-mitochondria contact sites (including Tom40 and the bridging protein Vps39/Vam6); to the kinase PINK1 and the

metabolite channel VDAC (porin); to the mitochondrial intermembrane space (IMS) protein import and assembly system (Mia40); to respiratory chain complexes, the $F_1F_O$-ATP synthase, and the fusion protein OPA1 of the IM; and to mtDNA nucleoids (with the mtDNA packaging factor, termed mitochondrial transcription factor A, TFAM)[256] of the matrix. Most components shown have been functionally conserved from yeast to humans; proteins that have been characterized in fungi only are indicated by a dashed border, whereas proteins that have been characterized in metazoans so far are bordered in red. In sum, ERMIONE forms a membrane-spanning system for the coordination of protein and lipid biogenesis, energetics, inheritance and quality control of mitochondria.



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