Trends in Biochemical Sciences

CellPress REVIEWS

Review

Cytosolic Events in the Biogenesis of Mitochondrial Proteins

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While targeting of proteins synthesized in the cytosol to any organelle is complex, mitochondria present the most challenging of destinations. First, import of nuclear-encoded proteins needs to be balanced with production of mitochondrial-encoded ones. Moreover, as mitochondria are divided into distinct subdomains, their proteins harbor a number of different targeting signals and biophysical properties. While translocation into the mitochondrial membranes has been well studied, the cytosolic steps of protein import remain poorly understood. Here, we review current knowledge on mRNA and protein targeting to mitochondria, as well as recent advances in our understanding of the cellular programs that respond to accumulation of mitochondrial precursor proteins in the cytosol, thus linking defects in targeting-capacity to signaling.

Highlights

Mitochondrial proteins synthetized in the cytosol can be targeted to mitochondria at different stages of gene expression: as mRNAs, ribosome-nascent chain complexes, or complete precursor proteins.

While almost all proteins use the same entry gate to the mitochondria, before and after it they can embark on different targeting and import pathways.

Delays in mitochondrial protein import or mistargeting to other organelles affect cellular homeostasis; hence, cells have evolved specific mechanisms to sense and counteract such situations.

Cytosolic chaperones promote mitochondrial protein import under normal conditions, as well as play a major role in stress response pathways associated with mitochondrial protein import defects.

A Two-Billion Year Relationship

The eukaryotic cell emerged following an integration between an archaeon and its bacterial symbiont. The host cell and the symbiont, which became a mitochondrion, have coevolved for so long that they are completely dependent on each other [1]. On one hand, modern cells rely on mitochondria for efficient production of ATP and vital cofactors, such as iron-sulfur clusters and heme. On the other hand, mitochondria have given up their autonomy for production of lipids and proteins to their host cells. This interdependence creates a constant crosstalk between mitochondria, the cytosol, and all other organelles through the flux of metabolites, involvement in common signaling pathways, and transport of newly synthetized proteins destined to mitochondria, herein termed 'precursor proteins', or 'precursors' [2].

Importing around 1000 different proteins into various mitochondrial locations is one of the most challenging tasks. The first step of **targeting** (see Glossary) involves the recognition and directional movement of the protein (or its encoding mRNA) from the cytosol to the surface of the correct organelle. Next, the protein can be handed over to the machinery responsible for its integration into the membranes or passage into the aqueous compartments of the organelle by **translocation** through protein channels or pores. The entire process of both targeting and translocation to mitochondria constitute mitochondrial protein import. The proteins that need to be imported to different compartments contain specific targeting signals that are recognized by both the targeting and translocation machineries (Box 1).

Protein translocation into mitochondria is well studied and it is clear that many different pathways exist to assist the correct translocation and integration of proteins to their mitochondrial subcompartments. All of them start at the **translocase of the outer membrane (TOM)** complex. The TOM complex consists of the β-barrel protein Tom40 that forms a translocation pore, receptor proteins Tom20 and Tom70 that recognize targeting signals on the precursors, Tom22 that can recognize precursors and also plays a structural role, and the small subunits Tom5, 6, and 7 that mediate complex assembly [3–7]. Since intramitochondrial routes have been reviewed extensively [8–16], they will not be addressed here.

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By contrast to translocation, targeting steps occurring in the cytosol cannot be studied as easily and hence have been less investigated and understood. Moreover, studies using gene deletion or downregulation were not successful in deciphering these steps, potentially due to redundancy of targeting factors [17]. Until now, few insights have been gleaned regarding targeting events of mitochondrial proteins while conceptually they can, and probably do, happen at multiple different stages of gene expression: before translation, during translation, and after translation (Figure 1). Yet, to date, it is still not clear for most mitochondrial proteins whether their targeting signal can be recognized directly by a receptor on mitochondria or whether they require a dedicated targeting factor. Moreover, dedicated targeting factors, such as the **signal recognition particle (SRP)** of the secretory pathway, have not been identified for mitochondrial proteins.

What is emerging clearly though is that cells have evolved a variety of quality control mechanisms that survey the protein on its way to mitochondria and activate various signaling and stress responses to deal with accumulation of precursor proteins in the cytosol. With new available biochemical, cell biological, and genetic tools, we believe that the time is ripe to revisit some of the major open questions in the field of mitochondrial protein targeting with the aim of reaching a detailed mechanistic understanding of these processes, at a level similar to that obtained for translocation.

mRNA Targeting

A path of a protein to its destination organelle can start even before translation by localization of its encoding mRNA. Usually, such mRNA targeting is mediated by a special sequence located in its

Box 1. Import Signals of Mitochondrial Precursors

Two bounding membranes of mitochondria create a variety of possible topologies for imported proteins (Figure I).

Soluble Matrix Proteins

These constitute ~50% of the mitochondrial proteome [146], with the majority harboring an N terminal cleavable mitochondrial targeting sequence (MTS; shown in red in Figure I). The MTS (10–100 amino acids long, positively charged peptide with a propensity to form an amphipathic α-helix [117,147]) is necessary and sufficient to direct proteins to the matrix. In addition to the MTS, some matrix proteins can contain internal MTS-like sequences (iMTSLs) that can bind OM receptors and help to keep precursors in an import-competent state [136]. However, a number of matrix proteins do not have a cleavable MTS and the signals they possess are poorly studied [148,149]. Furthermore, some MTS-containing proteins can be translocated into mitochondria without their MTS [24].

Inner Membrane (IM) Proteins

These consist of three groups: (i) mitochondrially encoded proteins synthesized on mitochondrial ribosomes that are inserted from the matrix side (not shown); (ii) metabolite carrier family proteins (shown in green) with a characteristic topology consisting of three modules (numbered 1–3), each of which is a hairpin with two TMDs and a matrix-exposed loop (each of these modules can contain the targeting information and the three modules cooperate to ensure efficient targeting [150]); and (iii) other proteins with various topologies that usually contain an MTS (red).

Intermembrane Space (IMS) Proteins

There are about 100 different proteins in the IMS, many of which are rather small (8–25 kDa) and contain conserved cysteine motifs that allow oxidative folding (shown in orange). Mia40 is a conserved oxidoreductase in the IMS that mediates the folding of these proteins and therefore their retention in the IMS [151]. Larger IMS proteins are usually made as precursors, with bipartite signals that consist of an N terminal MTS (red) followed by a TMD (cyan). Following arrest in the IM, these proteins (yellow-green) are cleaved C terminally of the TMD and released into the IMS [152].

Outer Membrane (OM) Proteins

Integral proteins of the OM can be embedded in different orientations and have a variable number of transmembrane α-helices or β-strands (shown in blue). β-Barrel proteins in eukaryotes can be found only in mitochondrial or chloroplast OMs (light blue). The signals that direct α-helical proteins to the OM are usually associated with the TMD (dark blue) and its flanking regions, but some of them can also contain a special N terminal targeting sequence [11,153]. β-Barrel proteins are targeted via a hydrophobic β-hairpin structural element [122].

Glossary

Chaperones: proteins that interact with unfolded or partially folded polypeptides to prevent their aggregation, assist their folding, and promote degradation in case of folding failure.
ER surface-mediated protein targeting: a pathway that captures unfolded mitochondrial precursor proteins on the ER surface and promotes their targeting to mitochondria.
J-domain: a structurally conserved helical domain that defines the family of heat shock proteins of 40 kDa (all related to bacterial DnaJ and also called Hsp40s, or J-proteins).
Metabolite carriers: a family of mitochondrial inner membrane proteins with conserved topology responsible for transfer of small molecules across this membrane.
Mitochondrial targeting sequence (MTS): an N terminal, positively charged, extension that has the potential to form amphiphilic helix and acts as a mitochondrial import sequence for many mitochondrial proteins. The MTS is mostly cleaved after import by a dedicated peptidase.
Nascent chain associated complex (NAC): a heterodimeric complex that is the first to interact with the nascent polypeptide upon exit from the ribosome tunnel and to protect this chain from aggregation.
Ribosome-nascent chain complex (RNC): a complex of an mRNA, translating ribosome and a polypeptide chain in the process of synthesis.
Ribosome profiling: an experimental technique used to map the distribution of translating ribosomes along mRNAs *in vivo*.
Signal recognition particle (SRP): a ribonucleoprotein complex responsible for the recognition of some ER import signals and targeting of such proteins to the ER.
Targeting: cytosolic steps of protein delivery to the destination organelle.
Translocase of the outer membrane (TOM): a complex in the mitochondrial outer membrane that recognizes mitochondrial precursor proteins and mediates their translocation through the outer membrane.
Translocation: the act of transport of a protein across a cellular membrane.
Ubiquilins: a family of proteins that can bind ubiquitinylated proteins via their ubiquitin-associated domain and

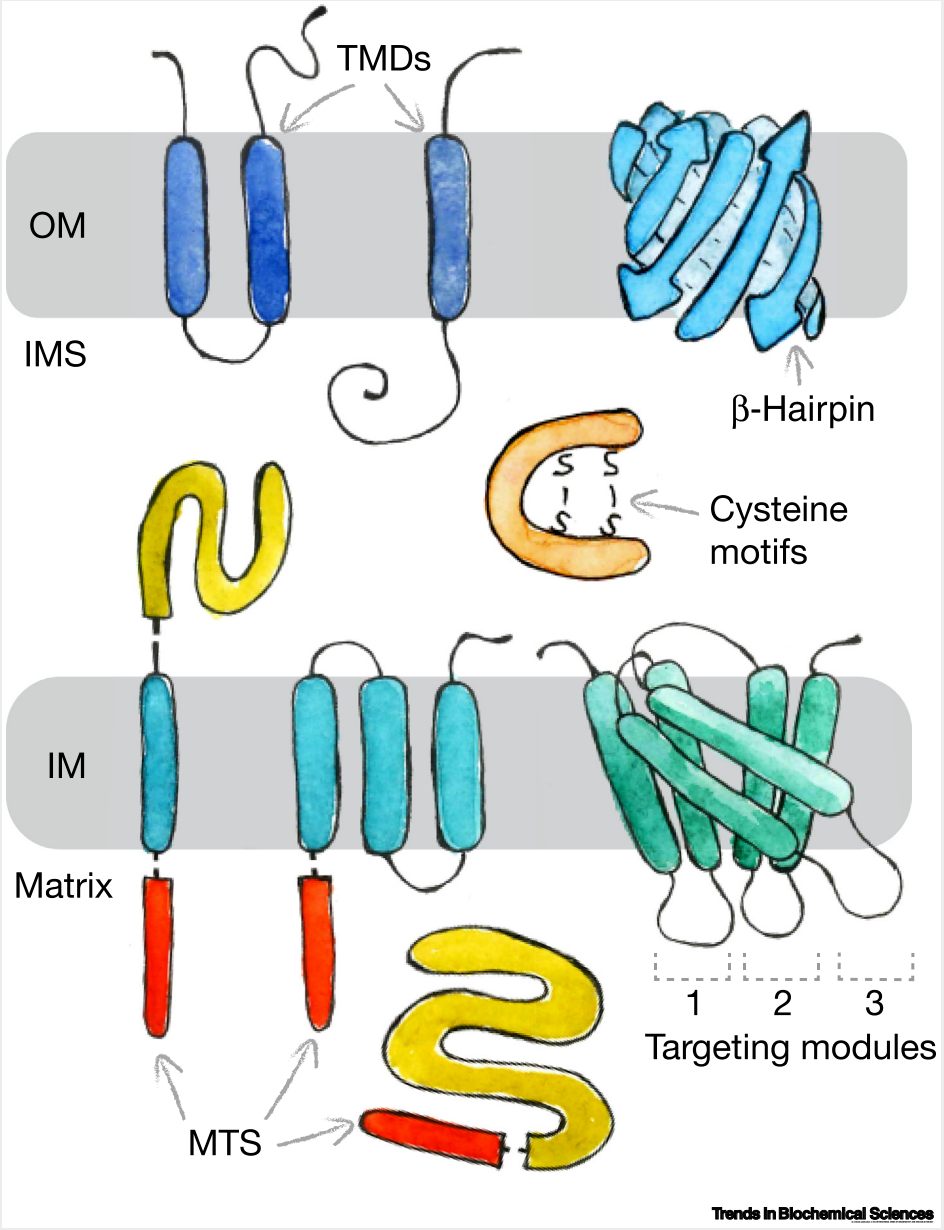

Figure I. Mitochondrial Proteins Harbor a Diversity of Signals That Allow Them to Be Sorted to the Correct Mitochondrial Subcompartment. (See box text for details). Abbreviations: IM, inner membrane; IMS, intermembrane space; MTS, mitochondrial targeting sequence; OM, outer membrane; TMD, transmembrane domain.

additionally bind the proteasome via a ubiquitin-like domain, thus delivering ubiquitinylated proteins for degradation.

Untranslated regions (UTRs): regions of an mRNA before the start codon (5′-UTR) and after the stop codon (3′-UTR).

untranslated regions (UTRs). Several mRNAs were directly shown to use this mechanism for mitochondrial targeting in *Saccharomyces cerevisiae* (from hereon called yeast) [18–22].

In yeast, targeting of mRNAs via their 3′- or 5′-UTRs is probably not absolutely required for the mitochondrial targeting of the encoded protein because generic 5′- and 3′-UTRs used in yeast whole-genomic libraries do not systematically perturb protein localization to mitochondria [23,24]. Still, in yeast, there is a well-studied RNA-binding protein that is associated with

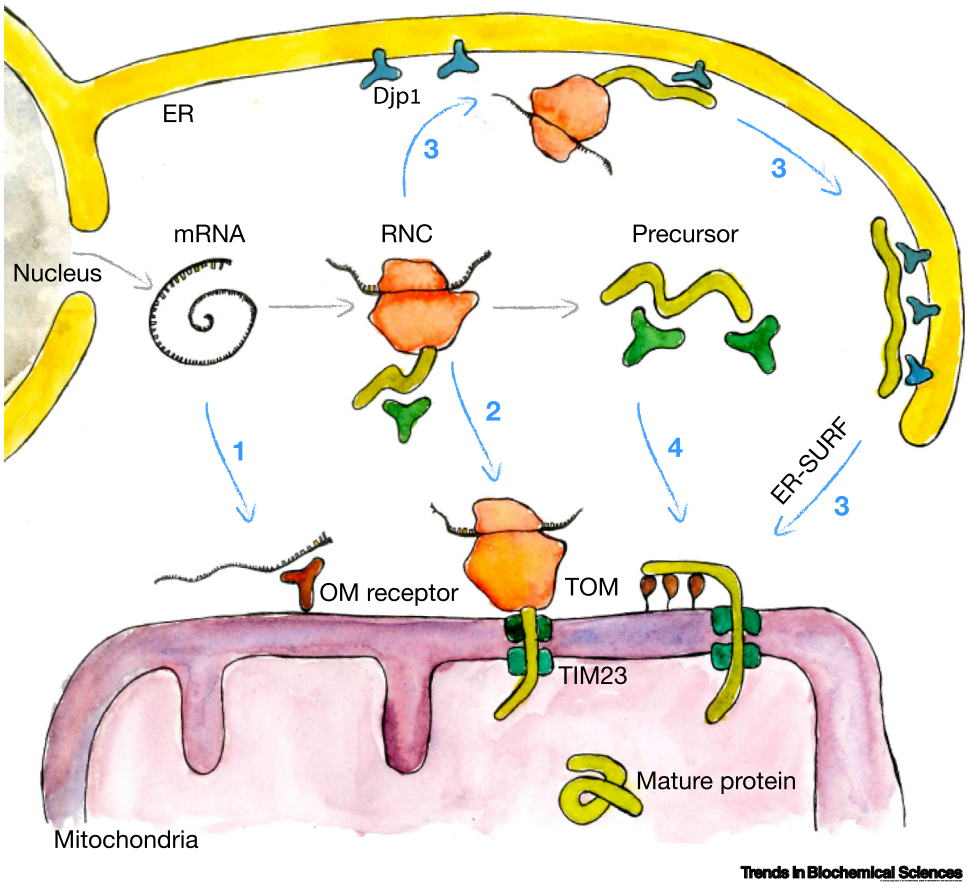

Figure 1. Targeting to Mitochondria Can Occur Pre-, Co-, or Post-Translationally. After mRNAs exit the nucleus, they can be targeted to the mitochondrial surface via RNA-binding proteins (pathway 1). Whether translation of such mRNAs can be specifically initiated on the mitochondrial surface remains unknown. If translation is initiated in the cytosol, ribosome-nascent chain complexes (RNCs) can be targeted to the outer membrane (OM) via targeting sequences (pathway 2). Alternatively, some of the RNCs can be targeted to the endoplasmic reticulum (ER) (pathway 3), complete translation in its proximity, and the precursor can be transferred to mitochondria via the ER surface-mediated protein targeting (ER-SURF) pathway. The substrate range of ER-SURF remains to be determined. If the precursor synthesis was completed in the cytosol, cytosolic factors can bind the precursor, keep it in an unfolded, import-competent, state, and facilitate its transfer to mitochondrial receptor proteins (pathway 4). Abbreviations: TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

mitochondria, Pumilio-homology domain family protein 3 (Puf3). Puf3 specifically binds mRNAs encoding proteins with mitochondria-related functions and modulates mitochondrial recruitment of some of them by binding a 3′-UTR motif [22,25–27]. It was therefore suggested that Puf3 promotes the targeting of mitochondrial mRNAs to the mitochondrial surface for proximal translation. Indeed, a case study of Cox17 [28,29] showed that its 3′-UTR confers localization to mitochondria in a Puf3-dependent way [18].

While the above seems to suggest a role for Puf3 in mRNA and therefore protein targeting, there is still a debate regarding its role. Most studies report that Puf3 is localized to the cytosol [23–25,30]. Only one work reported that Puf3 colocalizes with mitochondria where it can bind Mdm12 and Arp2 [31]. This finding pointed to a possible connection of mitochondrial protein-coding RNA regulation to mitochondria–endoplasmic reticulum (ER) contact sites and motility. Both deletion and overexpression of Puf3 do not significantly affect yeast growth on respiratory

media, as would have been expected for an important factor affecting mitochondrial protein import, but do cause slower growth on this media at elevated temperatures [31]. The phosphorylation state and stability of Puf3 is modulated by carbon source availability [31–33]. Maybe the most intriguing is the observation that upon *PUF3* deletion, when the mitochondrial association of *COX17* mRNA is lost, protein translocation is not affected but rather, stabilization of the mRNA occurs [18]. Moreover, the PUF domain alone is sufficient to promote *COX17* mRNA degradation, suggesting a role for mRNA turnover regulation rather than targeting for this specific mRNA.

But what about all other mitochondrial proteins and their transcripts? Whole-genome studies in yeast revealed different effects of Puf3 deletion on mRNA abundance, translation efficiency, and mitochondrial proteome abundance, highlighting its complex regulatory role [26,34,35]. Hence, to date, there is no unequivocal evidence that Puf3-mediated mRNA-localization is a part of the mitochondrial protein targeting process. Instead, Puf3 might help to regulate mRNA stability and translation and thus contribute to coordination of protein synthesis on cytosolic and mitochondrial ribosomes during the assembly of the oxidative phosphorylation (OXPHOS) components. Puf3 is probably not the only factor that can mediate UTR-dependent mRNA targeting to mitochondria but others have not yet been identified and studied.

What happens in mammals? A recent RNA-sequencing study using proximity labeling of RNAs with an engineered efficient alkaline peroxidase revealed that, in mammalian cells, there are many mRNAs localized to the mitochondrial outer membrane (OM) and no distinct class of proteins is enriched among them. Part of the RNAs are localized to mitochondria in a microtubule-dependent manner, some are translation-dependent (see later), and others are recruited independently of their translation, via their 3′-UTRs [36]. Hence, the localization of these mRNAs likely depends on RNA-binding proteins. Interestingly, this latter class of mRNAs was enriched with those encoding mitochondrial ribosome and OXPHOS components that require coordinated import and assembly [36]. However, the RNA-binding proteins mediating this recruitment have not yet been identified. Moreover, mRNA recruitment to mitochondria is not necessarily directly linked to translation since **ribosome profiling** experiments in yeast did not find the same RNAs that were localized to mitochondria using microarrays and fluorescent *in situ* hybridization [22,37,38].

Cotranslational Targeting

It is not clear whether translation of mRNAs targeted to mitochondria can also be initiated on the mitochondrial surface. Cotranslational protein targeting to mitochondria was first suggested since mitochondria-bound ribosomes were found translating RNAs of mitochondrial precursor proteins [39–42]. Later, this concept was expanded by microarray-hybridization studies of mRNAs associated with purified mitochondria [26,43], some of which depended on translation of the precursor protein. Interestingly, it appears that the nuclear-encoded precursor proteins translated from the mitochondria-bound mRNAs were mostly of bacterial origin and, in many cases, constituted large protein complex assembly factors, while structural subunits of these complexes encoded in the nuclear genomes were translated on cytosolic ribosomes [20,26,43]. By contrast to these microarray-based experiments, proximity-specific ribosome profiling revealed that, of all mitochondrial proteins, inner membrane (IM) proteins had the largest portion that was translated on membrane-proximal ribosomes with their exit tunnels oriented towards the OM. Thus, it seems that cotranslational import might be one of the mechanisms to promote biogenesis of hydrophobic membrane proteins [38].

Since the **mitochondrial targeting sequence (MTS)** is usually located at the N terminus of a translated protein and is synthetized first, it might engage with the receptors of the mitochondrial OM while the nascent chain is still attached to the ribosome, a structure which is termed a

Ribosome-Nascent Chain Complex (RNC). Indeed, localization of many mRNAs to mitochondria was shown to depend on their translation and the presence of the Tom20 receptor that binds MTSs [22,44]. If a transcript is very long or translation is slowed down by rare codons, RNCs that already synthetized an MTS may have time to associate with mitochondria [45]. However, it was proposed that some additional factors may specifically recruit RNCs to the mitochondrial OMs and act via binding the ribosome itself [46,47]. One such factor was identified as the **nascent chain associated complex (NAC)**.

NAC is one of the ribosome-associated protein biogenesis factors and is a heterodimer of α and β subunits [48,49]. Both α- and β-NAC bind the ribosome next to the exit tunnel [50]. Yeast have one α NAC (Egd2) and two alternative β-subunits (Egd1 and Btt1). Different subunits tend to interact with different substrate chains and show preference for proteins with certain functions or physico-chemical properties. Interestingly, the poorly expressed β-NAC Btt1 tends to bind nascent chains of mitochondrial precursor proteins [51]. NAC was suggested to promote cotranslational targeting to mitochondria acting via two possible mechanisms [52,53]. First, as was shown *in vitro* and *in vivo* in *Caenorhabditis elegans*, it promotes specificity by antagonizing unspecific targeting of mitochondrial proteins to the ER; it binds the ribosome next to the exit tunnel and prevents RNCs without a proper signal peptide to bind SRP or the ER translocon [50,54–56]. Second, for yeast cells, it has been suggested that NAC itself can recruit RNCs to mitochondria by binding the OM protein Om14 [57,58].

Different mechanisms for cotranslational translocation might have emerged in different organisms and probably depend on the relation between translation speed, import rate, and mitochondrial surface area. For example, a yeast cell has around 10^4 import sites on the OM and it needs to import proteins of total length of 5×10^9 amino acids to double the mitochondrial mass [59]. If all import proceeded cotranslationally (i.e., with the speed of translation; two to six amino acids per second) then the time required would be more than 10 hours, while in reality the yeast cell cycle takes less than 2 hours. This calculation suggests that the majority of the yeast mitochondrial proteome has to be imported post-translationally and might explain why the role of NAC for ensuring cotranslational import is not so vital in yeast compared with *C. elegans* or mammalian cells where these ratios can be different.

Based on the calculations above, it is reasonable to suggest that, in yeast, cotranslational import of the majority of the mitochondrial proteins must be specifically inhibited until the end of translation to reduce clogging of the import sites formed by the TOM complex. These preventive mechanisms of discrimination between co- and post-translational import under normal conditions are yet to be uncovered in yeast. However, there are several mechanisms that cells employ to either relieve clogging once it occurs or to deal with ensuing accumulation of precursor proteins in the cytosol (Box 2).

Additionally, in yeast, two constitutive mechanisms survey the translocons themselves and were termed the mitochondrial protein translocation-associated degradation (mitoTAD) and Vms1 pathways. The mitoTAD pathway relies on Ubx2, a protein with a function in ER-associated degradation. A pool of Ubx2 is localized to mitochondria and binds the TOM complex to recruit the Cdc48 ATPase that extracts stuck precursor proteins from the translocon [60]. Vms1 acts in case a clogged precursor is still attached to a stalled ribosome. The Vms1 pathway is required because the normal cytosolic ribosome quality control system cannot cope with RNCs occupied by stalled mitochondrial precursor proteins. Due to the presence of an MTS, nascent chains in such RNCs start translocation and are not available for extraction by the cytosolic ATPase Cdc48. Vms1 recognizes such ribosomes on the mitochondrial surface and releases the nascent chains to the mitochondrial matrix where they can be degraded [61,62].

A similar phenomenon linking mitochondrial dysfunction and translation quality control was uncovered in *Drosophila* and termed mitochondrial stress-induced translational termination impairment and protein carboxyl terminal extension (MISTERMINATE). In a model of Parkinson's disease, a deletion of PINK1, a gene involved in mitophagy (see [63] for review), caused mitochondrial dysfunction that lead to abnormal translation termination and ribosome stalling on the OM linked to the action of the cytosolic ribosome quality control system [64]. This effect was recapitulated in mammalian cell culture and could be suppressed by overexpression of ANZKF1, a homolog of yeast Vms1.

Post-Translational Targeting

Proteins that are targeted and translocated post-translationally spend some time as a pool of cytosolic precursors. Like other newly synthetized unfolded proteins, they interact with cytosolic **chaperones**. Among other factors, three heat shock protein (HSP) families have an important role in targeting and translocation of mitochondrial precursor proteins: chaperones of the Hsp40, Hsp70, and Hsp90 families (HSP of 40, 70, or 90 kDa, respectively) (Figure 2).

Cytosolic chaperones have been shown to promote mitochondrial protein import in three main ways [15]. First, as per their role, chaperones can keep precursors in an unfolded, import-competent state. Second, the chaperones can dock to mitochondrial receptors helping to transfer precursors to the TOM complex. Finally, chaperones can promote degradation of mitochondrial proteins that failed to translocate (Figure 2). This latter function is critical in case of abnormal cytosolic accumulation of mitochondrial precursor proteins (Box 2).

Box 2. The Response to Precursor Accumulation in the Cytosol

It is hypothesized that mitochondrial precursors spend only a short time in the cytosol, however, this may depend on the strength of their targeting signals. It was also shown that the accumulation of precursors in the cytosol is detrimental to cells and is associated with many disease conditions (reviewed in [154]).

Recent studies identified intracellular signaling networks that negate the accumulation of precursor proteins (Figure I; shown in yellow-green) or mitigate their detrimental effects [155]. Although a number of different acronyms were coined for these responses, such as mitochondrial unfolded protein response (UPR^{mt}) [156], mitochondrial compromised import response (mitoCPR) [157], unfolded protein response activated by mistargeting of proteins (UPRam) [158], and mitochondrial protein-induced stress (mPOS) [159], all these observed reactions may be aspects of one overarching cellular reaction. In yeast, these responses have been shown to consist of two branches: a cytosolic UPR through heat shock factor 1 (Hsf1; red arrows) that predominantly increases the levels and activities of chaperones (dark green) and the ubiquitin-proteasome system (UPRam, mPOS; green arrows) and the repression of the OXPHOS genes (UPR^{mt}; blue arrow) (probably through repression of the transcription factors Hap2/3/4/5 complex in yeast [160,161] or the ATFS-1/ATF4 function in animals [156]).

The Hsf1-mediated pathway elicits a number of processes. First, it induces chaperones and slows down translation. Hsf1 also triggers proteolysis via the transcription factor Rpn4 that controls proteasome abundance (this part of the pathway was described as UPRam [158]). In turn, Rpn4, via the transcription factor Pdr3, activates another branch of the response previously described as mitoCPR [157]. Pdr3 induces the expression of Cis1 that recruits the ATP-dependent dislocase Msp1 to the TOM complex [157]. Msp1 is an AAA-family protein that, under normal conditions, detects unassembled membrane proteins, including mislocalized endoplasmic reticulum- or peroxisome-targeted TA-proteins on the mitochondrial outer membrane and pulls them out for degradation or retargeting [162,163]. In the case of mitoCPR, Msp1 was hypothesized to clear clogged precursors from the TOM complex or to unfold the precursors and give them another chance to translocate [157].

Connections between mitochondrial and cytosolic proteostasis were also uncovered in *C. elegans*. Mitochondrial proteostasis in *C. elegans* is guarded by a UPR^{mt} pathway. It is triggered by the transcription factor ATFS-1, which has both an MTS and a nuclear localization sequence [156]. When mitochondrial function is disrupted, membrane potential drops and the relatively weak MTS of ATFS-1 is unable to stimulate efficient translocation to mitochondria, leading to nuclear localization where it activates expression of mitochondrial chaperones [164].

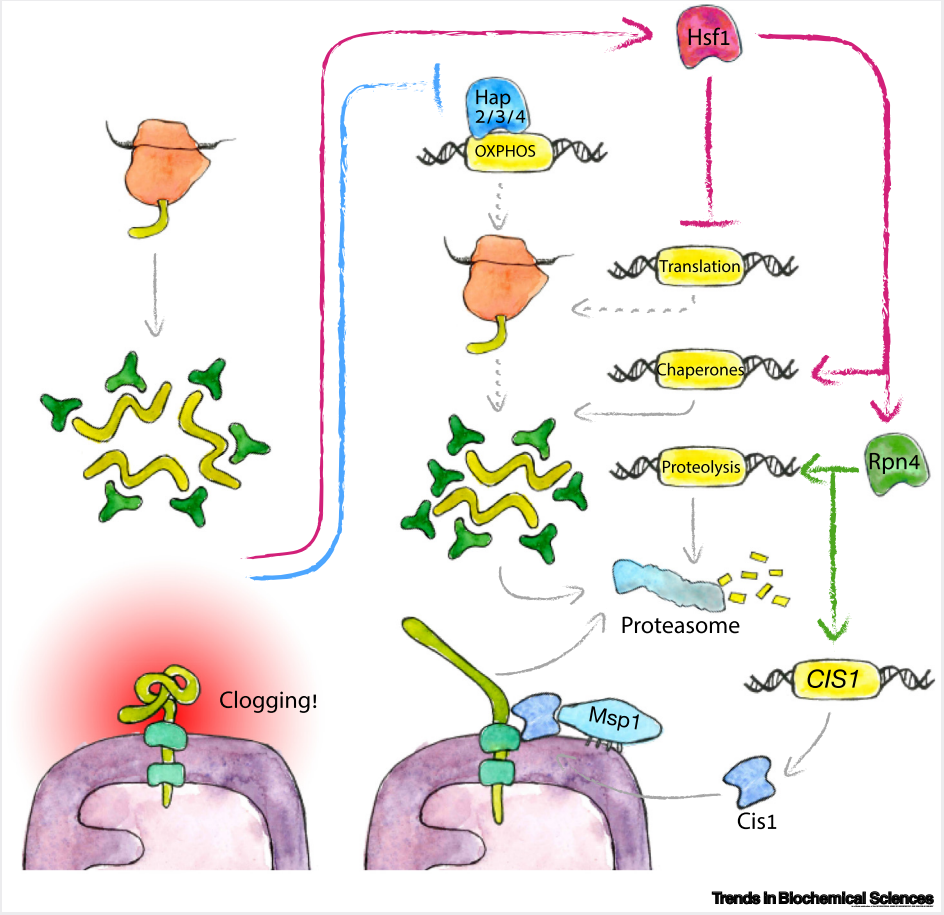

Figure I. Precursor Accumulation Triggers Cellular Responses That Coordinate Protein Synthesis, Targeting, Import, and Degradation to Optimize Mitochondrial Function and Protect the Cytosol. (See box text for details). Abbreviations: Hsf1, heat shock factor 1; OXPHOS, oxidative phosphorylation.

The Role of Hsp70 Chaperones in Targeting

In vitro, Hsp70 proteins are required to be present during precursor protein synthesis and were hypothesized to keep precursors in a soluble, unfolded state for translocation through the TOM and the translocase of the inner membrane complexes [65–67]. Indeed, depletion of two yeast Hsp70s, Ssa1 and Ssa2, leads to accumulation of unimported precursors in the cytosol [68,69]. This outcome could result from reduction in either import of proteins to mitochondria or their degradation in the cytosol; it was suggested that the MTS might be a signal that allows chaperones to discriminate between promoting substrate folding or keeping it unfolded [70,71].

Apart from keeping the precursors in an import-competent form, it was established that Hsp70s can directly dock onto the mitochondrial import receptor Tom70 in yeast and mammalian cells. The Hsp70 C terminal Glu-Glu-Val-Asp (EEVD) motif that is important for binding Hsp40s [72] also directly binds the tetratricopeptide (TPR) motifs of the Tom70 receptor and this binding is important for translocation of mitochondrial IM carrier and OM β-barrel proteins [67,73,74]. It is important to note that Hsp70s have broad substrate specificity and recognize cytosolic proteins as well as cargo of multiple other organelles. Hence, it is not clear what creates specificity for mitochondrial targeting. More selective binding might be determined

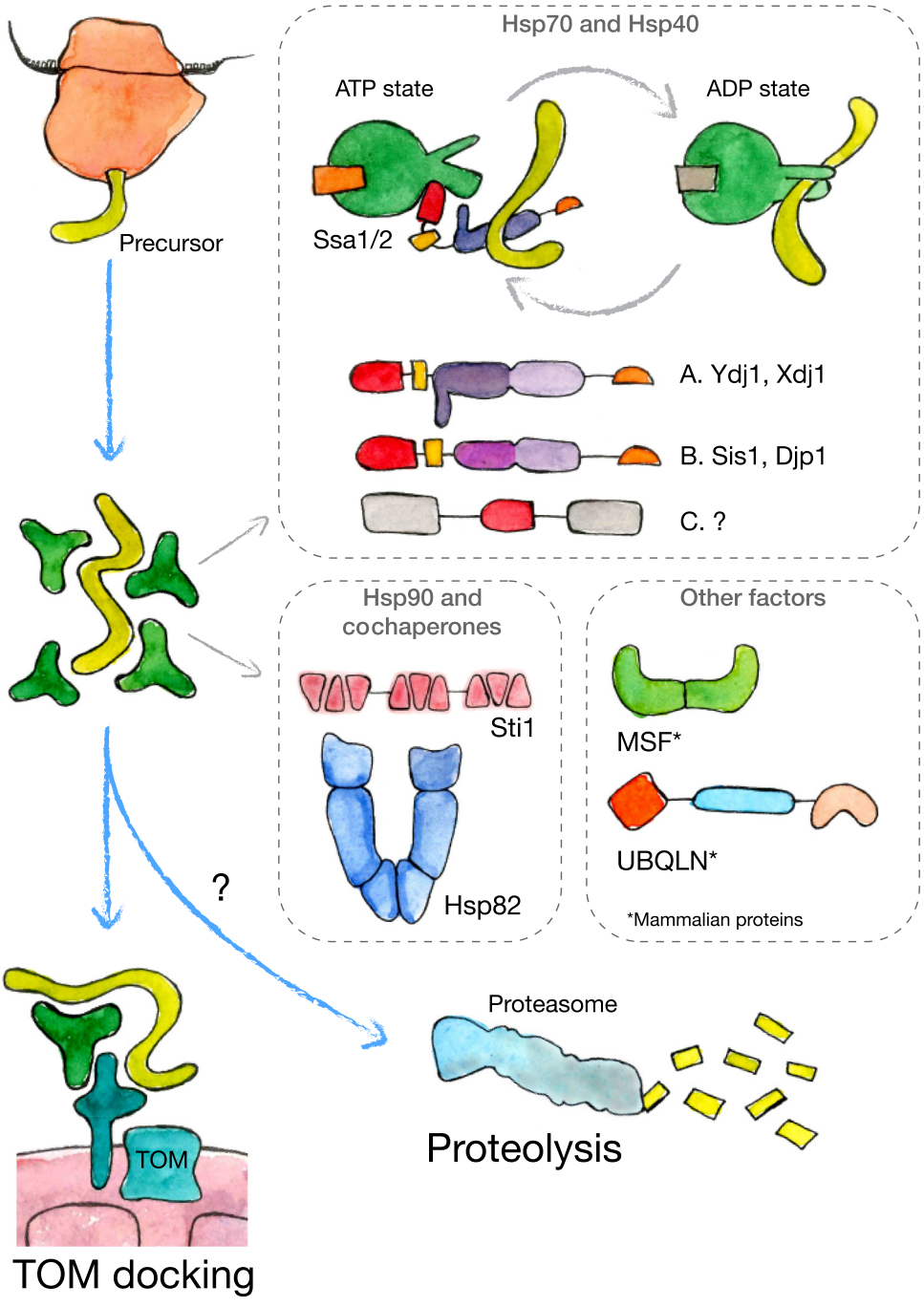

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Figure 2. Principles of Post-Translational Chaperone Function in Mitochondrial Precursor Protein Targeting. (Left) After the precursor (yellow-green) is released from the ribosome, several cytosolic factors (green) can keep it import-competent, facilitate its delivery to mitochondria by binding the outer membrane (OM) receptors (teal) and, potentially, promote proteasomal degradation of nontranslocated precursors. (Top right) Hsp70s (green) keep the precursors unfolded by cycling from a loosely bound ATP state to a tightly bound ADP state. Different structural classes of Hsp40s [A, B, and C; Hsp40 J-domain (red), Glu-Phe-rich domain (yellow), C terminal substrate-binding domain I with Zn-binding

(Figure legend continued at the bottom of the next page.)

by Hsp40s acting upstream of Hsp70. However, it is not clear whether Hsp70 and Hsp40 act consecutively or cooperatively during mitochondrial targeting.

The Role of Hsp40 Chaperones in Targeting

Hsp40s, also called J-proteins due to the presence of the common **J-domain**, act upstream of Hsp70s and are the main determinants of Hsp70 specificity by acting as modulators of their function. Hsp40s constitute a diverse group localized to different cellular compartments [75,76] and several have been implicated in mitochondrial targeting. A systematic study of the yeast cytosolic Hsp40s suggested that they can be divided into those with a more general function and those which might have evolved to perform more specific tasks (Figure 2) [77].

For example, Ydj1 is the most abundant DnaJ homolog in yeast. Ydj1 is farnesylated and localized to the cytosol, ER, and mitochondrial membranes [24,78–80]. *YDJ1* deletion causes a severe growth defect and affects both mitochondrial and ER targeting [77,81,82]. In the context of targeting, it was found to act together with Hsp70 [81–83] in enhancing *in vivo* import of ER-destined α-factor and aggregation-prone mitochondrial precursors such as Atp2 and the β-barrel protein porin [74,84]. Ydj1 could also bind *in vitro* translated β-barrel precursors and a synthetic β-hairpin peptide corresponding to the import signal for this type of protein [74]. In mammals, Ydj1 homologs DNAJA1, 2, and 4 were shown to assist mitochondrial import of matrix MTS-containing proteins *in vitro* and *in vivo* [85–87]. Hsp40s also form complexes with mitochondrial carrier precursors and Hsp70 and assist with the biogenesis of these precursors [85–88]. Coimmunoprecipitation with cytosolic domains of mitochondrial receptors revealed that Ydj1 prefers to bind Tom20 over Tom70, but the relevance of this interaction was not studied *in vivo* [89].

Xdj1, which is found only in Ascomycota, is structurally very similar to Ydj1, but an order of magnitude less abundant [78,90]. Xdj1 deletion causes respiratory growth reduction and TOM complex assembly defects [78,91]. Xdj1 is distributed between the cytosol and mitochondria where it might usher client proteins to Tom22 [91].

Sis1 might have a redundant role in protein targeting. Though it has a different domain composition compared with Ydj1 and Xdj1 [90] (Figure 2), its overexpression can still rescue Ydj1 deletion and, similar to the latter, it can also bind *in vitro* to Tom20 and precursors (β-barrel as well as matrix-destined) [74,81,89].

Another Hsp40, Djp1, was first described to play a role in the biogenesis of peroxisomes [92]. Recently, is was uncovered that Djp1 is a part of an unusual mitochondrial targeting pathway called **ER surface-mediated protein targeting** [93]. Hydrophobic precursors destined for the IM, such as Oxa1 and Coq2, can be captured by ER-localized Djp1 and later handed over to the TOM complex. In this mechanism, proteins on the ER surface are hypothesized to bind and chaperone hydrophobic precursors on their way to mitochondria. In support of this model, Djp1 was also found to be important for targeting of the mitochondrial transmembrane domains (TMD) containing OM protein Mim1 [74,94].

region (violet), C terminal substrate-binding domain I without Zn-binding region (magenta), C terminal substrate-binding domain II (light violet), dimerization domain (orange), variable domains (gray)] can deliver substrates to Hsp70s and thus define the specificity. Hsp40s of classes A and B were specifically implicated in facilitating targeting of mitochondrial proteins. No class C Hsp40s were found to be involved in targeting to date. (Bottom left) Hsp90s (Hsp82 and Hsc82 in yeast; blue) and their cochaperone Hsp70-Hsp90 organizing protein (HOP) (Sti1 in yeast; pink) can also bind different kinds of mitochondrial precursors and OM receptors. Mitochondrial import stimulation factor (MSF; green) and ubiquilins [bottom right; ubiquitin-like domain (red), medial substrate-binding domain (blue) and ubiquitin-associated domain (yellow)] do not belong to the HSP families and were studied in less detail. All protein names on the figure are from yeast, unless noted otherwise. Abbreviations: HSP, heat shock protein; TOM, translocase of the outer membrane.

The *in vivo* specificity of Hsp40s towards different mitochondrial precursors and how different types of chaperones coordinate binding and release of their substrates remains unknown.

The Role of Hsp90s and Their Cochaperones in Targeting

Hsp90s constitute another class of highly abundant chaperones found in all cell types. Unlike Hsp70s, Hsp90s do not recognize linear protein motifs but tend to bind proteins depending on their conformation or stability [95]. Around 20 cochaperones regulate Hsp90 function by regulating ATP hydrolysis, linking to the Hsp70 system, and delivering substrates. One of the most studied cochaperones is Hsp70-Hsp90 Organizing Protein (HOP). HOP can bind both Hsp70s and Hsp90s and facilitate substrate transfer from the former to the latter [96]. Hsp90s, too, can bind both cargo and OM receptors.

Yeast Hsp90 (called Hsp82 or Hsc82) associates *in vitro* with newly synthetized MTS-containing and hydrophobic precursors [67,87,88] including β-barrel proteins [74].

In vivo, murine Hsp90 binds to the precursor of adenine nucleotide carrier (ANT2) in a manner dependent on HOP [87].

Deletion of yeast HOP (named *STI1*) leads to fragmentation of mitochondrial network and synthetic growth phenotypes with deletions of OM import factors such as Tom20 and Mim1. Coimmunoprecipitation suggested that Sti1 can bind the Tom70 receptor [89]. Hsp90 itself can deliver hydrophobic precursors to the Tom70 receptor in mammalian mitochondria [73,97].

Additional Cytosolic Chaperones Involved in Mitochondrial Targeting

Several other cytosolic factors were implicated in mitochondrial precursors targeting. However, the precise function of these additional factors was not studied in detail and their role in targeting awaits further verification (Figure 2).

Ubiquilins

In mammalian cells, **ubiquilins** were identified as cytosolic interactors of the OM protein Omp25. Knockout of ubiquilins led to an accumulation of Omp25 precursors in the cytosol and to their reduced ubiquitination and degradation. Hence, it is not yet clear whether ubiquilins promote targeting, enable degradation of untargeted precursors, or both [98]. In mice, Ubiquilin 1 knockout caused B cell proliferation blockade as a result of cytosolic accumulation of mitochondrial precursors [99].

Mitochondria Import Stimulation Factor (MSF)

A heterodimer of 14-3-3 ζ/δ and ε was purified from rat cytosol based on affinity to the MTS of COX4 and named MSF [100,101]. In a homologous mammalian *in vitro* import system, MSF was shown to deliver precursor proteins to the Tom70 receptor [102,103]. The homologous yeast protein Bmh1, did not bind any common type of mitochondrial precursors [74]. So far, *in vivo* data to support a mitochondrial targeting role of MSF is still missing.

Aryl Hydrocarbon-Receptor Interacting Protein (AIP)

A two-hybrid screen for mammalian Tom20 interactors brought up AIP [104]. AIP is a Hsp90 interactor that consists of an N terminal peptidyl-prolyl cis/trans isomerase domain followed by several TPR repeats. In an *in vitro* system, AIP sustained precursors in an import-competent conformation and, *in vivo*, it promoted their import [104].

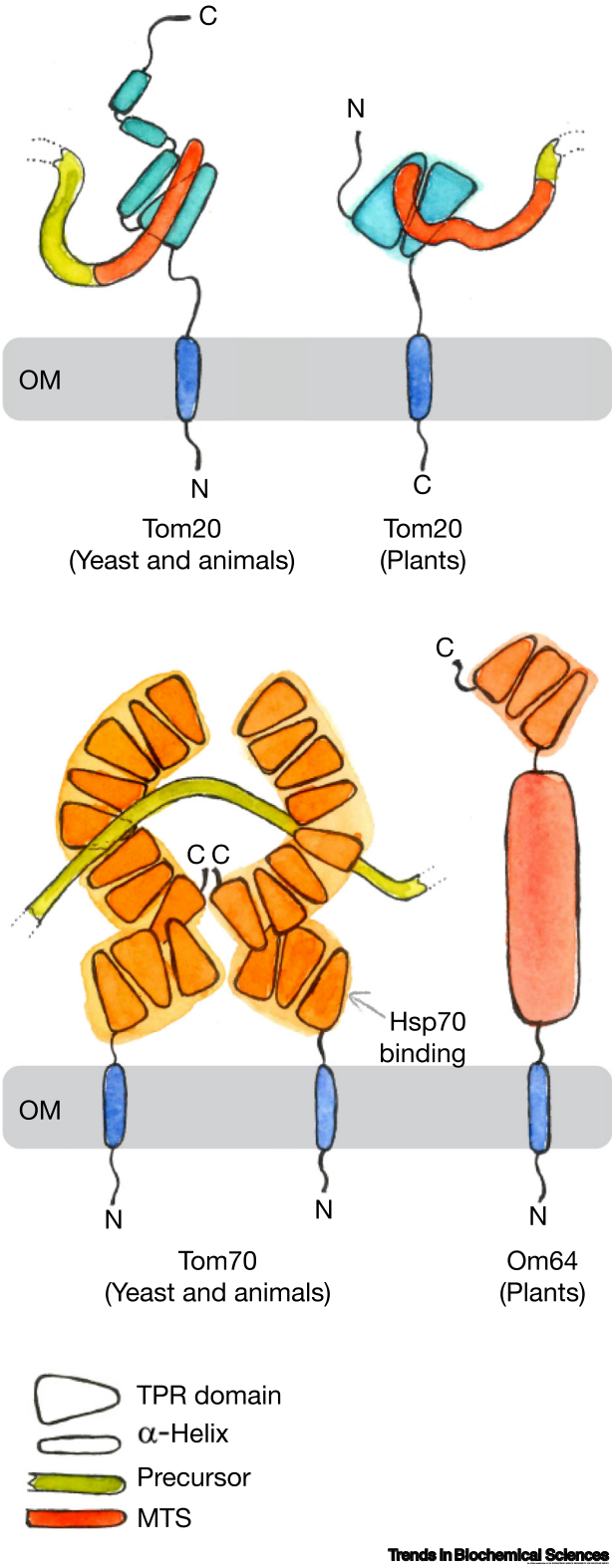

Figure 3. The Domain Architecture of Outer Membrane (OM) Receptors Promotes Their Capacity to Recognize Substrates. (Top) Tom20 receptors (teal and blue) in animals and fungi recognize mitochondrial targeting sequences (MTS) (orange) by a shallow groove formed by two α-helices of the tetratricopeptide (TPR) repeat and one additional helix [165–168]. Plants employ an analogous, unrelated Tom20 protein, which also uses TPR repeats to bind MTSs. (Bottom) Tom70 receptors (orange and blue) in fungi and animals form dimers. Each monomer is anchored in the OM via an N terminal α-helix that is followed by two cytosolic subdomains: the first three TPR repeats form the carboxylate clamp (proximal to the membrane) that can bind Hsp70s or Hsp90s and the remaining eight TPR repeats form a precursor-binding groove [73,132,169–171]. The Om64 protein of *Arabidopsis* plays a role analogous to that of Tom70 and likewise contains substrate-binding and chaperone-binding domains. Abbreviations: Hsp, heat shock protein; TOM, translocase of the outer membrane.

Presequence Binding Factor (PBF)

PBF is a yet unidentified protein factor only studied *in vitro* after being purified from rabbit reticulocyte lysate based on its binding to the MTS of a recombinant precursor of rat mitochondrial matrix ornithine carbamoyltransferase and promotion of its import into mitochondria [105].

OM Events

During the last step of targeting, precursor proteins are handed over to mitochondrial receptor proteins that are part of the translocation complexes. All mitochondrial precursor proteins destined for different suborganellar compartments have to cross the OM or be inserted into it. This process is mediated by the TOM complex [106,107], mitochondrial import (MIM) complex [108–110], and sorting and assembly machinery/translocase of outer membrane β-barrel proteins (SAM/TOB) complex [111–113]. Cryo-electron microscopy (cryo-EM) structures of the TOM complex revealed many details of how the precursors might traverse the OM, but the interaction of the pore components with Tom20 and Tom70 is not well understood [6,114,115]. The OM receptor proteins present a remarkable example of convergent evolution: different eukaryotic supergroups have evolved their own functional analogs of both Tom20 and Tom70 with different topologies (reviewed in [116]).

The Tom20 Receptor

Tom20 is the main receptor that recognizes MTSs [5,117–119] (Figure 3). Tom20 binds a relatively short motif of less than ten amino acids, while there are many MTSs that are much longer and can contain multiple motifs [117,120]. Such long MTSs might enhance translocation efficiency of the precursor by additional binding of Tom20 to one motif, while the previous motif of the same precursor is already transferred to the Tom40 pore [121]. The same substrate-binding groove interacts with the targeting signal of β-barrel precursors: a hydrophobic β-hairpin [122]. Plant Tom20 evolved independently and is anchored to the OM, unlike mammalian and fungal Tom20, via its the C terminus (Figure 3) [123,124].

The Tom70 Receptor

Tom70 is the main receptor for hydrophobic proteins without MTSs and a docking partner of the cytosolic chaperones Hsp70 and Hsp90 [5,73,125–127]. Tom70 also cooperates with the MIM complex for insertion of multispanning α-helical OM proteins [128,129]. Yeast have a paralogous receptor, Tom71, that is expressed at low levels [130,131]. Based on crystal structures, the two subdomains of Tom70 can have two conformations, open and closed. It was proposed that binding of cytosolic chaperones can promote transition to the open state that can accommodate client proteins [132]. In yeast, Tom70 is usually found in a dimeric form [3], however dimer formation is probably incompatible with binding both the chaperone and substrate [133]. Monomeric Tom70 might be an initial module that accepts substrate from the chaperones, while Tom70 dimerization might promote chaperone release. A similar function of promoting chaperone release from Tom70 was also proposed for Tom20 [134].

Tom70 binds distinct regions of the **metabolite carrier** precursors [127]. It is still not well understood how exactly the specificity towards these sequences is achieved and what are their characteristic features. Surprisingly, the substrate binding cleft of Tom70 can also bind an MTS [135]. Such an interaction and the recent discovery that Tom70 binds internal MTS sequences (iMTSLs), which exist in many mitochondrial precursor proteins, may explain why Tom70 can substitute for Tom20 loss. Aggregation-prone proteins apparently utilize iMTSLs to maintain their import competence [84,136]. Hence, in addition to its receptor function, Tom70 has also a cochaperone role as it cooperates with the cytosolic chaperone network to keep precursor proteins with iMTSLs in an import-competent state.

The Tom22 Receptor

Tom22 is an integral component of the TOM core complex that plays a structural role in organization of Tom40 pores but also contains precursor-binding receptor domains [6,114,137–140]. In fungi and mammals, Tom22 has acidic domains on both sides of the OM while the TMD is tightly bound to Tom40 [6,114]. The yeast Tom22 cytosolic domain does not seem to have a defined structure [124] but binds model precursors *in vitro* with a specificity that overlaps that of Tom20 and Tom70 [5,127]. *In vivo* experiments suggest that Tom20 and Tom22 might have similar specificity towards MTS-containing proteins and can even bind the same MTS from either hydrophobic or positively charged sides, respectively [141,142].

In summary, the two main types of mitochondrial receptors have overlapping functions but still have some specificity towards particular types of precursors and some single-span OM proteins [143–145]. Since the receptors can rescue deletions of each other and can bind a wide range of precursors, one might wonder why different eukaryotic lineages evolved exactly two types of receptors similar to Tom20 and Tom70 [116]. We may hypothesize that the main function of Tom20-like receptors is providing specificity by recognizing an MTS, while the main function of Tom70-like receptors is accepting unfolded passenger proteins from chaperones and keeping them in an import-competent state. If the Tom20 function is compromised, the specificity function mainly relies on the subsequent TOM components. In such a scenario, the overall import speed might be reduced, so the cell needs to rely on the chaperone system and Tom70 to deal with precursor proteins and guide them to mitochondria. This can explain why Tom70 with a mutated carboxylate clamp, which cannot bind chaperones, does not rescue the synthetic lethality of Tom20 and Tom70 double deletion in yeast [73]. Although the TOM receptors had been discovered 30 years ago and have been the focus of many studies, our understanding of their biochemical functions, specificity, and physiological role is still limited.

Concluding Remarks

Many cytosolic factors have been implicated in ensuring that mitochondrial precursor proteins safely reach their destination organelle while proteins of other organelles do not. However, for the most part, we lack a detailed mechanistic understanding of their function and it remains unclear if, and how many, additional factors or pathways remain to be discovered (see Outstanding Questions). The time is now ripe to revisit unanswered questions with new tools. Rapid progress in super-resolution and single molecule imaging, *in situ* cryo-EM, and proximity labeling coupled with mass-spectrometry or RNA-sequencing will help to uncover new pathways that precursors take in the cytosol and how their translation and import are organized and controlled in space and time.

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Outstanding Questions

Are there additional mechanisms for mRNA localization to mitochondria beyond Puf3? How is local translation on the outer membrane (OM) organized and controlled?

How are mitochondrial precursor proteins routed between co- and post-translational import pathways in different organisms and cell types? Are ribosomes encouraged or discouraged to synthetize mitochondrial proteins on the TOM complex?

Are there additional cytosolic factors that bind mitochondrial precursor proteins and what are the specificities of each cytosolic factor to their substrates *in vivo*? Are there dedicated targeting pathways remaining to be discovered?

How do cytosolic chaperones discriminate between substrates that need to be folded, kept unfolded during targeting, or degraded? What is the time difference between protein synthesis and translocation or degradation?

What are the exact roles of cytosolic factors in mitochondrial protein targeting? Do they just keep the substrates unfolded or do they contribute to the targeting directionality or specificity? Do the chaperones have a role in preventing mistargeting?

Where does the energy for conferring targeting specificity come from in case of mitochondria and how are precursors transferred from the cytosolic factors to outer membrane receptors?

Do the OM receptors act in a sequential or parallel manner and is this dependent on the specific substrates? Are the 'professional' receptors Tom20/Tom22 and Tom70/Tom71 supported by other OM proteins that expose low-affinity precursor binding sites?

Are the TOM receptors just passive binders that provide affinity sites or do they more actively help to unfold precursors and push them into the TOM pore, similar to what was proposed for the GTP-hydrolyzing translocases of chloroplasts (TOC) receptors on the OM of chloroplasts?

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