The Biogenesis of Mitochondrial Intermembrane Space Proteins

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ABSTRACT

The mitochondrial intermembrane space (IMS) houses a large spectrum of proteins with distinct and critical functions. Protein import into this mitochondrial sub-compartment is underpinned by an intriguing variety of pathways, many of which are still poorly understood. The constricted volume of the IMS and the topological segregation by the inner membrane cristae into a bulk area surrounded by the boundary inner membrane and the lumen within the cristae is an important factor that adds to the complexity of the protein import, folding and assembly processes. We discuss the main import pathways into the IMS, but also how IMS proteins are degraded or even retro-translocated to the cytosol in an integrated network of interactions that is necessary to maintain a healthy balance of IMS proteins under physiological and cellular stress conditions. We conclude this review by highlighting new and exciting perspectives in this area with a view to develop a better understanding of yet unknown, likely unconventional import pathways, how presequence-less proteins can be targeted and the basis for dual localisation in the IMS and the cytosol. Such knowledge is critical to understand the dynamic changes of the IMS proteome in response to stress, and particularly important for maintaining optimal mitochondrial fitness.

Keywords: mitochondria, protein folding, oxidative folding, intermembrane space, protein import

INTRODUCTION

The mitochondrial intermembrane space (IMS) is a small sub-compartment of only about 60 nanometres in diameter that is found between the outer and inner membranes of mitochondria (Frey and Mannella, 2000). Although small, the compartment is vital for mitochondrial function. The IMS acts as a crucial buffer between the cytosol and mitochondrial matrix through the exchange and maintenance of metabolites, lipids, proteins, metals and other enzyme cofactors necessary for mitochondrial function as well as redox regulation. IMS proteins play a role in the assembly of some of the respiratory chain complexes and help maintain the optimal activity of the respiratory chain, while some of them are critical for the initiation of apoptosis (Khalimonchuk and Winge, 2008; Bock and Tait, 2020).

The IMS comprises ~51 proteins in the yeast *S. cerevisiae* and ~127 proteins in humans (Vogtle *et al.*,2012; Hung *et al.*,2014). None of the proteins found in the IMS are encoded in the mitochondrial genome and therefore have to be imported into the organelle. Numerous diverse IMS protein import pathways have been discovered. The two most studied import pathways are the Mia40 oxidative folding pathway (also known as the disulfide relay pathway) and the stop-transfer pathway (Gasser *et al.*, 1982; Chacinska *et al.*, 2004). The import pathways of the IMS localised respiratory chain components cytochrome C and cytochrome C haem lyase are the only two other well-characterised IMS import pathways (Lill *et al.*, 1992; Mayer *et al.*, 1995). On the other hand, regulation and turnover of the IMS proteome is imperative to prevent protein misfolding and aggregation(Schreiner *et al.*, 2012a). Three proteostatic mechanisms have been discovered to regulate the IMS proteome. The inner membrane AAA- protease, Yme1 degrades IMS proteins (Leonhard *et al.*, 1996), whilst a

form of retro-translocation of proteins through the mitochondrial import gate, TOM (translocase of the outer membrane) into the cytosol has been uncovered for Mia40 substrates (Bragoszewski *et al.*, 2013). The cytosolic ubiquitin proteasome system has also been shown to degrade many IMS proteins (Bragoszewski *et al.*, 2015).

A part of the IMS proteome comprises dually localised proteins many of which localise to the cytosol. Only two proteins that localise dually both in the IMS and the cytosol have their import pathways characterised, Sod1 and Ccs1 (Gross *et al.*, 2011; Klöppel *et al.*, 2011; Reddehase et al., 2009; Field et al., 2003). The import pathways of other dually localised IMS proteins have yet to be uncovered.

This review will highlight IMS protein biogenesis focussing on the Mia40 disulfide relay, stop transfer, cytochrome *C* and cytochrome *C* haem lyase import pathways. Furthermore, we will present current knowledge of the biogenesis of dually-localised proteins in the IMS and finally we will discuss degradation within this compartment and retro-translocation into the cytosol coupled to degradation by the ubiquitin-proteasome system as two main aspects of the proteostasis of IMS proteins.

Protein import pathways into the IMS

The Mia40 import pathway

Protein import into the mitochondrial IMS is essential as it affects a variety of metabolic pathways, apoptosis and the oxidative phosphorylation in all organisms (for mammalian cells see Zamzami *et al.,* 1996; Briston *et al.,* 2018; Thomas, Esposito, *et al.,* 2019; Thomas *et al.,* 2019). Nevertheless, the first detailed analysis of the mitochondrial IMS proteome was done for the yeast *S. cerevisiae* annotating only about 50 proteins to be localized in the IMS (Vogtle *et al.,*2012). Most of those proteins display similar structural characteristics, as they are small proteins (< 25 kDa) with a simple structure, having a helix-loop-helix structure as their most common fold and conserved in mammalian cells (Banci *et al.,*2008, 2012). Additionally, most of the proteins of the IMS lack typical mitochondrial targeting sequences and follow therefore different import pathways into the mitochondria. Instead, a large portion of proteins destined for the IMS contain conserved cysteine residues, which allow their import via the mitochondrial IMS import and assembly (MIA) pathway.

The two major components of the MIA pathway are the oxidoreductase Mia40 and the FAD-dependent sulfhydryl oxidase Erv1 (Chacinska *et al.,* 2004; Hofmann *et al.,* 2005; Mesecke *et al.,* 2005; Rissler *et al.,* 2005; Banci *et al.,* 2009). After cytosolic translation and transport of the MIA substrates to the outer mitochondrial membrane, substrates are translocated through the TOM complex in an unfolded and reduced state (Sideris and Tokatlidis 2007). Already during translocation, substrates can interact with Mia40 within the IMS, which acts as a receptor driving the translocation direction towards the trans site of the TOM complex (Banci *et al.,* 2009; Banci *et al.,*2012; Peleh *et al.,*2016).

The yeast Mia40 itself is bound to the inner mitochondrial membrane via a N-terminal transmembrane domain, whilst the human Mia40 lacks this transmembrane domain and is completely soluble in the IMS. On the other hand, all Mia40 homologs contain six highly

conserved cysteine residues and are folded in a coiled-helix-coiled-helix (CHCH) domain, which is formed by two structural disulfide bonds connecting a twin CX9C motif that stabilize the protein. The CHCH domain forms a characteristic hydrophobic cleft that serves to bind substrates (Sideris et al., 2009; Banci et al., 2009; Kawano et al., 2009). Most of the Mia40 substrates contain a hydrophobic patch, which acts as an IMS targeting signal (ITS) or mitochondrial IMS-sorting signal (MISS) and facilitates the first interaction step of Mia40 to its substrates via hydrophobic stacking of the ITS/MISS within the cleft of Mia40 (Milenkovic et al., 2009; Sideris et al., 2009). The remaining two conserved cysteines of Mia40 are organized in a redox-active CPC motif that readily switches between a reduced and oxidized state. The CPC motif introduces the disulfide into the substrates (Banci et al., 2009; Kawano et al., 2009). The initial hydrophobic interaction between ITS/MISS and the cleft of Mia40 juxtaposes the substrate so that a transient intermolecular disulfide between Mia40 and the imported substrate is formed (Sideris et al., 2009; Koch and Schmid, 2014; Banci et al., 2010) involving the second cysteine of the Mia40 CPC motif and the docking cysteine of the ITS/MISS of the substrate. This transient intermolecular disulfide is subject to nucleophilic attack by another cysteine of the substrate, which results in the formation of an intramolecular disulfide within the substrate protein and its release from Mia40 (Figure 1A). This process results in folding of the substrate and trapping it in the IMS thus completing its import process (Banci et al., 2009)

However, disulfide introduction into the substrate leads to a reduction of Mia40 and thereby blockage of Mia40 for further import reactions. To maintain a vital MIA import pathway, Mia40 needs to be continuously re-oxidized, via interaction with Erv1 (Mesecke et al.,2005; Rissler et al.,2005; Banci et al., 2012; Banci et al., 2011). Erv1 is a sulfhydryl oxidase, which is present in the cell in a dimeric state and which requires a FAD cofactor for its complete redox-activity. Whilst interacting with reduced Mia40, Erv1 accepts electrons via a N-terminal

redox-active CXXC motif, transferring them onto a core disulfide and finally via the FAD cofactor onto a final electron acceptor (Bien et al., 2010; Lionaki et al., 2010). As a final electron acceptor under aerobic conditions, Erv1 can either transfer the electrons onto molecular oxygen, leading to hydrogen peroxide production within the IMS, or onto its presumably preferred acceptor cytochrome c, feeding the electrons into the respiratory chain (Bihlmaier et al.,2007; Dabir et al.,2007). Alternatively, Erv1 can use the fumarate reductase Osm1 as an electron acceptor under anaerobic conditions (Neal et al.,2017). Taken together, the MIA pathway is based on a disulfide relay system, in which electrons are transferred from the imported substrate onto Mia40 and Erv1 and finally an electron acceptor to re-oxidize Mia40 and therefore restore the import capacity of the MIA pathway.

Typical Mia40 substrates contain twin CX3C or CX9C motifs for disulfide formation within the IMS and are usually rather small (10 – 15 kDa) (Longen et al., 2009). An example for this family of proteins are the small TIM proteins. The small TIM proteins form different chaperone complexes, Tim9-Tim10 and Tim8-Tim13, which facilitate the transport of hydrophobic proteins destined for the inner or outer mitochondrial membranes through the soluble IMS (Koehler et al., 1998a; Koehler et al., 1998b; Paschen et al., 2000). Small Tim proteins are essential for protein import into the inner mitochondrial membrane and therefore crucial for the mitochondrial protein homeostasis. In addition to the typical Mia40 substrate motifs, also other substrates with different cysteine patterns were discovered. Mix23 displays CX14C/CX13C motifs for recognition in the MIA pathway, whereas other proteins like Erv1 itself are imported via a CXXC motif (Gabriel et al.,2007; Vogtle et al., 2012; Kallergi et al., 2013; Banci et al., 2013). Even though functional Mia40 depends on the interaction with endogenous Mia40 during its own import, Mia40 is not a conventional substrate of the MIA pathway in yeast. Moreover, it was discovered that the import of Mia40 takes place in three steps. First, Mia40 is inserted into the inner mitochondrial membrane by the TIM23 complex. Second, it interacts

with endogenous Mia40 to fold the core domain. Lastly, the CPC motif gets oxidized by interaction with Erv1 (Chatzi et al., 2013).

The stop-transfer import pathway

One of the first IMS import pathways discovered was the stop-transfer pathway that wa characterised in detail in yeast (Gasser et al., 1982; van Loon et al., 1986). Proteins that utilise this import pathway are the only known IMS proteins that contain cleavable pre-sequences similar to the matrix targeting pre-sequence. The exception of the stop-transfer signal is the presence of a transmembrane segment flanking the pre-sequence. The positively charged section of this bipartite pre-sequence is recognised by the TOM receptors Tom20 and Tom22 to facilitate its translocation across the outer membrane (Martin et al., 1991). The Tim50 receptor then feeds the positively charged, membrane potential ($\Delta\Psi$) dependent, region of the bipartite signal into the Tim23 channel (Martin et al., 1991; Meier et al., 2005). The hydrophobic transmembrane segment stalls the translocation in the Tim23 channel and prevents the precursor from complete import into the matrix; instead, the transmembrane segment is laterally released into the lipid bilayer (Glick et al., 1992; van der Laan et al., 2007; Botelho et al., 2011; Schendzielorz et al., 2018). This sorting process requires a specific conformation of the TIM23 complex comprising of Tim23, Tim17 and Tim21 (Chacinska et al., 2010; Schendzielorz et al., 2018). This form of the translocase called $TIM23^{SORT}$ is crucial for the lateral release of transmembrane segments into the lipid bilayer. Pam18, a subunit of the TIM23 complex, blocks the lateral release of transmembrane segments into the lipid bilayer and promotes matrix protein import. This Pam18-containing form of the TIM23 complex enhancing matrix-import is called $TIM23^{PAM}$ (Schendzielorz et al., 2018). The pre-sequence segment of the bipartite signal is usually cleaved by the matrix processing peptidase (MPP) (Daum et al., 1982; Mossmann et al., 2012). A second cleavage site exists between the

mature protein and the transmembrane segment. This motif is recognised and cleaved by the inner membrane proteases Imp1 and Imp2 to release the mature protein into the IMS (Figure 1B) (Gasser et al., 1982; Glick et al., 1992; Esser et al., 2004). The best characterised stop-transfer substrate, cytochrome *b*2, is released into the IMS through cleavage by Imp1 and also requires the mitochondrial matrix Hsp70 chaperone for its import and folding (Esaki et al., 1999). A second IMS protease, called Pcp1, is responsible for the cleavage of the stop-transfer substrate Mgm1 (OPA1 in humans), which is a mitochondrial GTPase required for mitochondrial dynamics (Herlan et al., 2004). Mgm1 is a unique stop-transfer protein as it resides in both the inner membrane and bulk IMS having a membrane-bound and a soluble form. The segregation of these two forms is thought to be regulated by ATP levels within the matrix and the topology of a second hydrophobic domain upstream of the stop-transfer signal. The yeast type 2C protein phosphatase Ptc5 is yet another IMS protein recently shown to be imported via the stop-transfer pathway and depends on Imp1 (Vogtle et al., 2012). Two key apoptotic proteins in humans also follow the stop-transfer pathway, namely the apoptosis inducing factor (Aif) and the second mitochondria-derived activator of caspases (Smac), although the peptidases that process these two proteins are yet to be elucidated (Herrmann and Hell, 2005). Cytochrome *C* peroxidase (Ccp1) is an IMS protein that has a N-terminal targeting signal that resembles the bipartite signal of the stop-transfer pathway (Maccecchini et al., 1979; Kaput et al., 1989). However, Ccp1 maturation was unaffected in an Imp1 mutant strain (Jan et al., 2000). Instead, the positively charged pre-sequence targeting signal guides Ccp1 to the inner membrane where the transmembrane domain arrests the protein. The haem cofactor is then assembled into the enzyme. Following this step, the matrix AAA protease subunits Yta10 and Yta12 cleave the transmembrane segment of the protein (Esser et al., 2002). Finally, release into the IMS of the core domain of Ccp1 is triggered by the cleavage of a short hydrophobic segment in Ccp1 by the protease Pcp1 (Esser et al., 2002).

Peroxiredoxin 1 import pathway

The yeast peroxiredoxin, Prx1, is a mitochondrial thioredoxin peroxidase that detoxifies H_2O_2 (Pedrajas et al., 2000). The enzyme is translated with a N-terminal matrix targeting sequence that is recognised and cleaved by the MPP via its R2 motif, a specific arginine configuration (R - X - X) recognised by MPP and is the site for cleavage. A second motif recognised by the mitochondrial intermediate peptidase Oct1 cleaves the remaining part of the presequence to allow release of the mature protein into the matrix (Figure 1C) (Mossmann et al., 2012; Gomes et al., 2017). Interestingly, Prx1 was shown to be dually localised between the matrix and the IMS of mitochondria (Gomes et al., 2017). The proposed import route into the IMS involves a very short hydrophobic segment present within Prx1 that triggers lateral release from the Tim23 into the inner membrane and cleavage by Imp2 (Figure 1C) (Gomes et al., 2017). This unconventional stop-transfer-like pathway facilitates segregation of Prx1 from the matrix to the IMS, although the exact regulation of this pathway is yet unclear. One could postulate that the selection between compartments occurs at the TIM23 level where the two conformations of TIM23 complexes are thought to operate. Prx1 imported through the $TIM23^{PAM}$ conformation is destined for the matrix whilst Prx1 imported through the $TIM23^{SORT}$ conformation is imported into the IMS.

Cytochrome C import pathway

Cytochrome *C* is a respiratory chain protein that resides on the IMS side of the inner membrane surface. The protein transfers electrons from respiratory complex III to complex IV. The release of cytochrome *C* into the cytosol is crucial for the initiation of the canonical intrinsic apoptotic signaling cascade (Liu et al., 1996). The TOM complex imports the non-haem bound form of cytochrome *C* (apocytochrome *C*) into the IMS where it then interacts

with cytochrome *C* haem lyase (CCHL) facilitating the integration of the haem cofactor into the apocytochrome *C* protein and release as functional cytochrome *C* (Figure 1D)(Mayer et al., 1995; Diekert et al., 2001; Wiedemann et al., 2003). How haem makes it from the matrix to the IMS is yet to be elucidated. Absence of the haem group within cytochrome *C* results in cytochrome *C* backsliding through the TOM complex and back into the cytosol. The exact translocation process through the TOM translocase is thought to differ from that of preproteins destined for the matrix (Diekert et al., 2001). Deletion of the Tom receptor proteins Tom20, Tom70, Tom5, Tom6 and Tom7 had no effect on the import of cytochrome *C* (Wiedemann et al., 2003). However, the Tom22 subunit was shown to be imperative for translocation into the IMS. This suggests the two key components required for cytochrome *C* translocation are the Tom40 channel-forming translocase and the Tom22 receptor (Wiedemann et al., 2003). The recognition signal present in cytochrome *C* that allows for translocation is so far not known.

Cytochrome C haem lyase import pathway

In yeast there are two Cytochrome *C* haem lyase (CCHL) proteins that are essential for integrating the haem cofactor into the c-type cytochromes; cytochrome *C* and cytochrome c_1 (Steiner et al.,1995). Both CCHL proteins reside in the IMS and are imported through a non-canonical import pathway. The CCHL enzymes do not contain an N-terminal pre-sequence and thus contain internal targeting information. At the stage of the outer membrane (OM), CCHL is imported through interaction with Tom20 of the TOM complex which recognises two internal targeting signals that are hydrophilic in nature (Mayer *et al.,* 1993; Künkele *et* al.,1998; Diekert et al.,1999). Once across the OM, CCHL rapidly folds before binding to the surface of the lipid bilayer of the inner membrane, which is also thought to require the internal targeting signal (Figure 1D) (Lill et al., 1992; Diekert et al.,1999; Herrmann and Hell,

2005). The import process of CCHL is ATP- and ΔΨ-independent, making this import process a relatively simple one in nature (Lill et al., 1992; Steiner et al., 1995). Whether any IMS proteins are involved in the translocation or the folding process of CCHL is yet unknown.

(Figure 1)

Proteostasis of intermembrane space proteins

Yme1 regulates much of the IMS proteome

The IMS is a small oxidising environment that is subject to a plethora of damage from the respiratory chain. Although there are some IMS-specific ATP-independent chaperone systems like the small Tim proteins (Petrakis et al., 2009) that protect against protein aggregation, there are no known ATP-dependent HSP (Heat-Shock Chaperone) systems present in the IMS to aid in the refolding of proteins. To this effect another proteostatic mechanism is used. The intermembrane space AAA protease, Yme1, is an inner membrane bound protein that faces the IMS and has ATP dependent proteolytic activity (Weber et al., 1996; van Dyck and Langer, 1999). Yme1 contains an N-terminal targeting signal that is imported through the TIM23 translocase, MPP cleaves the pre-sequence allowing for release of the transmembrane segment of Yme1 into the inner membrane (Hwang et al., 2007). Tim54, a subunit of the Tim22 complex, assembles Yme1 into a functional homo-hexameric protein (Hwang et al., 2007). Yme1 has been shown to be involved in protein quality control for three mitochondrial sub-compartments; the outer membrane, the IMS and the inner membrane (Baker et al., 2011; Baker et al., 2012; Wu et al., 2018). The structural basis of the proteolytic activity of Yme1 has been studied as a partial cryo-EM structure has been resolved (Puchades et al.. 2017). The catalytic M41 peptidase domain and the AAA+ ATPase domain reside in the IMS and are attached to the inner membrane by a transmembrane helix (Schreiner et al., 2012; Puchades et al., *2017*). ATP hydrolysis powers the proteolytic function of Yme1 by coordinating a ‘double spiral staircase’ of tyrosine residues to facilitate translocation of an unfolded substrate towards the negatively charged proteolytic active site(Puchades et al., 2017) . Several Mia40 substrate proteins are degraded via Yme1 when improperly folded. One such example is the small TIM proteins. Specifically, cysteine mutants of Tim9 or Tim10 that are incapable of

proper folding or forming the hetero-hexameric Tim9-Tim10 complex were shown to be degraded in a Yme1-dependent manner (Figure 2)(Baker et al., 2012; Spiller et al., 2015). It was later found that Tim9 can protect Tim10 against degradation by Yme1 (Spiller et al., 2015). The quality control mechanism that underpins the function of Yme1 is best shown in a yeast Yme1 deletion mutant (ΔYme1) grown at permissive temperatures (30°C). This results in a rather extended aggregation of several proteins from multiple mitochondrial compartments, including the IMS (Schreiner et al., 2012). Many IMS proteins, particularly Mia40 substrates and interactors, are found to aggregate including Erv1, Tim10, Cyc1,Cyc3, Aim13 and Atp23 (Schreiner et al., 2012). The aggregated proteins are detrimental to mitochondrial function which can be observed phenotypically as the ΔYme1 yeast strain struggles to grow under respiratory and non-permissive conditions (37°C). As the IMS is a well-balanced oxidising environment it is likely that Yme1 may be involved in redox quality control. In this respect, it is interesting that recombinantly expressed and functional Yme1 alters its conformation under hydrogen peroxide stress conditions (Brambley et al., 2019). This increases ATP binding to Yme1 potentially enabling increased proteolytic cleavage when the stress is resolved. This conformational change appears to be reversible suggesting that Yme1 adapts its proteolytic activity depending on the surrounding environment. Oxidative stress could potentially prime Yme1 for proteolytic activity when hydrogen peroxide stress occurs (Brambley et al., 2019), although such a mechanism has not yet been shown *in vivo*.

Interestingly, it has been reported that Yme1 possesses a chaperone-like function (Leonhard et al., 1999; Graef et al., 2007; Schreiner et al., 2012). *In vitro* experiments have shown that the AAA domain of Yme1 can specifically bind unfolded proteins to promote their refolding (Leonhard et al., 1999). In addition to this, *in vivo* evidence shows that Yme1 can act as a

chaperone to promote the assembly of Cox2 when the normal assembly factor, Cox18, is missing (Fiumera et al., 2009). To perform this function the Yme1 adaptor subunit Mgr3 needs to be absent from Yme1. Finally Yme1 has been shown to be involved in the folding of the phospholipid metabolism protein, Ups2, that resides in the IMS (Potting et al., 2010; Schreiner et al., 2012a). Although shown to be a substrate for the proteolytic activity of Yme1, there appears to be a chaperone-like function in the maintenance of folded Ups2. In humans Yme1 may play a large role in disease where the human homolog of Yme1, YME1L, has been shown to alter the mitochondrial proteome resulting in the promotion of pancreatic ductal cell adenocarcinoma cell proliferation (MacVicar et al., 2019). Yme1 has also been implicated in the import of the polynucleotide phosphorylase (PNPase) into the IMS. PNPase contains an N-terminal targeting signal that is targeted via the TIM23 translocase. Yme1 on the other hand assists the translocation of PNPase across the outer membrane and directs it to the TIM23 translocase where the N-terminal targeting signal is cleaved by MPP. Yme1 then facilitates release of PNPase into the IMS (Rainey et al., 2006).

Retro-translocation of IMS proteins

As previously described, Mia40 substrates make a transient disulfide bond with Mia40 which allows for disulfide transfer and folding of the substrate protein. Due to a transient covalent bond existing between substrate and import component this step in the import pathway is potentially liable to stalling of the substrate being imported (Mü et al., 2008; Habich et al., 2019). Hypothetically, reducing enzymes found within the IMS could perform the task of cleaving the intermolecular disulfide between the two proteins and allow Mia40 to bind another substrate. In yeast, the glutaredoxin or thioredoxin systems found to be present in the IMS could perform this task. In humans, the glutaredoxin system is shown to control the oxidation of Cox17, a Mia40 substrate (Habich et al., 2019). One quality control method to

remove some of these stalled Mia40 substrates is retro-translocation (Figure 2). In yeast, several Mia40 substrates are capable of escaping from the IMS when unfolded (Mü et al., 2008; Bragoszewski et al., 2015). Bragoszewski et al. (2013) show that certain Mia40 substrates can be retro-translocated through the TOM translocase, although the mechanism is yet to be elucidated. Once released into the cytosol the ubiquitin-proteasome system (UPS) is responsible for degrading the retro-translocated protein(Figure 2)(Bragoszewski et al., 2013, 2015). This process comes with its limitations as not all IMS proteins are capable of retro-translocation, this is likely due to topological and size constraints resulting in larger proteins incapable of being retro-translocated.

There have been several papers highlighting the role of the UPS in the degradation of Mia40 substrates in the cytosol (Bragoszewski et al., 2013, 2015; Habich et al., 2019; Mohanraj et al., 2019). It has been shown that the UPS can compete for the Mia40 substrates in the cytosol prior to import (Figure 2)(Mohanraj et al., 2019). This competition between import and degradation could be due to the slow kinetics during import of Mia40 substrates or it could be a mechanism to regulate the abundance of Mia40 substrates depending on cellular requirements. One example of the role kinetics plays in Mia40 substrate import is with the human Mia40 substrate COA7, a protein involved in the assembly of respiratory complex IV. It has been shown that in patient cells with a pathogenic variant of COA7 the import of the protein is much slower, resulting in degradation of the protein in the cytosol by the UPS (Mohanraj *et al.*, 2019). When the cells are treated with the proteasome inhibitor MG132, both protein import and complex IV activity are partially restored.

(Figure 2)

CONCLUSIONS AND FUTURE PERSPECTIVES

The mitochondrial IMS is a very constricted compartment with a much smaller volume than the mitochondrial matrix. The IMS-resident proteins constitute about 10% of the total mitochondrial proteome, they are very variable in their structures (ranging from simple folds like helix-turn-helix to more elaborate folds with co-factors like FAD, haem groups etc) and they are involved in a large spectrum of crucial functions (for example chaperones, lipid transfer proteins, assembly factors for the respiratory chain complexes etc). It is very intriguing that import of these proteins into the IMS, the process which defines their biogenesis, is ensured by several distinct import pathways. This is in contrast to import into the mitochondrial matrix, which relies on a single default pathway despite the far greater number of proteins residing in this compartment (two thirds of the mitochondrial proteins).

Proteins destined for the IMS have to cross only the outer membrane, presumably through the TOM complex, which is the common entry gate for all mitochondrial proteins as far as we know. Subsequent import and folding steps to ensure retention in the IMS do not require either ATP hydrolysis in the matrix or the inner membrane potential, which are the critical energy sources for import into the matrix and the inner membrane. The only exception to this is the stop-transfer pathway, in which the first step of translocation across the TIM23 complex is part of the matrix pathway. Consequently, protein-protein and protein-lipid interactions may play a crucial role in providing the necessary energy to render the protein import into the IMS a unidirectional process. The small volume and hence high local concentration of IMS proteins may provide the physiological context for such, normally low affinity non-covalent interactions that are concentration-dependent, to be productive. For the MIA pathway, the initial non-covalent hydrophobic binding of the preprotein to Mia40 is potentiated by the

subsequent covalent intermolecular disulfide bond created with Mia40. The presence of a large number of structurally and functionally different chaperones in the IMS (the Mia40, the Tim9-Tim10 complex and the Yme1) is also important for the folding and assembly in this compartment. The expanded specificity of Mia40 substrates, from an initial narrow set of a defined cysteine-motif (CXnC) to protein substrates with cysteines not organised in a specific motif or even to protein without cysteines, allows us to speculate that other IMS chaperones may also bind proteins beyond their hitherto established client proteins. In this respect it is not yet clear whether there is any overlap in binding substrates among these different chaperone systems in the IMS.

The recent identification of the mitochondrial contact site and cristae organizing system (MICOS complex) that, along with other proteins, controls the inner membrane architecture and formation of cristae (van der Laan et al., 2016) together with progress in our capacity to more precisely study the alterations and dynamics of the cristae (Stephan et al., 2019) provide an invaluable framework to understand in better detail the segregation of the IMS into the bulk IMS surrounded by the boundary IM and the lumen within the cristae. Although it is commonly thought that protein import occurs into the bulk IMS delineated by the boundary IM, it is not yet clear whether and how diffusion of small molecules and/or proteins between that and the cristae lumen occurs. Additionally, it is still poorly understood how alterations of the internal IM architecture may affect the biogenesis processes of IMS proteins.

In several respects, the interface between the mitochondria and the cytosol is not limited to the surface of the outer membrane but also the IMS itself due to the free diffusion of small molecules in and out the IMS into the cytosol via the OM porin channels. Proteins in the IMS

are therefore well positioned to play critical roles in signalling cues linking the mitochondria and the rest of the cell.

The import pathways for many IMS proteins remain unknown and it would be worthwhile studying in the future, particularly with a view to discovery of new fundamental principles for pre-sequence-less protein import and dual localisation of proteins. Teasing out such new import mechanisms would increase our knowledge in two important areas (i) the deciphering of how proteins negotiate the OM divide either through intricate translocation mechanisms across the TOM complex or by engaging other, yet unknown OM protein channels and (ii) new protein retention mechanisms in the IMS. Progressing our knowledge in terms of import pathways is clearly important, but future work should also shed light on how IMS proteins are degraded and/or exported into the cytosol so that an optimal balance in the IMS is maintained under physiological and stress conditions.

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FIGURE LEGENDS

Figure 1. The main protein import pathways into the intermembrane space (IMS) of mitochondria. Panels A-D depict the four most studied protein import pathways into the IMS. A: the MIA pathway, B: The stop-transfer pathway, C: the Prx1 pathway for dual localisaiton and D: the Cytochrome c/ Haem lyase import pathway. In each case, the step-wise import reaction is broken down to the individual stages. Details are referred in the main text.

Figure 2. Proteostasis of IMS proteins. The distinct pathways of (i) Yme1-dependent proteolysis or (ii) retro-translocation and Ubiquitin-Proteasome dependent degradation (UPS) are indicated. These are discussed in the main text.

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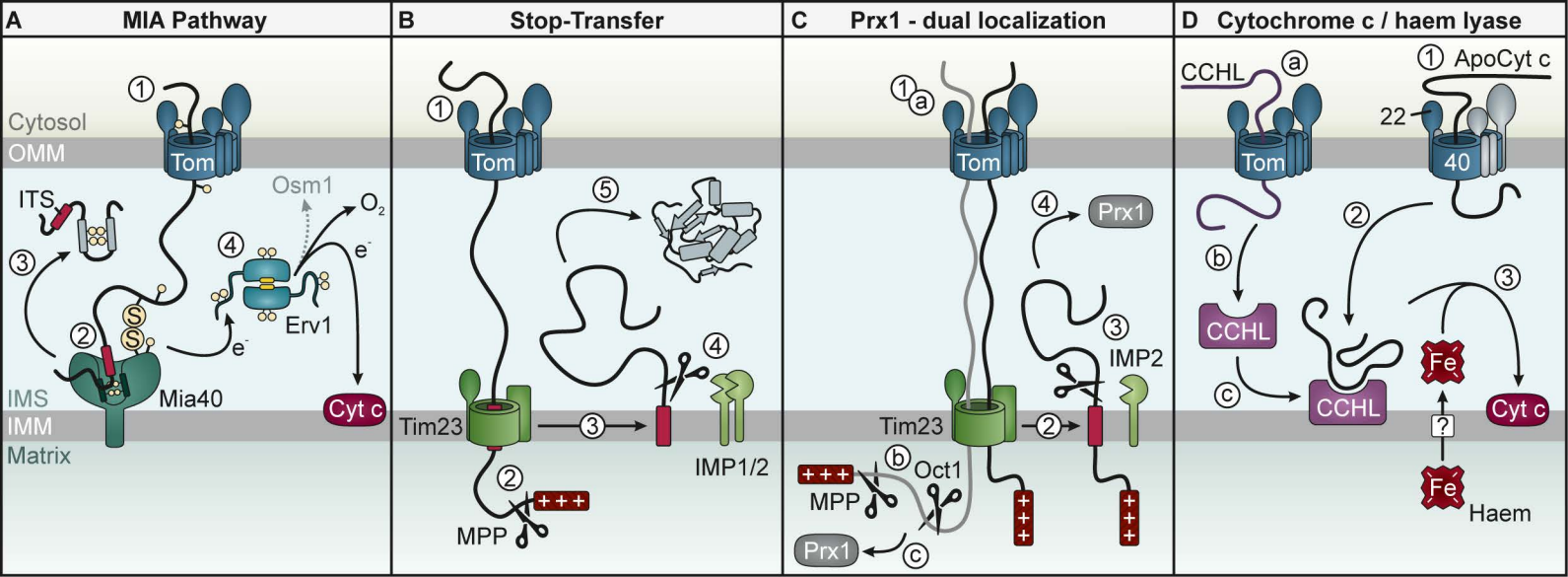
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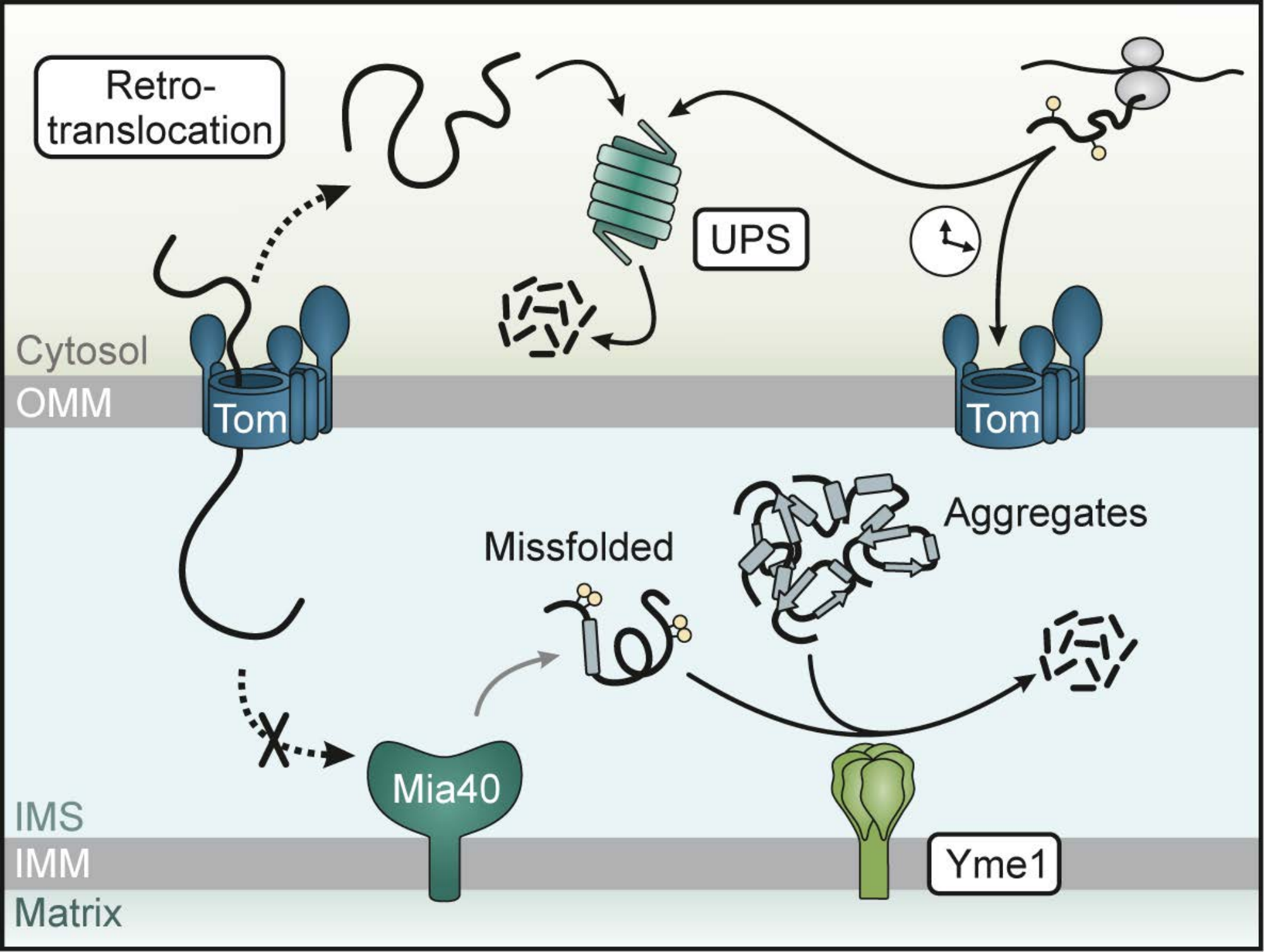
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