Review

Maria Clara Avendaño-Monsalve, José Carlos Ponce-Rojas and Soledad Funes*

From cytosol to mitochondria: the beginning of a protein journey

<https://doi.org/10.1515/hsz-2020-0110>

Received January 9, 2020; accepted February 24, 2020

Abstract: Mitochondrial protein import is one of the key processes during mitochondrial biogenesis that involves a series of events necessary for recognition and delivery of nucleus-encoded/cytosol-synthesized mitochondrial proteins into the organelle. The past research efforts have mainly unraveled how membrane translocases ensure the correct protein sorting within the different mitochondrial subcompartments. However, early steps of recognition and delivery remain relatively uncharacterized. In this review, we discuss our current understanding about the signals on mitochondrial proteins, as well as in the mRNAs encoding them, which with the help of cytosolic chaperones and membrane receptors support protein targeting to the organelle in order to avoid improper localization. In addition, we discuss recent findings that illustrate how mistargeting of mitochondrial proteins triggers stress responses, aiming to restore cellular homeostasis.

Keywords: heat shock proteins; mitochondrial protein import; mitochondrial stress response; mRNA targeting; ribosomal chaperones; yeast.

Introduction

Any living cell faces the challenge of properly sorting its proteome to specific destinations inside and outside the cell. The correct localization of proteins in cells aligns

with the compartmentalization process, required to optimize cellular chemical reactions by creating proper microenvironments and channeling between enzymes. However, protein sorting is defiant, and in many cases, mistargeted polypeptides can affect cell homeostasis. For this reason, cells have developed intricate systems to ensure the correct delivery of proteins to their final destinations. Such systems rely on two mechanisms to ensure correct protein targeting: (i) membrane receptors at the target organelle, exposing soluble domains to the cytosol that can couple with pore-forming proteins, which in turn allow translocation of substrates across the membrane into the organelle; and (ii) soluble factors in the cytosol that recognize substrates by identifying either amino acid sequences and/or secondary structures and keeping them in a partially unfolded state or certain elements on the mRNAs, which improve fidelity during protein transport.

The initial phases of protein transport into mitochondria correspond to the synthesis of precursors by cytosolic ribosomes. As soon as a nascent peptide emerges from the ribosomal exit tunnel, their unproductive association with other cellular components must be avoided to prevent protein misfolding or aggregation. Thereby, cytosolic chaperones bind and protect nascent proteins to assist their folding and to maintain precursor proteins in an import-competent conformation (Saibil, 2013). Chaperones can also act as recognition factors that promote the delivery of synthesized substrates to the required subcellular location. Substrate proteins often harbor targeting signals that are maintained accessible to be read due to the activity of chaperones and that determine the final localization of the protein.

The development and use of *in organello* import assays showed that protein transport to mitochondria can occur once protein synthesis is completed, that is, in a posttranslational manner (Harmey et al., 1977; Maccellini et al., 1979a,b; Reid and Schatz, 1982; Adrian et al., 1986; Bihlmaier et al., 2008). This is true for all studied matrix proteins or transporters such as the ATP/ADP carrier (Aac2 or AAC), which can be efficiently imported after full synthesis *in vitro*. However, an increasing

***Corresponding author: Soledad Funes**, Departamento de Genética Molecular Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Circuito Exterior s/n Ciudad Universitaria Coyoacán, México, Cd.Mx. 04510, Mexico, e-mail: sfunes@ifc.unam.mx. <https://orcid.org/0000-0003-1815-1977>

Maria Clara Avendaño-Monsalve: Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Circuito Exterior s/n Ciudad Universitaria Coyoacán, México, Cd.Mx. 04510, Mexico

José Carlos Ponce-Rojas: Department of Molecular, Cellular, and Developmental Biology, University of California at Santa Barbara, Santa Barbara, CA 93106-9625, USA

amount of experimental evidence coming from *in vivo* and *in vitro* assays, as well as electron microscopy, suggests that import can occur in a cotranslational manner (Kellems and Butow, 1972, 1974; Kellems et al., 1975; Knox et al., 1998; Kaltimbacher et al., 2006; Williams et al., 2014; Gold et al., 2017). In other words, soluble and membrane factors could promote that translationally active ribosomes come near the organelle's outer membrane (OM) to favor protein synthesis in the vicinity of the translocation pore. It has been suggested that the physicochemical traits of each mitochondrial protein, such as hydrophobicity or folding speed, could define their particular mechanisms required for import (Knox et al., 1998; Sass et al., 2003; Luk et al., 2005; Yogeve et al., 2007; Williams et al., 2014); however, experimental evidence is still lacking to further understand these early recognition steps of mitochondrial protein import.

The evolutionary origin of mitochondrial targeting machineries

Mitochondria are complex organelles – present in almost every eukaryotic cell – composed of two membranes, the OM and the inner membrane (IM) that delimitate two aqueous spaces; the intermembrane space (IMS); and the mitochondrial matrix (Palade, 1952). Such architecture is the result of their endosymbiotic origin, as these organelles arose from a bacterial ancestor (most likely an α -proteobacteria Roger et al., 2017) that was engulfed by archaea belonging to the superfamily Asgard (Zaremba-Niedzwiedzka et al., 2017; Spang et al., 2019). This event created a symbiotic connection in which the α -proteobacteria assumed selected metabolic functions, the most prominent being energy production, until they became mitochondria of modern eukaryotic cells. The permanent modification of their genomes was a crucial change that occurred in both cell types once fused. A significant fraction of the genome originally contained in the bacterial ancestor was lost, another portion migrated and fused to the host genome, and a small fraction was retained inside the organelle (which is now the mitochondrial genome). In consequence, the host adopted a portion of genes derived from the bacterial cell, which eventually generated the nuclear eukaryotic genome, which encodes more than 99% of the mitochondrial proteome.

Simultaneously with this genetic rearrangement, one of the most dramatic consequences of the endosymbiosis was the modification of the way proteins arrive to mitochondria and are distributed to the different mitochondrial subcompartments (Herrmann, 2003;

Chacinska et al., 2009). Hence, cytosolic and membrane factors evolved to identify those mitochondrial proteins now encoded in the nuclear genome and help them arrive to the organelle where they fulfill their biological function, and their role will be discussed in the following sections. Moreover, targeting signals had to be incorporated into the substrate proteins in order to ensure their identification and correct sorting within the cell. The most studied and understood mitochondrial targeting signals are those located at the N-terminus, called presequences, which are cleaved upon import into the matrix. These presequences have a length of 15 to 50 amino acids and form an amphipathic α helix possessing one side of positively charged residues and the other side rich in hydrophobic residues (Figure 1) (Chacinska et al., 2009; Garg and Gould, 2017).

Membrane receptors at the OM recognize targeting signals and assist the initial stages of protein targeting into mitochondria

Most mitochondrial precursor proteins enter into the organelle using the Translocase of the OM (TOM complex). Several studies addressed the structural arrangement of TOM, both in *Saccharomyces cerevisiae* and *Neurospora crassa*, and suggested that this complex associates in dimers or trimers (Model et al., 2002; Shiota et al., 2015; Bausewein et al., 2017; Araiso et al., 2019). Recently, a detailed description of the structural arrangement of the TOM complex was determined by cryo-electron microscopy (Tucker and Park, 2019; Araiso et al., 2019). It was observed that the basal arrangement of the complex involves dimers of the monomeric TOM, where every monomer includes the following proteins: Tom40, the β -barrel pore dedicated to translocate mitochondrial proteins; Tom22, which is a central receptor for preproteins harboring presequences; and the small subunits Tom5, Tom6, and Tom7, which are important for stabilization of the complex and for controlling its biogenesis depending on the phase of the cell cycle. Furthermore, it was determined that within the Tom40 pore, preproteins are sorted depending on their final destination by exposing different contact sites to chaperones at the IMS or to the TIM23 complex at the IM (Kiebler et al., 1990; Moczko et al., 1992; Künkele et al., 1998; Tucker and Park, 2019; Araiso et al., 2019; Sakaue et al., 2019). Additional studies have demonstrated that two receptors can associate with the basal

Figure 1: Mitochondrial protein import fidelity can be achieved in the cells by the concomitant action of elements at different stages of targeting. Most mitochondrial proteins are synthesized by cytosolic ribosomes (turquoise) and targeted to the organelle guided by different chaperones whose recognition and binding are essential to avoid mistargeting and possible aggregation. (A) For mitochondrial precursor proteins whose translation is completed in the cytosol, soluble chaperones from the Hsp70, Hsp40, and Hsp90 families (green, lime green, and violet) recognize them after their synthesis is completed and guide their way to the receptors of the TOM complex, thus allowing protein import. (B) Ribosomes translating mitochondrial proteins can be directed to the vicinity of the mitochondrial surface by the action of ribosomal chaperones such as NAC (yellow/green) and/or by proteins functioning as receptors for mRNAs encoding mitochondrial proteins (mMPs) such as Puf3 (purple).

TOM monomer: Tom20 and Tom70; however, their association is weaker than that of Tom22 (Shiota et al., 2015; Sakaue et al., 2019). All three receptors, Tom20, Tom70, and Tom22, expose cytosolic domains required for the recognition of mitochondrial substrates and their delivery to Tom40. These receptors will be discussed in the following sections.

Tom20 recognizes precursors harboring mitochondrial targeting signals

Tom20 is considered the master receptor of TOM because of its capacity to recognize a broad repertoire of precursors, mainly those possessing a cleavable presequence. Work done in *S. cerevisiae* demonstrated that Tom20 can receive different substrates, such as CoxIV, Cyt_c, and reporter constructs, such as Su9-DHFR (presequence of F₀-ATPase subunit 9 of *N. crassa* fused with mouse dihydrofolate reductase) and b2Δ-DHFR (presequence of Cytb2 of *S. cerevisiae* lacking 19 amino acids fused to DHFR). However, Tom20 can also recognize precursors with internal signals such as the phosphate carrier (PiC) (Brix et al., 1997, 1999). Just as Tom20, the homolog in *N. crassa* Mom19

also recognizes mitochondrial proteins with editable presequences and internal signals, examples of the latter are Su9-DHFR, F₁-ATPase subunit β, and the Fe/S protein of the bc₁ complex (Moczko et al., 1994). Recently, it was described that Tom20 also recognizes a β-hairpin signal sequence present at the C-terminus of β-barrel protein residents of the OM (Jores et al., 2016). In binding to partially unfolded precursors, Tom20 prevents the aggregation of these proteins before their import to mitochondria, in which Tom20 functions as a chaperone (Yano et al., 2003).

Tom20 is anchored to the OM by an α-helical transmembrane domain (TMD) and exposes its C-terminal domain to the cytosol. This domain contains a motif of tetratricopeptide repeats (TPR), which is important for its association with cytosolic chaperones such as the 70- and 90-heat shock protein families (Hsp70 and Hsp90) that will be discussed later in detail. These chaperones and Tom20 act sequentially during protein transport to mitochondria (Söllner et al., 1989; Harkness et al., 1994; Moczko et al., 1994). In addition, the cytosolic domain of Tom20 can directly bind to the presequence in mitochondrial precursors through negatively charged patches that interact with the positively charged side of the amphipathic presequence

(Brix et al., 1997) or by means of two hydrophobic patches that can also interact with the presequence (Rimmer et al., 2011). This suggests that Tom20 has at least two different sites for recognition of presequences, probably due to their amphipathicity (Abe et al., 2000; Rimmer et al., 2011). The crucial role of *TOM20* is illustrated by the compromised fitness of a *tom20* null mutant in *S. cerevisiae* (Harkness et al., 1994; Moczko et al., 1994), also indicating partial functional redundancy with other mitochondrial receptors, which will be discussed below.

Tom70 is the receptor in charge of recognizing carrier proteins of the IM

The second receptor of the TOM complex is Tom70 that recognizes substrates containing hydrophobic regions such as β -barrel proteins of the OM like Porin (Moczko et al., 1994), IMS proteins like Cyt_c (Brix et al., 1997), and IM carriers like AAC and PiC (Hines et al., 1990; Söllner et al., 1990; Hines and Schatz, 1993; Brix et al., 1997, 1999; Melin et al., 2015). In yeast, Tom70 has a less abundant paralog, Tom71, which is also believed to support protein recognition (Schlossmann et al., 1996; Burri et al., 2006). Tom70 interacts with hydrophobic precursors by the identification of internal noncleavable signal sequences and delivers them to the receptor Tom22 (Hines et al., 1990; Steger et al., 1990; Hines and Schatz, 1993; Schlossmann et al., 1994; Haucke et al., 1996; Brix et al., 1997, 1999; Komiya et al., 1997). Similar to Tom20, Tom70 is anchored to the OM via its N-terminus, whereas the soluble C-terminal domain faces the cytosol. The latter domain contains 4 to 11 TPR motifs, which constitute the region of interaction with cytosolic chaperones Hsp70 and Hsp90, with mitochondrial precursors, and with Tom20 (Komiya et al., 1997; Young et al., 2003; Melin et al., 2015). Tom70 also recognizes nonhydrophobic internal presequence-like signal sequences (internal MTS) broadening the protein spectrum whose import is influenced by this receptor (Backes et al., 2018). In this way, Tom70 has a role as cochaperone able to bind these proteins with internal signals and maintaining them in an import-competent state. Tom70 and Tom20 display partial functional redundancy as yeast null mutants on either gene (i.e. *TOM70* or *TOM20*) compromise cell fitness, but a double mutant is synthetic lethal (Ramage et al., 1993).

The receptor where precursor proteins converge: Tom22

The third receptor of the TOM complex is Tom22, which exposes its N- and C-terminal domains to the cytosol

and to the IMS, respectively (Kiebler et al., 1993; Araiso et al., 2019; Tucker and Park, 2019). Tom22 also recognizes and binds the presequence of precursors (Kiebler et al., 1993; Mayer et al., 1995; Brix et al., 1997, 1999; Yamano et al., 2008) simultaneously with Tom20 but at opposite sides of the presequence (Yamano et al., 2008; Rimmer et al., 2011; Shiota et al., 2011). The IMS domain of Tom22 makes part of the *trans* presequence-binding site for precursor translocation along with the loop between β 1 and β 2 sheets of Tom40 and the IMS loop of Tom7 (Araiso et al., 2019; Tucker and Park, 2019). This receiving motif promotes the transfer of precursors from Tom40 to Tim50, which is the receptor for incoming proteins at the TIM23 complex (Bolliger et al., 1995; Moczko et al., 1997; Shiota et al., 2011; Araiso et al., 2019). Tom22 thus mediates the transfer of precursors to complexes located in the IMS or the IM depending on the nature of the incoming polypeptide. The relevance of Tom22 for mitochondrial import is supported by the lethal phenotype of a *TOM22* null mutant (Lithgow et al., 1994; Hönlinger et al., 1995).

Noncanonical receptors support protein transport into mitochondria

Several proteins facilitate membrane insertion of OM proteins but do not require the pore formed by Tom40. One of these is Mim1, a protein anchored to the OM by a single transmembrane helix that contains two GXXXG motifs that allow its oligomerization, a step required for the membrane insertion of incoming proteins such as Tom20 and Tom22 (Waizenegger et al., 2005; Becker et al., 2008; Popov-Čeleketić et al., 2008; Dimmer and Rapaport, 2010). Similarly, the SAM complex (discussed below) enhances the import and assembly of TOM subunits Tom22, Tom20, and Tom6 (Stojanovski et al., 2007; Thornton et al., 2010). The detailed mechanism for the latter remains unclear.

Cytosolic chaperones assist initial phases of mitochondrial protein transport

Family of Hsp

Cytosolic chaperones are one of the main protein families that support protein transport (Deshaies et al., 1988; Young et al., 2003; Fan et al., 2006). Hsp40, Hsp70, and

Hsp90, respectively, recognize and bind nascent peptides as they are synthesized by ribosomes or when protein synthesis is completed (Figure 1A). Heat shock proteins enhance protein transport by maintaining substrates in an import-competent state (i.e. partially unfolded) and deliver them to the mitochondrial surface to the specific receptors localized at the OM. In particular, the cytosolic C-terminus of Tom70 interacts with the EEVD motifs in Hsp70 or Hsp90 before guiding the substrates to the pore of the translocase (Young et al., 2003; Wu and Sha, 2006; Li et al., 2009; Zanphorlin et al., 2016). The relevance of the interaction between Hsp70 or Hsp90 and Tom70 has been demonstrated for hydrophobic substrates such as the reporter construct Cyb2⁽¹⁻⁸⁷⁾-DHFR, the phosphate and the AAC carriers in yeast, and the mitochondrial peptide transporter in mammals (Young et al., 2003; Hoseini et al., 2016). It remains unknown how chaperones can discriminate between substrates directed to different organelles and how they participate in more than one route of transport. An example of this is Ssa1, an Hsp70 from the stress subfamily of proteins A (SSA), which impacts the biogenesis of proteins from both the endoplasmic reticulum (ER) and mitochondria. Indeed, yeast temperature-sensitive *SSA1* mutants accumulate Atp2 as well as α -factor and carboxypeptidase Y precursors, suggesting the role of Ssa1 for proper sorting (Becker et al., 1996). However, whether the lack of function of SSA affects either protein sorting directly or the proteasomal degradation of the untargeted precursors remains undetermined.

Moreover, ribosome profiling studies in yeast have shown that the Ssb subfamily of Hsp70s (Ssb1 and Ssb2) associates to the exit tunnel of ribosomes translating nearly 80% of all mitochondrial proteins. Therefore, it is possible that Ssb binds to the mitochondrial nascent chains directly at the exit tunnel and keeps them in an unfolded state ready to engage the TOM complex for import (Döring et al., 2017; Stein et al., 2019). Furthermore, the cytosolic ring-shaped chaperonin TRiC/CCT also binds mitochondrial precursors cotranslationally without competing with Ssb; thereby, it has been also proposed to enhance mitochondrial protein targeting. However, more work needs to be done to elucidate TRiC relevance for mitochondrial biogenesis (Stein et al., 2019).

The function of the cytosolic chaperones mentioned previously depends on the formation of complexes with cochaperones of the Hsp40 family. Such is the case for Hsp40-related DnaJ chaperones from the DJA family in mammals and cochaperones Sti1, Ydj1, Xdj1, and Djp1 in yeast (Bhangoo et al., 2007; Papić et al., 2013; Hoseini et al., 2016; Opaliński et al., 2018). However, in some cases, Hsp40s promote protein import without association to Hsp70 or Hsp90. One example of this is Xdj1, a protein that

physically interacts with the receptor Tom22 and promotes import of hydrophobic precursors such as Oxa1 by delivering them to Tom22. Xdj1 also promotes the assembly of the TOM complex by enhancing import of TOM subunits such as Tom22 (Opaliński et al., 2018). A similar function is suggested for Djp1, as it displays specific physical interaction with Tom70. Indeed, Djp1 and Tom70 are required for the proper targeting of Mim1 to mitochondria (Papić et al., 2013; Opaliński et al., 2018). Hsp40s also assist delivery of β -barrel proteins such as porin, Tom40, and Sam50 to the OM along with SSA Hsp70s (Jores et al., 2018).

Further investigations are required to better understand the impact each specific chaperone-cochaperone pair has on protein transport. One possibility would be that different associations increase the variety of substrates they can bind while simultaneously giving specificity for the route of transport. Another mutually nonexclusive possibility is that they reside in different locations of the cell such as in the cytosol, on the surfaces of the ER and of mitochondria, and in the nucleus.

The nascent polypeptide-associated complex (NAC)

In addition to cytosolic Hsps, NAC (Nascent polypeptide-Associated Complex) also facilitates mitochondrial protein transport (Figure 1). NAC is a chaperone that assembles in heterodimers (an α and a β subunit) or homodimers (specifically in archaea, where NAC is a homodimer of α subunits). In yeast, there are two genes encoding β -subunits (*EGD1*, which encodes for subunit β , and *BTT1*, which encodes for subunit β'), so that six NAC dimers could be formed. These nonidentical dimers differ in their substrate spectrum as they bind to ribosomes translating different proteins. However, the presence of the six dimers has not directly been proven and is only inferred from work made in mutants lacking two out of the three NAC genes (Hu and Ronne, 1994; Shi et al., 1995; del Alamo et al., 2011). NAC binds cytosolic ribosomes and nascent polypeptides simultaneously, by inserting its positively charged patch located in the N-terminal of subunit β into the ribosomal exit tunnel, contacting nascent polypeptides of at least two amino acids long, close to the ribosomal peptidyl-transfer center. In contrast, subunit α remains at the ribosomal surface and associates with nascent polypeptides when they exit the tunnel. NAC thus functions as a ribosome plug that escorts nascent polypeptides (Reimann et al., 1999; Wegrzyn et al., 2006; Gamerdinger et al., 2019).

It was initially suggested that NAC supports protein sorting to mitochondria as yeast null mutants of NAC show decreased amounts of proteins within the organelle

(George et al., 1998, 2002; Yogev et al., 2007). Additionally, depletion of NAC during *in vitro* import reactions results in less efficient translocation of substrates into mitochondria (Fünfschilling and Rospert, 1999). Furthermore, pull-down experiments of stalled ribosomes and genetic screens suggested that $\alpha\beta'$ and $\beta\beta'$ -NAC dimers bind preferentially mitochondrial proteins, whereas the $\alpha\beta$ -NAC binds cytosolic proteins (del Alamo et al., 2011; Ponce-Rojas et al., 2017). However, both $\alpha\beta$ - and $\alpha\beta'$ -NAC complexes are relevant for mitochondrial protein transport as they bind receptors at the OM: Om14 and Sam37, respectively (Figure 1B, Lesnik et al., 2014; Ponce-Rojas et al., 2017). The relevance of NAC for cell homeostasis is reflected by the fact that the deletion of either of the genes encoding NAC subunits results in a lethal phenotype in mouse, nematodes, and flies (Deng and Behringer, 1995; Markesich et al., 2000; Bloss et al., 2003). Strikingly, in the budding yeast *S. cerevisiae*, deletion of the three genes encoding NAC subunits has no effect on the viability of the organism (Reimann et al., 1999; Ponce-Rojas et al., 2017), which illustrates the buffering capacity of the sorting machineries that orchestrate protein transport to mitochondria. How all these factors associate and organize in protein targeting is unknown, and further studies are necessary to understand the detailed biological implication of each chaperone and receptor pairs to the protein targeting process.

Om14 and Sam37 constitute a docking mitochondrial platform for the ribosomal chaperone NAC

The recruitment of NAC and translationally active ribosomes to the vicinity of the OM has been related to the function of Om14 and Sam37. Om14 is one of the most abundant proteins in yeast OM that spans the membrane three times, with its N-terminus facing the cytosol and its C-terminus in the IMS (Burri et al., 2006). This protein functions as a receptor for $\alpha\beta$ -NAC and associates with cytosolic ribosomal proteins (Lauffer et al., 2012; Lesnik et al., 2014). It has been shown that its absence decreases the number of active ribosomes associated with the mitochondrial surface (Lesnik et al., 2014).

Sam37 is one of the subunits of the SAM complex (Sorting and Assembly Machinery), which mediates the insertion of β -barrels into the OM after they have been imported into the organelle crossing the TOM complex and transiently bound to chaperones at the IMS. The central subunit of SAM is Sam50, a membrane-embedded β -barrel protein (Kozjak et al., 2003; Paschen et al., 2003) that

interacts with the peripheral proteins Sam35 and Sam37 (Kozjak et al., 2003; Wiedemann et al., 2003; Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004). Furthermore, it was reported that Sam37 wires the transient association between the TOM and SAM complexes during the insertion of β -barrel proteins (Wenz et al., 2015). It has been also proposed that this protein can participate in the transport of other types of incoming proteins via its cytosolic domain (Stojanovski et al., 2007). Initial biochemical evidence indicated that Sam37 associates with Tom70 and was therefore considered a receptor of the TOM complex; however, it is currently not understood if this association occurs only with Sam37 or if it requires the entire SAM complex. Also, it was shown that blocking the cytosolic domain of Sam37 hampers the transport of proteins such as Aac2 and Cyt_c to the mitochondria (Gratzer et al., 1995). Recently, Sam37 was identified as the binding partner for the $\alpha\beta'$ -NAC complex. The relevance of this physical interaction is reflected in the compromised fitness of a yeast mutant lacking both components that exhibits a decreased import efficiency of the mitochondrial precursors Oxa1, Nfs1, and Sod2 (Ponce-Rojas et al., 2017). These findings suggest a relevant role for both Om14 and Sam37 in early steps of recognition during mitochondrial import, probably by facilitating the approach of translationally active ribosomes to the vicinity of mitochondria (Figure 1B). How these factors are interconnected with each other and other chaperones and receptors in the whole import apparatus is currently not understood.

Mitochondrial localization of mRNAs is important for protein import

Localization of mRNAs is a strategy to ensure that protein synthesis can occur at the site where the encoded polypeptide is needed (Jan et al., 2014; Williams et al., 2014; Buxbaum et al., 2015). Initially, the addition of the antibiotic cycloheximide, which stalls translation without losing the mRNA-ribosome-nascent polypeptide association, allowed the coisolation of translationally active cytosolic ribosomes with purified mitochondria and was observed at the vicinity of the OM, in particular at the OM-IM contact sites (Kellems and Butow, 1972, 1974; Kellems et al., 1974, 1975). The localization of clustered ribosomes at the mitochondrial surface was confirmed by cryo-tomography through the specific binding with

the TOM complex in a nascent-chain-dependent manner (Gold et al., 2017).

mRNAs encoding mitochondrial proteins (mMPs) have also been observed associated with the mitochondrial surface. The analysis of these mMPs enriched at the mitochondrial fractions indicated that their protein products originated from a prokaryotic origin, whereas mMPs of eukaryotic origin were found preferentially at free-cytosolic ribosomes, suggesting that cotranslational acting signals could contribute to ensure the fidelity of the import process before specialized import machineries were fully developed at the mitochondrial membranes (Suissa and Schatz, 1982; Marc et al., 2002). Furthermore, it was observed that polysomes associated at the OM carry preferentially mRNAs encoding IM proteins, supporting the notion that cotranslational import of hydrophobic membrane proteins prevents mislocalization and aggregation in aqueous environments (Williams et al., 2014). As these experiments have been performed in the presence of cycloheximide, whether anchoring of ribosomes with the translocation machinery at the OM is required for import remains undetermined. To elucidate if mRNA association to mitochondria depends on translation, a comparison was made in mammalian HEK293T treated with cycloheximide or with puromycin. These results allowed identifying a subpopulation of mRNAs that remains associated with mitochondria in the absence of intact ribosomes. Among these mMPs are those encoding components of the mitochondrial ribosome and OXPHOS components (Fazal et al., 2019).

mRNA molecules carry signals important for their mitochondrial localization

mRNA trafficking can modulate both spatial and temporal localization of the encoded protein. Such mechanisms involve signals within the mRNA (*cis*-acting elements) and proteins (or protein complexes, *trans*-acting elements) that recognize such signals and determine their localization. For mMPs, it was suggested that there is a signature that recruits them onto the organelle. This signal could either be a particular nucleotide sequence contained within the mRNA or a secondary or tertiary structure formed by the mRNA molecule. Up to date, neither the sequence nor the structure has been precisely determined. However, it was proposed that the mechanisms of recognition are evolutionarily conserved at least between yeast and humans, as human mMPs expressed in yeast cells can localize to mitochondria (Sylvestre et al., 2003).

First approaches indicated that the 3'-untranslated region (3'-UTR) of mRNAs is important for their localization at the mitochondrial surface as this sequence is sufficient to direct nonmitochondrial mRNAs to the OM (Marc et al., 2002; Margeot et al., 2002; Liu and Liu, 2007). Indeed, absence of the 3'-UTR decreases import efficiency of certain substrates such as Atp2 and Oxa1, and their precursor proteins are accumulated outside the mitochondria (Gadir et al., 2011; Zabezhinsky et al., 2016). The region between nucleotides 50 and 150 downstream of the stop codon of *ATP2* mRNA proved to be sufficient to localize such mRNA to the vicinity of mitochondria (Margeot et al., 2002). Comparative analysis of different 3'-UTRs found structural elements and/or particular nucleotide sequences that could function as signals for localization, i.e. the bulged stem-loop structure (Min2 element, Liu and Liu, 2007) and the 10-nt element 5'-CYTGAAATA-3', where Y is C or T, which is present in 56 mMPs (Figure 2, *ATP2* mRNA, Anderson and Parker, 2000).

Even though the influence of the 3'-UTR has been proven important for localization of mRNAs, it also requires the translation of the presequence (Figure 2, *OXA1* mRNA), probably to ensure engagement of the translation machinery to the TOM complex to enable mitochondrial import. The 3'-UTR-mediated localization of mRNAs could also depend on the nature of the encoded protein. One possible feature is the presence of TMDs and their hydrophobicity level, which could be misread as hydrophobic ER signals (Zabezhinsky et al., 2016). In such cases, proper targeting to mitochondria would be ensured *in vivo* only if all the appropriate signals are read; otherwise, proteins could be misdirected to other organelles such as the ER. Imbalances in protein transport specificity could be used by the cell as a mechanism for monitoring metabolic states, and that may trigger stress responses such as the Unfolded Protein Response (UPR).

Puf3: a protein that determines the fate of mMPs

An mRNA-binding protein (mRBP) that has been proposed as a *trans* element for the recognition of mMPs is Puf3. This protein belongs to the PUF (Pumilio and FBF) family of proteins, which are highly conserved among eukaryotes. PUF proteins modulate posttranscriptionally the expression of different mRNA molecules through the recognition of specific *cis*-regulatory elements. Their RNA-binding domain is composed of eight consecutive α -helical PUF repeats that adopt a crescent-shaped structure (reviewed in Wang et al., 2018). Each PUF repeat recognizes a specific

Figure 2: Different signals at mMPs favor their localization at the OM.

Mitochondrial localization of mRNAs can be influenced by signals at the 3'-UTR of mMPs such as CYTGTAATA (where Y represents C or T, Min2 element found in *ATP2* mRNA) or UGUUAUUAU (Puf3 recognition sequence), which can be recognized by mRBPs located at the OM such as Puf3 (purple). In addition, other regions both at the 3'-UTR and at the region encoding the presequence (MTS) are relevant for mitochondrial localization of mMPs such as *OXA1*. This transport is also linked to components of the COPI vesicles.

nucleotide on the mRNA target, thereby the recognition sites are composed of eight nucleotides on the bound mRNA (Wang et al., 2001, 2002).

In yeast, there are six PUF proteins, from which Puf3 is involved in several aspects of mitochondrial biogenesis such as decay of mMPs and mitochondrial dynamics through its binding with Mdm12 (Olivas and Parker, 2000; García-Rodríguez et al., 2007; Quenault et al., 2011). Puf3 associates to the cytosolic surface of the OM and binds preferentially mMPs through the recognition of the nucleotide sequence 5'-UGUAUUAU-3' on their 3'-UTR (Figure 2, Gerber et al., 2004; Kershaw et al., 2015). Furthermore, the presence of the Puf3 recognition site was associated with perimitochondrial localization of the mRNAs carrying it, and thereby Puf3 was proposed as a key component that situates mRNAs to the vicinity of the OM, hence guiding cotranslational mitochondrial import (Saint-Georges et al., 2008). However, not all mMPs possess a Puf3-binding domain, and therefore they have been classified

into two classes depending on the presence or absence of this characteristic.

It has been shown that Puf3 protein levels decrease under respiratory conditions (García-Rodríguez et al., 2007); in addition, Puf3 also enhances degradation of mMPs under fermentative conditions, whereas in respiratory conditions the translation of those mRNAs is favored (Miller et al., 2014; Lee and Tu, 2015) probably as a mechanism to tune protein synthesis. Furthermore, recent ribosome profiling data showed that Puf3-mRNA targets are stabilized in the absence of Puf3, but their translation efficiency is decreased, suggesting that in addition to the role that Puf3 plays during mRNA stability, they can also participate – directly or through its interaction partners – in the translational capacity shifts required during metabolic changes of the cell, in particular from fermentative to nonfermentative metabolism (Wang et al., 2019).

It is clear that Puf3 is vital for the regulation of mitochondrial biogenesis; however, our understanding of

its precise role during mitochondrial import remains far from being completely described.

Tom20 cooperates with Puf3 in the localization of mMPs

Besides its canonical role for protein recognition, Tom20 was found to be important for proper localization of mRNAs in the vicinity of mitochondria. In yeast mutants lacking *TOM20*, some mRNAs lose their mitochondrial localization. The absence of *TOM20* also results in an increased expression of *PUF3*, which points toward an idea that Tom20 and Puf3 could play redundant functions only for mMP localization. Furthermore, a mutant lacking both genes is unable to grow on media with non-fermentable carbon sources, strengthening the idea that these two components cooperate for mitochondrial biogenesis (Eliyahu et al., 2010; Gadir et al., 2011).

Coupling vesicle transport with mRNA localization: the role of coat complex I

Coat Complex I (COPI) is a molecular machinery involved in enabling vesicle formation and selecting the cargo that has to be transported within the formed vesicles in the secretory pathway. The role of COPI has been more closely involved in retrieval from the Golgi apparatus to the ER and intra-Golgi transport, endosomal transport, lipid homeostasis, and mRNA transport (reviewed in Arakel and Schwappach, 2018). However, it was observed that temporal inactivation of Sec27, essential β -coat protein of the COPI coatomer, results in loss of mitochondrial localization of mMPs such as *OXA1*, *CBS1*, *FIS1*, *IMG1*, *RSM25*, and *MDM10* independently on the protein synthesis (Figure 2, Slobodin and Gerst, 2010; Zabezhinsky et al., 2016). Two *cis*-acting elements in *OXA1* mRNA were found to be relevant for localization depending on COPI: the encoded presequence and a TMD region. Alterations on these *cis*-elements resulted in a mislocalization of the mMPs to the ER. As COPI has not been involved yet on vesicle/lipid trafficking to mitochondria, mMP targeting by COPI could be a process independent on vesicle formation and probably relies on the COPI subunits themselves; however, the precise mechanism of mRNA/COPI recognition and binding remains unclear (Zabezhinsky et al., 2016). Disruption of COPI function also affects mitochondrial morphology, respiration, membrane potential, and protein import. However, the pleiotropic effects of

COPI on mitochondrial biogenesis could be either direct or indirect as the result of mRNA mislocalization.

mRBPs related to mitochondrial biogenesis without a clear influence on mitochondrial protein targeting

Clu1 is an mRBP initially reported to be part of the translation initiation factor 3 (eIF3); however, it is not essential for translation, and its absence produces a phenotype of fragmented mitochondria in yeast (Zhu et al., 1997; Vornlocher et al., 1999; Mitchell et al., 2013). The mammalian homolog, CLUH, is a cytosolic RBP that associates with mitochondria and binds several mMPs such as components of the respiratory chain and the tricarboxylic acid cycle (TCA) and the fatty acid and amino acid metabolisms, as well as components of the mitochondrial dynamics and mitophagy (Gao et al., 2014). It has been proposed that CLUH protects target mRNAs from degradation during their transport to the OM and is an important player during posttranscriptional reprogramming to increase the oxidative capacity of mitochondria (Schatton and Rugarli, 2019).

In *Drosophila melanogaster*, the OM protein MDI (Mitochondrial DNA Insufficient) binds to the RNA-binding protein Larp (La-related protein) and recruits it to the mitochondrial surface (Zhang et al., 2016). Larp belongs to a conserved superfamily of eukaryotic proteins that bind RNA molecules and are involved in different stages of RNA biogenesis such as processing, maturation, localization, and regulation of mRNA translation (reviewed in Dock-Bregeon et al., 2019). The elimination of MDI leads to a delocalization of Larp from the mitochondrial surface to the cytosol and to a decreased protein synthesis in the vicinity of mitochondria without affecting general translation. The absence of MDI reduces the presence of 64 nuclear-encoded mitochondrial proteins, including 21 subunits of the mitochondrial ribosome, 23 subunits of the oxidative phosphorylation (OXPHOS) complexes, and the mitochondrial transcription factor TFAM (Zhang et al., 2016).

Cellular responses to mistargeting and mitochondrial protein accumulation

Although it was previously suggested, it is only in recent years that special attention was drawn to the cellular

responses triggered by a failure in the recognition, sorting, and import of mitochondrial proteins (Figure 3).

It has been observed that any decrease in mitochondrial protein import efficiency causes a transcriptional and translational rewiring, reducing the amount of ribosomal proteins (i.e. an attenuation of cytosolic translation) and increasing proteasomal activity. This kind of cellular response was observed to be triggered by clogging

of the TOM complex, by malfunction of Mia40 (a protein relevant for sorting and folding of IMS proteins), or by depleting the membrane potential (essential to mitochondrial import into the IM and matrix). Such response was called UPRam (UPR activated by mistargeting of proteins) or mPOS (mitochondrial precursor overaccumulation stress) (Figure 3A, blue arrows; Wang and Chen, 2015; Wrobel et al., 2015; Boos et al., 2019). The aforementioned

Figure 3: Mistargeting of mitochondrial proteins triggers diverse responses.

(A) Clogging of the TOM complex by saturation with incoming proteins activates changes in the transcriptional program of the cell via the activation of Hsf1 (a modulator of the cytosolic translation), Rpn4 (an activator of proteasomal genes), and Pdr3. One of the genes upregulated by Pdr3 is *CIS1* that encodes for the cytosolic protein Cis1. This protein is directed to the mitochondrial surface where the OM AAA-protease Msp1 is also recruited to the vicinity of the compromised TOM complex. Trapped substrates are then ubiquitinated (red circles) and directed to degradation by the proteasome (red arrows). Msp1 is also involved in the identification of mistargeted tail-anchored proteins at the OM and facilitates their transfer to the ER membrane where they are then ubiquitinated by Doa10 and transferred to the proteasome with the help of Cdc48 (turquoise arrows). An impairment or slowdown in mitochondrial protein import leads to an accumulation of precursor proteins in the cytosol, which triggers the activation of UPRam/mPOS and a translational rewiring that in turn initiates the synthesis of the ubiquitin-proteasome system to degrade the accumulated proteins. In contrast, synthesis of ribosomal proteins and proteins involved in translation is downregulated. (B) Nascent polypeptide-Associated Complex determines accuracy during sorting of mitochondrial proteins to the organelle. Malfunctions in this early recognition lead to mistargeting of mitochondrial proteins to the ER caused by SRP recognition of their hydrophobic segments. This mislocalization activates the UPR in the ER lumen. (C) Mitochondrial proteins mistargeted to the surface of the ER are recognized by the Hsp40 chaperone Djpl and redirected to the TOM complex (pink arrows). Tail-anchored proteins that have to be localized to the OM are identified in the cytosol by ubiquilins, which are able to either regulate their targeting to the OM or mark them for proteasomal degradation (purple arrows).

adaptive transcriptional program involves the activation of transcriptional factors Hsf1 (inducing the heat shock response) and Rpn4 (inducing proteasome synthesis), as well as the partial inactivation of the HAP complex to mute the expression of OXPHOS genes (Boos et al., 2019). This response demonstrates that when protein transport to mitochondria is halted, protein synthesis is attenuated, and proteasomal degradation increases until clogging of the TOM complex is solved, illustrating how protective responses coordinate and connect to reestablish mitochondrial protein import.

Another response was discovered with the accumulation of proteins containing bipartite signals such as Cox5a (i.e. IM proteins possessing a presequence followed by a TMD), which causes a transcriptional reprogramming that depends on the transcriptional factor Pdr3. One of the Pdr3 targets is *CIS1*, whose encoded protein is found associated with mitochondria where it recruits the AAA-protease Msp1 to the vicinity of the TOM complex. There, Msp1 helps to pull out clogged precursors, which are then ubiquitinated and degraded by the proteasome. This system is called mitochondrial compromised protein-import response or mitoCPR (Figure 3A, red arrows; Weidberg and Amon, 2018). Msp1 has also been related to the surveillance of mislocalized tail-anchored proteins at the OM and facilitates their transfer to the ER. There, the ubiquitin ligase Doa10 and its partners Ubc7, Ubc5, and Cue1 ubiquitinate the tail-anchored protein before it is pulled out of the ER membrane by the cytosolic AAA-ATPase Cdc48 and directed for proteasomal degradation (Figure 3A, turquoise arrows; Matsumoto et al., 2019). Interestingly, the key transcriptional factor for the mitoCPR Pdr3 is one of the targets of Rpn4 (Boos et al., 2019), which could indicate an interconnection between the described responses.

TOM complex clogging activates a specific response that involves Ubx2, a dual localized protein in the ER and mitochondria, which senses the accumulation of precursor proteins at the TOM complex and then recruits the AAA-ATPase Cdc48. There, Cdc48 removes the accumulated proteins, which are then degraded by the proteasome. This pathway, called mitoTAD (mitochondrial protein translocation-associated degradation), ensures that the Tom40 channel is available for translocation of mitochondrial precursors (Mårtensson et al., 2019).

As mentioned earlier, the ribosomal chaperone NAC ensures mitochondrial targeting fidelity avoiding inappropriate entrance of non-ER resident proteins to this organelle. Such vital task was demonstrated by studies in *Caenorhabditis elegans*, where NAC knockdown results in an aberrant mistargeting of mitochondrial

proteins to the ER independently of the signal recognition particle (SRP). As a consequence, there is an overload in the ER capability and mitochondrial malfunction, which triggers a UPR in both organelles, affecting the whole organism homeostasis (Figure 3B; Gamerdinger et al., 2015, 2019). Similarly, SRP acts as counterpart to NAC providing specificity to protein targeting to the ER, thereby avoiding mislocalization of ER proteins into mitochondria (Costa et al., 2018).

Even with the existence of the described responses to alterations on the import systems, mistargeting to other organelles such as the ER still occurs. To rescue these proteins and redirect them to mitochondria, two different mechanisms have been described to date. One involves Djp1, an Hsp40 chaperone that can be localized both to the ER and the cytosol. Djp1 senses mislocalized mitochondrial proteins at the ER surface and returns them to the TOM complex. The discovery of this ER-SURF pathway (ER surface retrieval pathway) supports the notion of buffering systems outside mitochondria that ensure proper mitochondrial transport (Figure 3C, pink arrows; Hansen et al., 2018). Additionally, a recently discovered group of chaperones, the ubiquilins, prevents the aggregation of tail-anchored mitochondrial proteins in the cytosol. The principal function of ubiquilins is to sense if those proteins can be retargeted to the OM or to facilitate their degradation by the proteasome (Figure 3C, violet arrows, Itakura et al., 2016).

The discovery of the different mechanisms by which cells can respond to alterations in mitochondrial protein targeting and import is encouraging toward a more integrative model that explains how mitochondrial protein targeting is finely tuned in response to metabolic changes or stress conditions.

An integrative view on mitochondrial protein recognition in the cytosol

Over the last decades, two models have been proposed for explaining mitochondrial protein import: posttranslational and cotranslational. Different components, such as chaperones, signals on the substrate proteins (i.e. presequence, hydrophobic TMDs, among others), or the signals on the mMPs, were described independently and with the limitations of studying only a handful of mitochondrial proteins. However, few efforts have been made to experimentally address the study of the elements that

mediate early stages of protein import, thereby neglecting the relevance of cotranslational acting elements *in vivo*, even for proteins that have been described to be imported posttranslationally *in vitro*. Furthermore, a comprehensive analysis is crucial to determine the precise role of posttranslational and cotranslational elements, either separately or simultaneously, to better understand how proteins are targeted to the mitochondria.

In this review, we sought to expose and integrate all the different variables that have been considered throughout the years and that must work at early steps of recognition, transport, and targeting for mitochondrial proteins (Figure 1). We therefore propose a model where a translationally active ribosome associated with ribosomal chaperones, such as NAC, identifies the synthesis of a new mitochondrial protein and helps to discriminate between a posttranslational and a cotranslational pathway. If the first is favored, the ribosome completes protein synthesis, and Hsp chaperones associate with the newly synthesized protein and escort it to the TOM complex (Figure 1A). Otherwise, Sam37 and Om14 function as receptors for NAC, thereby facilitating the approach of the translationally active ribosome to the vicinity of the TOM complex coupling mitochondrial translation with import (Figure 1B). Even though Om14 is known to be present only in yeast, it is possible that yet unidentified factors could mediate a similar function in other eukaryotes. We do not conceive these two systems as mutually exclusive *in vivo*, as some of the described components have been reported to have a role in both of them. It seems very plausible that the collection of signals at both the mRNA and protein levels, as well as the nature of the mitochondrial protein and the metabolic state of a cell, determines the factors required for transport of each mitochondrial protein. There are still several questions unanswered before we can fully understand the precise mechanisms underlying mitochondrial protein targeting *in vivo*, but the recent genome-wide analyses are shedding some light on the general mechanisms of recognition and targeting of mitochondrial proteins.

Acknowledgments: Our work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT, CB 237344) and the Dirección General de Asuntos del Personal Académico (DGAPA-UNAM, IN207518) to S.F. M.C.A.M. is a graduate student from Programa de Doctorado en Ciencias Bioquímicas, Universidad Nacional Autónoma de México (UNAM), and she is supported by CONACyT (464718). We would like to thank Johannes M. Herrmann, Diego González-Halphen, and Mauricio Cárdenas-Rodríguez for critically reading this manuscript.

References

- Abe, Y., Shodai, T., Muto, T., Mihara, K., Torii, H., Nishikawa, S., Endo, T., and Kohda, D. (2000). Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20. *Cell* 100, 551–560.
- Adrian, G., McCammon, M., Montgomery, D., and Douglas, M. (1986). Sequences required for delivery and localization of the ADP/ATP translocator to the mitochondrial inner membrane. *Mol. Cell. Biol.* 6, 626–634.
- Anderson, J.S. and Parker, R. (2000). Computational identification of *cis*-acting elements affecting post-transcriptional control of gene expression in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 28, 1604–1617.
- Araiso, Y., Tsutsumi, A., Qiu, J., Imai, K., Shiota, T., Song, J., Lindau, C., Wenz, L.-S., Sakaue, H., Yunoki, K., et al. (2019). Structure of the mitochondrial import gate reveals distinct preprotein paths. *Nature* 575, 395–401.
- Arakel, E.C. and Schwappach, B. (2018). Formation of COPI-coated vesicles at a glance. *J. Cell Sci.* 131, jcs209890.
- Backes, S., Hess, S., Boos, F., Woellhaf, M.W., Gödel, S., Jung, M., Mühlhaus, T., and Herrmann, J.M. (2018). Tom70 enhances mitochondrial preprotein import efficiency by binding to internal targeting sequences. *J. Cell Biol.* 217, jcb.201708044.
- Bausewein, T., Mills, D.J., Langer, J.D., Nitschke, B., Nussberger, S., and Kühlbrandt, W. (2017). Cryo-EM structure of the TOM core complex from *Neurospora crassa*. *Cell* 170, 693–700.
- Becker, J., Walter, W., Yan, W., and Craig, E. (1996). Functional interaction of cytosolic Hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation *in vivo*. *Mol. Cell. Biol.* 16, 4378–4386.
- Becker, T., Pfannschmidt, S., Guiard, B., Stojanovski, D., Milenkovic, D., Kutik, S., Pfanner, N., Meisinger, C., and Wiedemann, N. (2008). Biogenesis of the mitochondrial TOM complex Mim1 promotes insertion and assembly of signal-anchored receptors. *J. Biol. Chem.* 283, 120–127.
- Bhangoo, M.K., Tzankov, S., Fan, A., Dejgaard, K., Thomas, D.Y., and Young, J.C. (2007). Multiple 40-kDa heat-shock protein chaperones function in Tom70-dependent mitochondrial import. *Mol. Biol. Cell* 18, 3414–3428.
- Bihlmaier, K., Bien, M., and Herrmann, J.M. (2008). *In vivo* import of proteins into isolated mitochondria. In: *Methods in Molecular Biology*, Vol. 457: Membrane Trafficking, A. Vancura, ed. (Totowa, NJ, USA: Humana Press), pp. 85–94.
- Bloss, T.A., Witze, E.S., and Rothman, J.H. (2003). Suppression of CED-3-independent apoptosis by mitochondrial betaNAC in *Caenorhabditis elegans*. *Nature* 424, 1066–1071.
- Bolliger, L., Junne, T., Schatz, G., and Lithgow, T. (1995). Acidic receptor domains on both sides of the outer membrane mediate translocation of precursor proteins into yeast mitochondria. *EMBO J.* 14, 6318–6326.
- Boos, F., Krämer, L., Groh, C., Jung, F., Haberkant, P., Stein, F., Wollweber, F., Gackstatter, A., Zöller, E., van der Laan, M., et al. (2019). Mitochondrial protein-induced stress triggers a global adaptive transcriptional programme. *Nat. Cell Biol.* 21, 442–451.
- Brix, J., Dietmeier, K., and Pfanner, N. (1997). Differential recognition of preproteins by the purified cytosolic domains of the mitochondrial import receptors Tom20, Tom22, and Tom70. *J. Biol. Chem.* 272, 20730–20735.

- Brix, J., Rüdiger, S., Bukau, B., Schneider-Mergener, J., and Pfanner, N. (1999). Distribution of binding sequences for the mitochondrial import receptors Tom20, Tom22, and Tom70 in a presequence-carrying preprotein and a non-cleavable preprotein. *J. Biol. Chem.* 274, 16522–16530.
- Burri, L., Vascotto, K., Gentle, I.E., Chan, N.C., Beilharz, T., Stapleton, D.I., Ramage, L., and Lithgow, T. (2006). Integral membrane proteins in the mitochondrial outer membrane of *Saccharomyces cerevisiae*. *FEBS J.* 273, 1507–1515.
- Buxbaum, A.R., Haimovich, G., and Singer, R.H. (2015). In the right place at the right time: visualizing and understanding mRNA localization. *Nat. Rev. Mol. Cell Biol.* 16, 95–109.
- Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009). Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138, 628–644.
- Costa, E.A., Subramanian, K., Nunnari, J., and Weissman, J.S. (2018). Defining the physiological role of SRP in protein-targeting efficiency and specificity. *Science* 359, 689–692.
- del Alamo, M., Hogan, D.J., Pechmann, S., Albanese, V., Brown, P.O., and Frydman, J. (2011). Defining the specificity of cotranslationally acting chaperones by systematic analysis of mRNAs associated with ribosome-nascent chain complexes. *PLoS Biol.* 9, e1001100.
- Deng, J. and Behringer, R.R. (1995). An insertional mutation in the *BTF3* transcription factor gene leads to an early postimplantation lethality in mice. *Transgenic Res.* 4, 264–269.
- Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A., and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* 332, 800–805.
- Dimmer, K. and Rapaport, D. (2010). The enigmatic role of Mim1 in mitochondrial biogenesis. *Eur. J. Cell Biol.* 89, 212–215.
- Dock-Bregeon, A., Lewis, K.A., and Conte, M.R. (2019). The La-related proteins: structures and interactions of a versatile superfamily of RNA binding proteins. *RNA Biol.*, DOI:10.1080/15476286.2019.1695712.
- Döring K., Ahmed N., Riemer T., Suresh H.G., Vainshtein Y., Habich M., Riemer J., Mayer M.P., O'Brien E.P., Kramer G., and Bukau B. (2017). Profiling Ssb-nascent chain interactions reveals principles of Hsp70-assisted folding. *Cell* 170, 298–311.
- Eliyahu, E., Pnueli, L., Melamed, D., Scherrer, T., Gerber, A.P., Pines, O., Rapaport, D., and Arava, Y. (2010). Tom20 mediates localization of mRNAs to mitochondria in a translation-dependent manner. *Mol. Cell Biol.* 30, 284–294.
- Fan, A.C., Bhangoo, M.K., and Young, J.C. (2006). Hsp90 functions in the targeting and outer membrane translocation steps of Tom70-mediated mitochondrial import. *J. Biol. Chem.* 281, 33313–33324.
- Fazal, F.M., Han, S., Parker, K.R., Kaewsapsak, P., Xu, J., Boettiger, A.N., Chang, H.Y., and Ting, A.Y. (2019). Atlas of subcellular RNA localization revealed by APEX-Seq. *Cell* 178, 473–490.
- Fünfschilling, U. and Rospert, S. (1999). Nascent polypeptide-associated complex stimulates protein import into yeast mitochondria. *Mol. Biol. Cell* 10, 3289–3299.
- Gadir, N., Haim-Vilmsky, L., Kraut-Cohen, J., and Gerst, J.E. (2011). Localization of mRNAs coding for mitochondrial proteins in the yeast *Saccharomyces cerevisiae*. *RNA* 17, 1551–1565.
- Gamerding, M., Hanebuth, M., Frickey, T., and Deuerling, E. (2015). The principle of antagonism ensures protein targeting specificity at the endoplasmic reticulum. *Science* 348, 201–207.
- Gamerding, M., Kobayashi, K., Wallisch, A., Kreft, S.G., Sailer, C., Schlömer, R., Sachs, N., Jomaa, A., Stengel, F., Ban, N., et al. (2019). Early scanning of nascent polypeptides inside the ribosomal tunnel by NAC. *Mol. Cell* 75, 996–1006.
- Gao, J., Schatton, D., Martinelli, P., Hansen, H., Pla-Martin, D., Barth, E., Becker, C., Altmueller, J., Frommolt, P., Sardiello, M., et al. (2014). CLUH regulates mitochondrial biogenesis by binding mRNAs of nuclear-encoded mitochondrial proteins. *J. Cell Biol.* 207, 213–223.
- Garcia, M., Darzacq, X., Delaveau, T., Jourden, L., Singer, R., and Jacq, C. (2007). Mitochondria-associated yeast mRNAs and the biogenesis of molecular complexes. *Mol. Biol. Cell.* 18, 362–368.
- García-Rodríguez, L.J., Gay, A., and Pon, L.A. (2007). Puf3p, a Pumilio family RNA binding protein, localizes to mitochondria and regulates mitochondrial biogenesis and motility in budding yeast. *J. Cell Biol.* 176, 197–207.
- Garg, S.G. and Gould, S.B. (2017). The role of charge in protein targeting evolution. *Trends Cell Biol.* 27, 894–905.
- George, R., Beddoe, T., Landl, K., and Lithgow, T. (1998). The yeast nascent polypeptide-associated complex initiates protein targeting to mitochondria *in vivo*. *Proc. Natl. Acad. Sci. USA* 95, 2296–2301.
- George, R., Walsh, P., Beddoe, T., and Lithgow, T. (2002). The nascent polypeptide-associated complex (NAC) promotes interaction of ribosomes with the mitochondrial surface *in vivo*. *FEBS Lett.* 516, 213–216.
- Gerber, A.P., Herschlag, D., and Brown, P.O. (2004). Extensive association of functionally and cytotopically related mRNAs with Puf family RNA-binding proteins in yeast. *PLoS Biol.* 2, E79.
- Gold, V.A., Chrosicki, P., Bragoszewski, P., and Chacinska, A. (2017). Visualization of cytosolic ribosomes on the surface of mitochondria by electron cryo-tomography. *EMBO Rep.* 18, 1786–1800.
- Gratzer, S., Lithgow, T., Bauer, R., Lamping, E., Paltauf, F., Kohlwein, S., Haucke, V., Junne, T., Schatz, G., and Horst, M. (1995). Mas37p, a novel receptor subunit for protein import into mitochondria. *J. Cell Biol.* 129, 25–34.
- Hansen, K.G., Aviram, N., Laborenz, J., Bibi, C., Meyer, M., Spang, A., Schuldiner, M., and Herrmann, J.M. (2018). An ER surface retrieval pathway safeguards the import of mitochondrial membrane proteins in yeast. *Science* 361, 1118–1122.
- Harkness, T., Nargang, F., van der Klei, I., Neupert, W., and Lill, R. (1994). A crucial role of the mitochondrial protein import receptor MOM19 for the biogenesis of mitochondria. *J. Cell Biol.* 124, 637–648.
- Harmey, M.A., Hallermayer, G., Korb, H., and Neupert, W. (1977). Transport of cytoplasmically synthesized proteins into the mitochondria in a cell free system from *Neurospora crassa*. *Eur. J. Biochem.* 81, 533–544.
- Haucke, V., Horst, M., Schatz, G., and Lithgow, T. (1996). The Mas20p and Mas70p subunits of the protein import receptor of yeast mitochondria interact via the tetratricopeptide repeat motif in Mas20p: evidence for a single hetero-oligomeric receptor. *EMBO J.* 15, 1231–1237.
- Herrmann, J.M. (2003). Converting bacteria to organelles: evolution of mitochondrial protein sorting. *Trends Microbiol.* 11, 74–79.
- Hines, V. and Schatz, G. (1993). Precursor binding to yeast mitochondria. A general role for the outer membrane protein Mas70p. *J. Biol. Chem.* 268, 449–454.
- Hines, V., Brandt, A., Griffiths, G., Horstmann, H., Brüttsch, H., and Schatz, G. (1990). Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70. *EMBO J.* 9, 3191–3200.

- Hönlinger, A., Kübrich, M., Moczek, M., Gärtner, F., Mallet, L., Bussereau, F., Eckerskorn, C., Lottspeich, F., Dietmeier, K., and Jacquet, M. (1995). The mitochondrial receptor complex: Mom22 is essential for cell viability and directly interacts with preproteins. *Mol. Cell. Biol.* 15, 3382–3389.
- Hoseini, H., Pandey, S., Jores, T., Schmitt, A., Franz-Wachtel, M., Macek, B., Buchner, J., Dimmer, K., and Rapaport, D. (2016). The cytosolic cochaperone Sti1 is relevant for mitochondrial biogenesis and morphology. *FEBS J.* 283, 3338–3352.
- Hu, G.-Z. and Ronne, H. (1994). Yeast BTF3 protein is encoded by duplicated genes and inhibits the expression of some genes in vivo. *Nucleic Acids Res.* 22, 2740–2743.
- Ishikawa, D., Yamamoto, H., Tamura, Y., Moritoh, K., and Endo, T. (2004). Two novel proteins in the mitochondrial outer membrane mediate β -barrel protein assembly. *J. Cell Biol.* 166, 621–627.
- Itakura, E., Zavodszky, E., Shao, S., Wohlever, M.L., Keenan, R.J., and Hegde, R.S. (2016). Ubiquilins chaperone and triage mitochondrial membrane proteins for degradation. *Mol. Cell* 63, 21–33.
- Jan, C.H., Williams, C.C., and Weissman, J.S. (2014). Principles of ER cotranslational translocation revealed by proximity-specific ribosome profiling. *Science* 346, 1257521.
- Jiang, H., Guan, W., and Gu, Z. (2010). Tinkering evolution of post-transcriptional RNA regulons: Puf3p in fungi as an example. *PLoS Genet.* 6, e1001030.
- Jores, T., Klinger, A., Groß, L.E., Kawano, S., Flinner, N., Duchardt-Ferner, E., Wöhrner, J., Kalbacher, H., Endo, T., Schleiff, E., et al. (2016). Characterization of the targeting signal in mitochondrial β -barrel proteins. *Nat. Commun.* 7, 12036.
- Jores, T., Lawatschek, J., Beke, V., Franz-Wachtel, M., Yunoki, K., Fitzgerald, J.C., Macek, B., Endo, T., Kalbacher, H., Buchner, J., et al. (2018). Cytosolic Hsp70 and Hsp40 chaperones enable the biogenesis of mitochondrial β -barrel proteins. *J. Cell Biol.* 217, 3091–3108.
- Kaltimbacher, V., Bonnet, C., Lecoeuvre, G., Forster, V., Sahel, J.-A., and Corral-Debrinski, M. (2006). mRNA localization to the mitochondrial surface allows the efficient translocation inside the organelle of a nuclear recoded ATP6 protein. *RNA* 12, 1408–1417.
- Kellems, R. and Butow, R. (1972). Cytoplasmic-type 80 S ribosomes associated with yeast mitochondria. I. Evidence for ribosome binding sites on yeast mitochondria. *J. Biol. Chem.* 247, 8043–8050.
- Kellems, R. and Butow, R. (1974). Cytoplasmic type 80 S ribosomes associated with yeast mitochondria. III. Changes in the amount of bound ribosomes in response to changes in metabolic state. *J. Biol. Chem.* 249, 3304–3310.
- Kellems, R., Allison, V., and Butow, R. (1974). Cytoplasmic type 80S ribosomes associated with yeast mitochondria. II. Evidence for the association of cytoplasmic ribosomes with the outer mitochondrial membrane *in situ*. *J. Biol. Chem.* 249, 3297–3303.
- Kellems, R., Allison, V., and Butow, R. (1975). Cytoplasmic type 80S ribosomes associated with yeast mitochondria. IV. Attachment of ribosomes to the outer membrane of isolated mitochondria. *J. Cell Biol.* 65, 1–14.
- Kershaw, C.J., Costello, J.L., Talavera, D., Rowe, W., Castelli, L.M., Sims, P.F., Grant, C.M., Ashe, M.P., Hubbard, S.J., and Pavitt, G.D. (2015). Integrated multi-omics analyses reveal the pleiotropic nature of the control of gene expression by Puf3p. *Sci. Rep.* 5, 15518.
- Kiebler, M., Pfaller, R., Söllner, T., Griffiths, G., Horstmann, H., Pfanner, N., and Neupert, W. (1990). Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. *Nature* 348, 610–616.
- Kiebler, M., Keil, P., Schneider, H., van der Klei, I.J., Pfanner, N., and Neupert, W. (1993). The mitochondrial receptor complex: a central role of MOM22 in mediating preprotein transfer from receptors to the general insertion pore. *Cell* 74, 483–492.
- Knox, C., Sass, E., Neupert, W., and Pines, O. (1998). Import into mitochondria, folding and retrograde movement of fumarate in yeast. *J. Biol. Chem.* 273, 25587–25593.
- Komiya, T., Rospert, S., Schatz, G., and Mihara, K. (1997). Binding of mitochondrial precursor proteins to the cytoplasmic domains of the import receptors Tom70 and Tom20 is determined by cytoplasmic chaperones. *EMBO J.* 16, 4267–4275.
- Kozjak, V., Wiedemann, N., Milenkovic, D., Lohaus, C., Meyer, H.E., Guiard, B., Meisinger, C., and Pfanner, N. (2003). An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. *J. Biol. Chem.* 278, 48520–48523.
- Künkele, K.-P., Juin, P., Pompa, C., Nargang, F.E., Henry, J.-P., Neupert, W., Lill, R., and Thieffry, M. (1998). The isolated complex of the translocase of the outer membrane of mitochondria. Characterization of the cation-selective and voltage-gated preprotein-conducting pore. *J. Biol. Chem.* 273, 31032–31039.
- Lauffer, S., Mäbert, K., Czupalla, C., Pursche, T., Hoflack, B., Rödel, G., and Krause-Buchholz, U. (2012). *Saccharomyces cerevisiae* porin pore forms complexes with mitochondrial outer membrane proteins Om14p and Om45p. *J. Biol. Chem.* 287, 17447–17458.
- Lee, C.-D. and Tu, B.P. (2015). Glucose-regulated phosphorylation of the PUF protein Puf3 regulates the translational fate of its bound mRNAs and association with RNA granules. *Cell Rep.* 11, 1638–1650.
- Lesnik, C., Cohen, Y., Atir-Lande, A., Schuldiner, M., and Arava, Y. (2014). OM14 is a mitochondrial receptor for cytosolic ribosomes that supports co-translational import into mitochondria. *Nat. Commun.* 5, 5711.
- Li, J., Qian, X., Hu, J., and Sha, B. (2009). Molecular Chaperone Hsp70/Hsp90 prepares the mitochondrial outer membrane translocon receptor Tom71 for preprotein loading. *J. Biol. Chem.* 284, 23852–23859.
- Lithgow, T., Junne, T., Suda, K., Gratzer, S., and Schatz, G. (1994). The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast. *Proc. Natl. Acad. Sci. USA* 91, 11973–11977.
- Liu, J.M. and Liu, D.R. (2007). Discovery of a mRNA mitochondrial localization element in *Saccharomyces cerevisiae* by nonhomologous random recombination and *in vivo* selection. *Nucleic Acids Res.* 35, 6750–6761.
- Luk, E., Yang, M., Jensen, L.T., Bourbonnais, Y., and Culotta, V. (2005). Manganese activation of superoxide dismutase 2 in the mitochondria of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280, 22715–22720.
- Maccacchini, M., Rudin, Y., Blobel, G., and Schatz, G. (1979a). Import of proteins into mitochondria: precursor forms of the extramitochondrially made F1-ATPase subunits in yeast. *Proc. Natl. Acad. Sci. USA* 76, 343–347.
- Maccacchini, M., Rudin, Y., and Schatz, G. (1979b). Transport of proteins across the mitochondrial outer membrane. A precursor form of the cytoplasmically made intermembrane enzyme cytochrome c peroxidase. *J. Biol. Chem.* 254, 7468–7471.

- Marc, P., Margeot, A., Devaux, F., Blugeon, C., Debrinski, M., and Jacq, C. (2002). Genome-wide analysis of mRNAs targeted to yeast mitochondria. *EMBO Rep.* 3, 159–164.
- Margeot, A., Blugeon, C., Sylvestre, J., Vialette, S., Jacq, C., and Debrinski, M. (2002). In *Saccharomyces cerevisiae*, ATP2 mRNA sorting to the vicinity of mitochondria is essential for respiratory function. *EMBO J.* 21, 6893–6904.
- Markesich, D., Gajewski, K., Nazimiec, M., and Beckingham, K. (2000). Bicaudal encodes the *Drosophila* beta NAC homolog, a component of the ribosomal translational machinery. *Development* 127, 559–572.
- Mårtensson, C.U., Priesnitz, C., Song, J., Ellenrieder, L., Doan, K., Boos, F., Floerchinger, A., Zufall, N., Oeljeklaus, S., Warscheid, B., et al. (2019). Mitochondrial protein translocation-associated degradation. *Nature* 569, 679–683.
- Matsumoto, S., Nakatsukasa, K., Kakuta, C., Tamura, Y., Esaki, M., and Endo, T. (2019). Msp1 clears mistargeted proteins by facilitating their transfer from mitochondria to the ER. *Mol. Cell* 76, 191–205.
- Mayer, A., Nargang, F., Neupert, W., and Lill, R. (1995). MOM22 is a receptor for mitochondrial targeting sequences and cooperates with MOM19. *EMBO J.* 14, 4204–4211.
- Melin, J., Kilisch, M., Neumann, P., Lytovchenko, O., Gomkale, R., Schendzielorz, A., Schmidt, B., Liepold, T., Ficner, R., Jahn, O., et al. (2015). A presequence-binding groove in Tom70 supports import of Mdl1 into mitochondria. *Biochim. Biophys. Acta* 1853, 1850–1859.
- Milenkovic, D., Kozjak, V., Wiedemann, N., Lohaus, C., Meyer, H.E., Guiard, B., Pfanner, N., and Meisinger, C. (2004). Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. *J. Biol. Chem.* 279, 22781–22785.
- Miller, M.A., Russo, J., Fischer, A.D., Leban, F.A., and Olivas, W.M. (2014). Carbon source-dependent alteration of Puf3p activity mediates rapid changes in the stabilities of mRNAs involved in mitochondrial function. *Nucleic Acids Res.* 42, 3954–3970.
- Mitchell, S.F., Jain, S., She, M., and Parker, R. (2013). Global analysis of yeast mRNPs. *Nat. Struct. Mol. Biol.* 20, 127–133.
- Moczko, M., Dietmeier, K., Söllner, T., Segui, B., Steger, H.F., Neupert, W., and Pfanner, N. (1992). Identification of the mitochondrial receptor complex in *Saccharomyces cerevisiae*. *FEBS Lett.* 310, 265–268.
- Moczko, M., Ehmann, B., Gärtner, F., Hönlinger, A., Schäfer, E., and Pfanner, N. (1994). Deletion of the receptor MOM19 strongly impairs import of cleavable preproteins into *Saccharomyces cerevisiae* mitochondria. *J. Biol. Chem.* 269, 9045–9051.
- Moczko, M., Bömer, U., Kübrich, M., Zufall, N., Hönlinger, A., and Pfanner, N. (1997). The intermembrane space domain of mitochondrial Tom22 functions as a trans binding site for preproteins with N-terminal targeting sequences. *Mol. Cell. Biol.* 17, 6574–6584.
- Model, K., Prinz, T., Ruiz, T., Radermacher, M., Krimmer, T., Kühlbrandt, W., Pfanner, N., and Meisinger, C. (2002). Protein translocase of the outer mitochondrial membrane: role of import receptors in the structural organization of the TOM complex. *J. Mol. Biol.* 316, 657–666.
- Olivas, W. and Parker, R. (2000). The Puf3 protein is a transcript-specific regulator of mRNA degradation in yeast. *EMBO J.* 19, 6602–6611.
- Opaliński, Ł., Song, J., Priesnitz, C., Wenz, L.S., Oeljeklaus, S., Warscheid, B., Pfanner, N., and Becker, T. (2018). Recruitment of cytosolic J-proteins by TOM receptors promotes mitochondrial protein biogenesis. *Cell Rep.* 25, 2036–2043.
- Palade, G.E. (1952). The fine structure of mitochondria. *Anat. Rec.* 114, 427–451.
- Papić, D., Elbaz-Alon, Y., Koerdt, S., Leopold, K., Worm, D., Jung, M., Schuldiner, M., and Rapaport, D. (2013). The role of Djpl in import of the mitochondrial protein Mim1 demonstrates specificity between a cochaperone and its substrate protein. *Mol. Cell. Biol.* 33, 4083–4094.
- Paschen, S.A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D., and Neupert, W. (2003). Evolutionary conservation of biogenesis of beta-barrel membrane proteins. *Nature* 426, 862–866.
- Ponce-Rojas, J., Avendaño-Monsalve, M., Yañez-Falcón, A., Jaimes-Miranda, F., Garay, E., Torres-Quiroz, F., Deluna, A., and Funes, S. (2017). $\alpha\beta$ -NAC cooperates with Sam37 to mediate early stages of mitochondrial protein import. *FEBS J.* 284, 814–830.
- Popov-Čeleketić, J., Waizenegger, T., and Rapaport, D. (2008). Mim1 functions in an oligomeric form to facilitate the integration of Tom20 into the mitochondrial outer membrane. *J. Mol. Biol.* 376, 671–680.
- Quenault, T., Lithgow, T., and Traven, A. (2011). PUF proteins: repression, activation and mRNA localization. *Trends Cell Biol.* 21, 104–112.
- Ramage, L., Junne, T., Hahne, K., Lithgow, T., and Schatz, G. (1993). Functional cooperation of mitochondrial protein import receptors in yeast. *EMBO J.* 12, 4115–4123.
- Reid, G. and Schatz, G. (1982). Import of proteins into mitochondria. Yeast cells grown in the presence of carbonyl cyanide m-chlorophenylhydrazone accumulate massive amounts of some mitochondrial precursor polypeptides. *J. Biol. Chem.* 257, 13056–13061.
- Reimann, B., Bradsher, J., Franke, J., Hartmann, E., Wiedmann, M., Prehn, S., and Wiedmann, B. (1999). Initial characterization of the nascent polypeptide-associated complex in yeast. *Yeast* 15, 397–407.
- Rimmer, K.A., Foo, J., Ng, A., Petrie, E.J., Shilling, P.J., Perry, A.J., Mertens, H.D., Lithgow, T., Mulhern, T.D., and Gooley, P.R. (2011). Recognition of mitochondrial targeting sequences by the import receptors Tom20 and Tom22. *J. Mol. Biol.* 405, 804–818.
- Roger, A.J., Muñoz-Gómez, S.A., and Kamikawa, R. (2017). The origin and diversification of mitochondria. *Curr. Biol.* 27, 1177–1192.
- Saibil, H. (2013). Chaperone machines for protein folding, unfolding and disaggregation. *Nat. Rev. Mol. Cell Biol.* 14, 630–642.
- Saint-Georges, Y., Garcia, M., Delaveau, T., Jourdain, L., Crom, S., Lemoine, S., Tanty, V., Devaux, F., and Jacq, C. (2008). Yeast mitochondrial biogenesis: a role for the PUF RNA-binding protein Puf3p in mRNA localization. *PLoS One* 3, e2293.
- Sakaue, H., Shiota, T., Ishizaka, N., Kawano, S., Tamura, Y., Tan, K., Imai, K., Motono, C., Hirokawa, T., Taki, K., et al. (2019). Porin associates with Tom22 to regulate the mitochondrial protein gate assembly. *Mol. Cell* 73, 1044–1055.
- Sass, E., Karniely, S., and Pines, O. (2003). Folding of fumarate during mitochondrial import determines its dual targeting in yeast. *J. Biol. Chem.* 278, 45109–45116.
- Schatton, D. and Rugarli, E.I. (2019). A concert of RNA-binding proteins coordinates mitochondrial function. *Crit. Rev. Biochem. Biol.* 53, 652–666.

- Schlossmann, J., Dietmeier, K., Pfanner, N., and Neupert, W. (1994). Specific recognition of mitochondrial preproteins by the cytosolic domain of the import receptor MOM72. *J. Biol. Chem.* 269, 11893–11901.
- Schlossmann, J., Lill, R., Neupert, W., and Court, D.A. (1996). Tom71, a novel homologue of the mitochondrial preprotein receptor Tom70. *J. Biol. Chem.* 271, 17890–17895.
- Shi, X., Parthun, M.R., and Jaehning, J.A. (1995). The yeast *EGD2* gene encodes a homologue of the α NAC subunit of the human nascent-polypeptide-associated complex. *Gene* 165, 199–202.
- Shiota, T., Mabuchi, H., Tanaka-Yamano, S., Yamano, K., and Endo, T. (2011). *In vivo* protein-interaction mapping of a mitochondrial translocator protein Tom22 at work. *Proc. Natl. Acad. Sci. USA* 108, 15179–15183.
- Shiota, T., Imai, K., Qiu, J., Hewitt, V.L., Tan, K., Shen, H.-H., Sakiyama, N., Fukasawa, Y., Hayat, S., Kamiya, M., et al. (2015). Molecular architecture of the active mitochondrial protein gate. *Science* 349, 1544–1548.
- Slobodin, B. and Gerst, J.E. (2010). A novel mRNA affinity purification technique for the identification of interacting proteins and transcripts in ribonucleoprotein complexes. *RNA* 16, 2277–2290.
- Söllner, T., Griffiths, G., Pfaller, R., Pfanner, N., and Neupert, W. (1989). MOM19, an import receptor for mitochondrial precursor proteins. *Cell* 59, 1061–1070.
- Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W. (1990). A mitochondrial import receptor for the ADP/ATP carrier. *Cell* 62, 107–115.
- Spang, A., Stairs, C.W., Dombrowski, N., Eme, L., Lombard, J., Caceres, E.F., Greening, C., Baker, B.J., and Ettema, T.J. (2019). Proposal of the reverse flow model for the origin of the eukaryotic cell based on comparative analyses of Asgard archaeal metabolism. *Nat. Microbiol.* 4, 1138–1148.
- Steger, H., Söllner, T., Kiebler, M., Dietmeier, K., Pfaller, R., Trülsch, K., Tropschug, M., Neupert, W., and Pfanner, N. (1990). Import of ADP/ATP carrier into mitochondria: two receptors act in parallel. *J. Cell Biol.* 111, 2353–2363.
- Stein K.C., Kriel A., and Frydman J. (2019). Nascent polypeptide domain topology and elongation rate direct the cotranslational hierarchy of Hsp70 and TRIC/CCT. *Mol. Cell* 75, 1117–1130.
- Stojanovski, D., Guiard, B., Kozjak-Pavlovic, V., Pfanner, N., and Meisinger, C. (2007). Alternative function for the mitochondrial SAM complex in biogenesis of α -helical TOM proteins. *J. Cell Biol.* 179, 881–893.
- Suissa, M. and Schatz, G. (1982). Import of proteins into mitochondria. Translatable mRNAs for imported mitochondrial proteins are present in free as well as mitochondria-bound cytoplasmic polysomes. *J. Biol. Chem.* 257, 13048–13055.
- Sylvestre, J., Margeot, A., Jacq, C., Dujardin, G., and Corral-Debrinski, M. (2003). The role of the 3' untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells. *Mol. Biol. Cell* 14, 3848–3856.
- Thornton, N., Stroud, D.A., Milenkovic, D., Guiard, B., Pfanner, N., and Becker, T. (2010). Two modular forms of the mitochondrial sorting and assembly machinery are involved in biogenesis of α -helical outer membrane proteins. *J. Mol. Biol.* 396, 540–549.
- Tucker, K. and Park, E. (2019). Cryo-EM structure of the mitochondrial protein-import channel TOM complex at near-atomic resolution. *Nat. Struct. Mol. Biol.* 26, 1158–1166.
- Vornlocher, H.-P., Hanachi, P., Ribeiro, S., and Hershey, J.W. (1999). A 110-kilodalton subunit of translation initiation factor eIF3 and an associated 135-kilodalton protein are encoded by the *Saccharomyces cerevisiae* TIF32 and TIF31 genes. *J. Biol. Chem.* 274, 16802–16812.
- Waizenegger, T., Habib, S.J., Lech, M., Mokranjac, D., Paschen, S.A., Hell, K., Neupert, W., and Rapaport, D. (2004). Tob38, a novel essential component in the biogenesis of beta-barrel proteins of mitochondria. *EMBO Rep.* 5, 704–709.
- Waizenegger, T., Schmitt, S., Zivkovic, J., Neupert, W., and Rapaport, D. (2005). Mim1, a protein required for the assembly of the TOM complex of mitochondria. *EMBO Rep.* 6, 57–62.
- Wang, X. and Chen, X. (2015). A cytosolic network suppressing mitochondria-mediated proteostatic stress and cell death. *Nature* 524, 481–484.
- Wang, X., Zamore, P.D., and Hall, T. (2001). Crystal structure of a Pumilio homology domain. *Mol. Cell* 7, 855–865.
- Wang, X., McLachlan, J., Zamore, P.D., and Hall, T. (2002). Modular recognition of RNA by a human Pumilio-homology domain. *Cell* 110, 501–512.
- Wang, M., Ogé, L., Perez-Garcia, M.-D., Hamama, L., and Sakr, S. (2018). The PUF protein family: overview on PUF RNA targets, biological functions, and post transcriptional regulation. *Int. J. Mol. Sci.* 19, 410.
- Wang, Z., Sun, X., Wee, J., Guo, X., and Gu, Z. (2019). Novel insights into global translational regulation through Pumilio family RNA-binding protein Puf3p revealed by ribosomal profiling. *Curr. Genet.* 65, 201–212.
- Wegrzyn, R.D., Hofmann, D., Merz, F., Nikolay, R., Rauch, T., Graf, C., and Deuerling, E. (2006). A conserved motif is prerequisite for the interaction of NAC with ribosomal protein L23 and nascent chains. *J. Biol. Chem.* 281, 2847–2857.
- Weidberg, H. and Amon, A. (2018). MitoCPR – a surveillance pathway that protects mitochondria in response to protein import stress. *Science* 360, eaan4146.
- Wenz, L.-S., Ellenrieder, L., Qiu, J., Bohnert, M., Zufall, N., van der Laan, M., Pfanner, N., Wiedemann, N., and Becker, T. (2015). Sam37 is crucial for formation of the mitochondrial TOM-SAM supercomplex, thereby promoting β -barrel biogenesis. *J. Cell Biol.* 210, 1047–1054.
- Wiedemann, N., Kozjak, V., Chacinska, A., Schönfisch, B., Rospert, S., Ryan, M.T., Pfanner, N., and Meisinger, C. (2003). Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature* 424, 565–571.
- Williams, C.C., Jan, C.H., and Weissman, J.S. (2014). Targeting and plasticity of mitochondrial proteins revealed by proximity-specific ribosome profiling. *Science* 346, 748–751.
- Wrobel, L., Topf, U., Bragoszewski, P., Wiese, S., Sztolsztener, M.E., Oeljeklaus, S., Varabyova, A., Lirski, M., Chroscicki, P., Mroczek, S., et al. (2015). Mistargeted mitochondrial proteins activate a proteostatic response in the cytosol. *Nature* 524, 485–488.
- Wu, Y. and Sha, B. (2006). Crystal structure of yeast mitochondrial outer membrane translocon member Tom70p. *Nat. Struct. Mol. Biol.* 13, 589–593.
- Yamano, K., Yatsukawa, Y., Esaki, M., Hobbs, A.E., Jensen, R.E., and Endo, T. (2008). Tom20 and Tom22 share the common signal recognition pathway in mitochondrial protein import. *J. Biol. Chem.* 283, 3799–3807.

- Yano, M., Terada, K., and Mori, M. (2003). Mitochondrial import receptors Tom20 and Tom22 have chaperone-like activity. *J. Biol. Chem.* 279, 10808–10813.
- Yogev, O., Karniely, S., and Pines, O. (2007). Translation-coupled translocation of yeast fumarate into mitochondria *in vivo*. *J. Biol. Chem.* 282, 29222–29229.
- Young, J.C., Hoogenraad, N.J., and Hartl, F.U. (2003). Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. *Cell* 112, 41–50.
- Zabehzinsky, D., Slobodin, B., Rapaport, D., and Gerst, J.E. (2016). An essential role for COPI in mRNA localization to mitochondria and mitochondrial function. *Cell Rep.* 15, 540–549.
- Zanphorlin, L.M., Lima, T.B., Wong, M.J., Balbuena, T.S., Minetti, C.A., Remeta, D.P., Young, J.C., Barbosa, L.R., Gozzo, F.C., and Ramos, C.H. (2016). Heat shock protein 90 kDa (Hsp90) has a second functional interaction site with the mitochondrial import receptor Tom70. *J. Biol. Chem.* 291, 18620–18631.
- Zaremba-Niedzwiedzka, K., Caceres, E.F., Saw, J.H., Bäckström, D., Juzokaite, L., Vancaester, E., Seitz, K.W., Anantharaman, K., Starnawski, P., Kjeldsen, K.U., et al. (2017). Asgard archaea illuminate the origin of eukaryotic cellular complexity. *Nature* 541, 353–358.
- Zhang, Y., Chen, Y., Gucek, M., and Xu, H. (2016). The mitochondrial outer membrane protein MDI promotes local protein synthesis and mtDNA replication. *EMBO J.* 35, 1045–1057.
- Zhu, Q., Hulen, D., Liu, T., and Clarke, M. (1997). The *cluA*-mutant of *Dictyostelium* identifies a novel class of proteins required for dispersion of mitochondria. *Proc. Natl. Acad. Sci. USA* 94, 7308–7313.

José Carlos Ponce-Rojas

Department of Molecular, Cellular, and Developmental Biology, University of California at Santa Barbara, Santa Barbara, CA 93106-9625, USA

José Carlos Ponce-Rojas earned his PhD at UNAM studying protein targeting to the mitochondria. Currently, he is a postdoctoral fellow at the University of California at Santa Barbara, California, investigating how stress responses arise in eukaryotic cells to stabilize internal composition imbalances.

Soledad Funes

Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Circuito Exterior s/n Ciudad Universitaria Coyoacán, México, Cd.Mx. 04510, Mexico, sfunes@ifc.unam.mx, <https://orcid.org/0000-0003-1815-1977>

Soledad Funes is an associate professor in the Department of Molecular Genetics of the Cell Physiology Institute at UNAM. Her research focuses on different aspects of mitochondrial biogenesis including the function of the NAC complex during mitochondrial protein import.

Bionotes

Maria Clara Avendaño-Monsalve

Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Circuito Exterior s/n Ciudad Universitaria Coyoacán, México, Cd.Mx. 04510, Mexico

Maria Clara Avendaño-Monsalve was born in Medellín, Colombia. She is a Biological Engineer by the National University of Colombia-Campus Medellín. She moved to Mexico City and earned a master's degree in Biochemical Sciences by the National Autonomous University of Mexico (UNAM). She is currently doing a PhD at the same university studying how mitochondrial proteins arrive to the mitochondrial surface with the help of the ribosomal-associated chaperone NAC.