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Assembling the mitochondrial outer membrane

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The general preprotein translocase of the outer mitochondrial membrane (TOM complex) transports virtually all mitochondrial precursor proteins, but cannot assemble outer-membrane precursors into functional complexes. A recently discovered sorting and assembly machinery (SAM complex) is essential for integration and assembly of outer-membrane proteins, revealing unexpected connections to mitochondrial evolution and morphology.

Mitochondria are not only of central importance for the bioenergetics of eukaryotic cells but have critical functions in the metabolism of amino acids and lipids, in the biosynthesis of heme and Fe-S clusters, and in apoptosis[1–4]. Mitochondria contain about 800 (yeast)[1] to 1,500 different proteins (human)[2]. Although mitochondria contain a complete genetic system in their innermost compartment, the matrix, only ~1% of mitochondrial proteins are encoded by the organellar genome and synthesized in the matrix.

The vast majority of mitochondrial proteins are encoded by nuclear genes and synthesized as precursor proteins on cytosolic ribosomes[5–9]. Two major pathways of protein transport into mitochondria have been characterized in detail: the presequence pathway for import of matrix proteins and the carrier pathway for import of hydrophobic inner membrane proteins (**Fig. 1**). Recent studies, however, led to the identification of a novel pathway that is dedicated to the sorting and assembly of mitochondrial outer-membrane proteins, in particular of a class of proteins anchored in the membrane by transmembrane β-strands. This review summarizes the known mechanisms of mitochondrial protein import and then focuses on the discovery of the sorting and assembly machinery of the outer membrane (SAM complex) and its implications for protein assembly, mitochondrial evolution and morphology.

Two major pathways of mitochondrial protein import

Mitochondrial precursor proteins contain targeting signals that are recognized by receptors of the translocase of the outer mitochondrial membrane (TOM complex) (**Fig. 1**). The classical targeting signal is at the N terminus. This presequence forms a positively charged amphipathic α-helix and is imported into the matrix[5–9]. Upon interaction with Tom receptors, the presequences direct the translocation of the preproteins through the import channel of the TOM complex to the presequence translocase of the inner mitochondrial membrane (TIM23 complex). The presequence translocase-associated motor (PAM) drives the completion of protein translocation into the matrix, where the mitochondrial processing peptidase (MPP) removes the presequences. Some proteins of the inner membrane are also synthesized with a presequence. Those precursor proteins use the presequence import pathway with the TOM complex and the TIM23 complex and are guided into the inner membrane by a hydrophobic sorting sequence that typically follows the positively charged presequence[10,11].

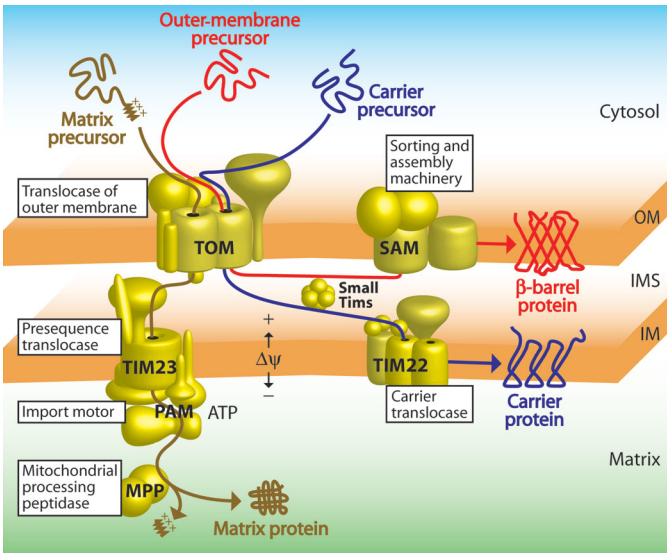

Figure 1 Protein import pathways into mitochondria. Most mitochondrial proteins are synthesized on cytosolic ribosomes and imported post-translationally. Cytosolic chaperones (not shown) guide the precursor proteins to the central mitochondrial entry gate, the translocase of the outer membrane (TOM complex). Then the precursor proteins follow different import pathways. Presequence-carrying proteins, such as most matrix proteins, are translocated by the presequence translocase of the inner membrane (TIM23 complex) in a membrane potential (Δψ)-dependent manner. The ATP-driven presequence translocase-associated motor (PAM) is required for the completion of protein translocation into the matrix, where the mitochondrial processing peptidase (MPP) removes the presequence. Noncleavable hydrophobic inner-membrane proteins, such as the carrier proteins, are guided by complexes of small Tim proteins across the intermembrane space (IMS) and are inserted into the inner membrane (IM) by the Δψ-driven carrier translocase (TIM22 complex). The precursors of β-barrel proteins of the outer membrane (OM) are transferred from the TOM complex to the sorting and assembly machinery (SAM complex) with the help of small Tim proteins of the IMS.

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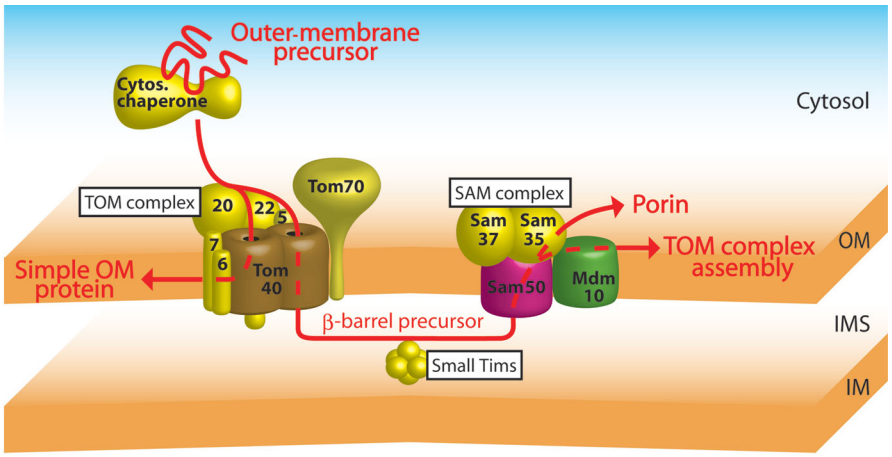

Figure 2 The protein import and sorting machineries of the mitochondrial outer membrane. All outer-membrane (OM) proteins are synthesized in the cytosol. Molecular chaperones bind to the precursor proteins and prevent their aggregation. The precursor proteins are recognized by the translocase of the outer membrane (TOM complex). The TOM complex consists of three receptors (Tom20, Tom22 and Tom70), the channel-forming protein Tom40 and three small Tom proteins (Tom5, Tom6 and Tom7). Simple OM proteins with single transmembrane segments can be directly imported into the OM with the help of the TOM complex. The precursors of OM proteins with a more complicated topology, such as β-barrel proteins, are translocated through the TOM complex to the intermembrane space (IMS) side. Small Tim proteins guide the precursor proteins to the sorting and assembly machinery (SAM complex), where the insertion into the OM takes place. Sam50 forms the core of the SAM complex and cooperates with Sam35 and Sam37 in the integration of β-barrel proteins such as porin and Tom40. However, the precursor of Tom40 additionally requires the function of Mdm10 that promotes the assembly of Tom40 with other Tom subunits to the mature TOM complex. IM, inner membrane.

Many mitochondrial proteins, however, are synthesized without a cleavable extension but contain internal targeting signals. Major representatives of this second group of precursor proteins are hydrophobic inner-membrane proteins such as the large family of metabolite translocators (carrier proteins)[5,6,8,9]. The carrier proteins are imported by a different mechanism than the cleavable presequence-carrying proteins[12]. Although the cleavable preproteins and the noncleavable carrier proteins are both imported by the TOM complex, the mode of translocation is different. Presequence proteins are translocated through the TOM complex as linear polypeptide chains, guided by the N-terminal presequence, whereas the carrier precursors traverse the TOM channel with a middle portion first (in a loop formation)[13]. After passing through the TOM complex, the import route of the carrier precursors diverges from the presequence route and uses a specific machinery of the intermembrane space and inner membrane (**Fig. 1**). A class of intermembrane-space proteins, termed small Tim proteins, bind the carrier precursors and protect the hydrophobic segments during transfer across the aqueous intermembrane space[5,6,8,9,14]. The small Tim proteins thus function in a chaperone-like manner and deliver the carrier precursors to the membrane-integrated carrier translocase of the inner membrane (TIM22 complex)[15].

Identification of a new protein biogenesis pathway

The proteins of the mitochondrial inner membrane contain transmembrane α-helices like the proteins that are present in most other membranes of a eukaryotic cell, including the endoplasmic reticulum, the nuclear envelope, the Golgi and post-Golgi compartments of the secretory pathway and the plasma membrane. The mitochondrial outer membrane represents an exception because it contains proteins that are anchored in the membrane by multiple β-strands[16–18]. Little has been known about the import and membrane integration of those β-barrel proteins.

All mitochondrial outer-membrane proteins are synthesized as noncleavable precursor proteins on cytosolic ribosomes. Because the TOM complex functions as the central entry gate for virtually all nuclear-encoded mitochondrial precursor proteins, it had been assumed that this complex is also responsible for the sorting and assembly of outer-membrane proteins, including the precursors of the TOM complex itself[19–22]. The TOM complex has a size of ~450 kDa and consists of seven different subunits[23,24] (**Fig. 2**). Tom20, Tom22 and Tom70 function as receptors for the various classes of mitochondrial pre-cursor proteins[25,26] (numbers refer to the approximate molecular mass of a component in kDa). The core of the TOM complex is made up of Tom40 that forms the protein-conducting channel across the outer membrane and thus is essential for cell viability[27,28]. A small Tom protein, Tom5, supports the insertion of precursor proteins into the Tom40 channel[29] while two other small Tom proteins, Tom6 and Tom7, influence the stability of the TOM complex[30]. Upon synthesis in the cytosol, the precursors of Tom proteins are recognized by the pre-existing TOM complex; that is, the mature TOM complex is required for importing its own precursor proteins[19,21,30–33].

Recent studies, however, revealed that the TOM complex was not sufficient to integrate β-barrel proteins into the outer membrane and was not able to assemble its own precursor proteins into functional complexes. The discovery of the new sorting and assembly pathway with the SAM complex occurred in two steps. The model substrate was the precursor of the β-barrel protein Tom40. The precursor of Tom40 is initially recognized by the Tom receptors on the mitochondrial surface[19,30,32]. Unexpectedly, the Tom40 precursor is not directly integrated into the TOM complex but uses the pre-existing Tom40 channel for translocation to the *trans* side of the outer membrane—that is, to the intermembrane space[30,34] (**Fig. 2**). The Tom40 precursor then assembles via several intermediate steps into a mature TOM complex. This initial observation suggested the presence of a novel assembly pathway for outer-membrane proteins[30].

The second step for the discovery of the SAM complex was made by the analysis of yeast mutant mitochondria that lacked individual outer-membrane proteins. Mitochondria lacking the protein Sam37 (formerly named Mas37 or Tom37) were strongly impaired in the assembly of Tom40, whereas the two known import pathways, the presequence pathway and the carrier pathway, were not affected[34]. Sam37 was identified about a decade ago. Yeast cells lacking Sam37 showed pleiotropic effects in phospholipid metabolism, protein import and a genetic interaction with Tom proteins[35], leading to the name Tom37. Subsequently, it was demonstrated that the protein was not present in the TOM complex and did not act as an import receptor[36], so its function was unclear. It is now established that Sam37 is the founding subunit of the SAM complex of the mitochondrial outer membrane[34] (**Fig. 2**). Mitochondria lacking Sam37 are impaired in the assembly pathway not only of Tom40 but also of

further β-barrel proteins, including the most abundant outer-membrane protein, porin. Sam37 is present in a 200-kDa SAM core complex that is crucial for the membrane integration of mitochondrial β-barrel proteins, whereas simple outer-membrane proteins that contain a single α-helical transmembrane segment can be inserted into the membrane with the aid of the TOM complex alone[34]. The crucial role of Sam37 for sorting and assembly of outer-membrane proteins explains the various pleiotropic effects that had been observed in mutant cells lacking Sam37.

The SAM complex is essential for cell viability

Two additional subunits of the SAM core complex have been found and each is essential for cell viability. The first, Sam50 (Tob55/Omp85) was identified by three different experimental strategies: purification of the SAM complex[37], proteomic analysis of the mitochondrial outer membrane[38] and sequence comparison of bacterial and mitochondrial proteins[39]. The second, Sam35 (Tob38) was identified by isolation of the SAM complex[40,41]. Sam50 is an integral outer-membrane protein with a predicted β-barrel domain, whereas Sam35 behaves as peripheral membrane protein that is exposed on the mitochondrial surface[37–41] (**Fig. 2**). Conditional yeast mutants of Sam50 or Sam35 are defective in the assembly of β-barrel outer-membrane proteins. Sam50 and Sam35 thus show the same substrate specificity as Sam37 (refs. 34,37–41). Sam50 is thought to represent the central subunit of the SAM complex. Purified Sam50 has been reported to form ring-shaped particles with a large channel[38]. Sam50 contains an N-terminal domain that is probably exposed to the intermembrane space and may represent the interaction site for β-barrel precursors before their insertion into the SAM complex. The exact molecular functions of Sam35, Sam37 and Sam50 are still unknown but the genetic and biochemical experiments demonstrate that all three proteins have to cooperate for an efficient membrane integration of outer-membrane β-barrel precursors[34,37–41].

Together with Tom40, three proteins of the mitochondrial outer membrane are now known that are strictly essential for cell viability. The presence of two essential proteins, Sam50 and Sam35, in the SAM complex emphasizes the central role of the new complex in mitochondrial biogenesis.

Protein transfer from TOM to SAM

How are the precursors of β-barrel proteins transferred from the TOM complex to the SAM complex? A removal of intermembrane space proteins by opening of the outer membrane strongly impairs the transfer of β-barrel precursors to the SAM complex, whereas simple outer-membrane proteins with an α-helical membrane anchor, like Tom20, are efficiently assembled[42]. Thus intermembrane space components are probably involved in the transfer of β-barrel precursors from TOM to SAM. Indeed, the complexes of small Tim proteins, which have been known for their role in transfer of inner-membrane precursors from the TOM complex to the TIM22 complex[5,8,9,14], also function in the biogenesis of outer-membrane proteins[42,43] (**Fig. 2**), explaining the previous observation that porin assembly depends on an intact intermembrane space[44]. Remarkably, each of the small Tim complexes, the essential Tim9–Tim10 complex and the non-essential Tim8–Tim13 complex, promote the transfer of β-barrel precursors to the SAM complex. The chaperone-like functions of the small Tim proteins probably shield hydrophobic segments of the precursor proteins against aggregation in the aqueous intermembrane space and thus keep the β-barrel precursors in a competent state for insertion into the outer membrane via the SAM complex.

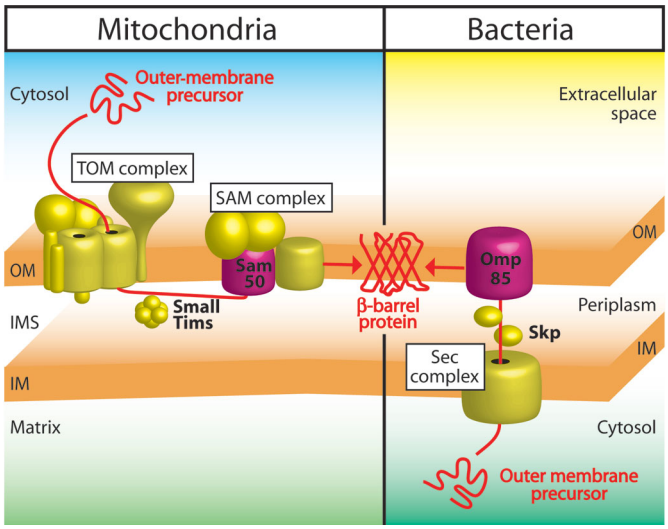

Figure 3 Conservation of β-barrel insertion into the outer membranes of mitochondria and Gram-negative bacteria. Sam50 of the mitochondrial SAM complex contains a large membrane-embedded domain that is homologous to a domain of the protein Omp85 of the bacterial outer membrane (OM). Although the initial transport steps of OM β-barrel proteins are different for mitochondria (via the TOM complex of the OM) and bacteria (via the Sec complex of the inner membrane (IM)), the proteins are inserted into the OM from the same direction (from the mitochondrial intermembrane space (IMS) and the bacterial periplasm). Chaperone-like proteins guide the proteins through the aqueous IMS (small Tim proteins) and periplasm (Skp), respectively. Protein integration into the OM is then mediated by the conserved Sam50/Omp85. Omp85 may be part of a multisubunit complex[47] (not shown) like the SAM complex; however, further subunits of this putative Omp85 complex have not been identified so far.

A conserved pathway for protein assembly

The analysis of the primary structure of Sam50 revealed a striking similarity to the outer-membrane protein Omp85 of Gram-negative bacteria[37–39]. Sam50 contains a C-terminal β-barrel domain of ~30 kDa that is conserved from bacteria to man. Actually, Sam50 is the first transport component of the mitochondrial outer membrane that exhibits substantial homology to a bacterial protein. Different functions have been reported for bacterial Omp85, participating in either the insertion of β-barrel proteins into the bacterial outer membrane[45] or the transport of lipids to the outer membrane[46]. In light of the role of Sam50 in mitochondrial protein insertion, it seems likely that bacterial Omp85 also functions in protein export (**Fig. 3**).

Because mitochondrial β-barrel precursors are exposed to the intermembrane space before being inserted into the SAM complex, the direction of insertion has also been conserved from bacteria to eukaryotic cells. In mitochondria and bacteria, the hydrophobic β-barrel precursors are transferred through an aqueous space, the intermembrane space or the periplasm, respectively. In both cases, chaperone-like molecules have been found that are critical for the transfer of the precursor proteins, the small Tim proteins of mitochondria, the chaperone Skp of bacteria and possibly additional chaperones[42,43,47–49] (**Fig. 3**). Though the small Tim proteins and Skp are not homologous to each other, the principle of chaperone-assisted transfer of precursors to Sam50/Omp85 has been conserved.

Interestingly, bacterial Omp85 is also homologous to the channel-forming protein Toc75 of chloroplasts[39,47,50]. There are four genes in *Arabidopsis thaliana* that could encode forms of Toc75. Toc75-III is the central component of the TOC protein import complex of the

chloroplast outer envelope and thus transports precursor proteins in the opposite direction than Sam50/Omp85 (ref. 50). However, chloroplasts contain a further isoform, Toc75-V, which is more closely related in sequence to the outer-membrane protein SynToc75 of the chloroplastic ancestors cyanobacteria, to bacterial Omp85 and to mitochondrial Sam50 (refs. 39,51). Toc75-V is not present in the isolated TOC import complex of chloroplasts. It may thus represent the equivalent of Sam50 in a putative protein sorting and assembly machinery of the chloroplast outer envelope.

Connecting protein assembly and mitochondrial morphology

The SAM complex revealed an unexpected connection between two fields that had thus far been regarded as different areas: protein assembly and mitochondrial morphology. Upon purification of the SAM complex, the β-barrel protein Mdm10 was found as the fourth subunit[52]. The 56-kDa protein Mdm10 had been previously identified by its role in maintaining mitochondrial distribution and morphology[53]. Mdm10 associates with the 200-kDa SAM core complex to form a larger complex of 350 kDa (ref. 52).

Whereas the three subunits of the SAM core complex, Sam35, Sam37 and Sam50, are generally required for the biogenesis of β-barrel proteins of the outer membrane, Mdm10 plays a specific role in the assembly of the TOM complex (**Fig. 2**). Mdm10 is required for the late stages of Tom40 assembly. After insertion of the Tom40 precursor into the outer membrane by the SAM core complex, the precursor stably associates with the small protein Tom5. Then the other Tom subunits, in particular Tom6, Tom7 and the central receptor Tom22, bind to Tom40 in a sequential manner that depends on the presence of Mdm10 (ref. 52). Mdm10 seems to function as a scaffold where the late steps of assembly of the multisubunit TOM complex take place. Because Tom22 and at least some of the small Tom proteins do not form β-barrel structures but contain α-helical transmembrane segments[16,24], Mdm10 broadens the substrate spectrum of the large SAM complex so that the β-barrel protein Tom40 can interact with α-helical proteins[52].

Yeast cells lacking Mdm10 show defects in both assembly of the TOM complex and mitochondrial morphology[52,53]. This raises the question of whether the primary function of Mdm10 is protein assembly or maintenance of mitochondrial morphology. Whereas the mitochondria of wild-type yeast cells form a tubular network, cells lacking Mdm10 contain large, spherical mitochondria[53]. Yeast mutants of the TOM complex or of subunits of the SAM core complex have similar aberrant mitochondrial shapes; in other words, the morphology defects are not specific for Mdm10 alone but are found with various mutant mitochondria that are defective in assembly of the TOM complex. Thus, the alteration of mitochondrial shape is probably a secondary effect of the loss of Mdm10 because defects in TOM assembly will lead to impaired import of many outer-membrane proteins, including proteins directly required for maintaining mitochondrial morphology. It is also conceivable that Mdm10 plays a dual role—that is, functions in the protein assembly machinery[52] and in the machinery controlling mitochondrial distribution and morphology[54].

Conclusions and perspectives

The identification and characterization of the SAM pathway led to several surprising twists in the analysis of organelle biogenesis. Pleiotropic effects and seemingly unrelated functions of mitochondrial proteins such as Sam37 and Mdm10 can now be explained by a central role in membrane protein assembly. The conservation of Sam50 from bacteria to man provides important insight into the evolution of the mitochondrial outer membrane and the development of its protein biogenesis system. A protein transport system of the Sam50/Omp85-type was already present in the mitochondrial ancestor while the TOM complex was developed in the eukaryotic cell. The Sam50 system may thus have carried out general functions in protein import and export in the beginning of mitochondrial development. Chloroplasts contain two Omp85-type proteins, the protein import channel Toc75-III and the more bacterial-like isoform Toc75-V[50,51]. It will be interesting to see whether Toc75-V is required for the integration of β-barrel proteins into the chloroplast outer envelope.

The identification of a novel protein-sorting pathway raises important questions that will be the subject of future research. The driving forces for protein translocation and insertion at the outer membrane are largely unknown. The ATP-dependence of the import of porin is probably due to the release of the precursor from cytosolic chaperones[55] whereas the energetics of translocation via TOM complex and SAM complex have not been addressed so far. Different models are possible to explain how the SAM complex promotes the membrane integration of β-barrel proteins. A channel formed by Sam50 may mediate the insertion of precursor proteins and then release them laterally into the lipid phase of the membrane. However, Sam50 is a β-barrel protein itself and the lateral opening of a channel formed by β-strands would be energetically unfavorable because several hydrogen bonds must be broken, whereas the lateral release of α-helices by α-helical translocases (such as in the mitochondrial inner membrane or the endoplasmic reticulum) mainly depends on the dissociation of van der Waals interactions[17,18]. It is conceivable that other subunits of the SAM complex like Sam35 and Sam37 are crucial in regulating the function and opening of Sam50 in an energy-dependent manner. Alternatively, the SAM complex may function in a scaffold-like manner to facilitate the insertion of proteins at the protein-lipid interface[39]. Sam50 may serve additional functions and, for example, participate in formation of the still elusive mitochondrial channel that is responsible for release of intermembrane-space proteins during apoptosis[4,9,38]. Interestingly, Sam37 and Sam35 of yeast mitochondria are related to the mammalian mitochondrial outer-membrane proteins metaxin 1 and metaxin 2, respectively[40,56]. The two metaxins have sequence similarity and interact with each other[56]. Metaxin 1 was reported to be required for tumor necrosis factor–induced cell death[57], leading to the speculation that the SAM complex may be involved in apoptosis[40,58].

What is the folding state of the precursor proteins during TOM translocation and transfer through the intermembrane space? Possibly part of the β-barrel domain is already preformed[21,32] and the precursor polypeptide chain is inserted into the outer membrane in a block-wise manner. The available evidence indicates that the precursor proteins are still associated with the outer membrane when they are transferred from TOM to SAM. It is unclear whether further intermembrane space factors, chaperones or outer-membrane components are necessary for transfer from TOM to SAM, and which role is played by the Sam50 domain that is located in the intermembrane space.

The surprising connection between protein assembly and mitochondrial morphology raises the possibility that the presence of Mdm10 in a mitochondrial assembly machinery is just the tip of an iceberg. Genetic and cell biological screens have revealed a large number of proteins that are directly or indirectly involved in the maintenance of mitochondrial morphology[59,60]. We speculate that other so-called morphology components may also play a role in the assembly of mitochondrial protein complexes.

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COMPETING INTERESTS STATEMENT

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