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Review

The ins and outs of the intermembrane space: Diverse mechanisms and evolutionary rewiring of mitochondrial protein import routes ☆

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ABSTRACT

Background: Mitochondrial biogenesis is an essential process in all eukaryotes. Import of proteins from the cytosol into mitochondria is a key step in organelle biogenesis. Recent evidence suggests that a given mitochondrial protein does not take the same import route in all organisms, suggesting that pathways of mitochondrial protein import can be rewired through evolution. Examples of this process so far involve proteins destined to the mitochondrial intermembrane space (IMS).

Scope of review: Here we review the components, substrates and energy sources of the known mechanisms of protein import into the IMS. We discuss evolutionary rewiring of the IMS import routes, focusing on the example of the lactate utilisation enzyme cytochrome b_2 (Cyb2) in the model yeast *Saccharomyces cerevisiae* and the human fungal pathogen *Candida albicans*.

Major conclusions: There are multiple import pathways used for protein entry into the IMS and they form a network capable of importing a diverse range of substrates. These pathways have been rewired, possibly in response to environmental pressures, such as those found in the niches in the human body inhabited by *C. albicans*.

General significance: We propose that evolutionary rewiring of mitochondrial import pathways can adjust the metabolic fitness of a given species to their environmental niche. This article is part of a Special Issue entitled Frontiers of Mitochondrial.

1. Introduction

Mitochondria are organelles that play a central role in metabolism and energy production. Mitochondrial activity and biogenesis is regulated in response to a variety of intracellular and environmental cues [1], including nutrient availability [2], oxidative stress [3] and presence of toxic compounds [4]. In order for mitochondria to replicate and adapt rapidly to their environment, they must be populated with functional proteins in their correct compartments. With the vast majority of mitochondrial proteins encoded in the nuclear genome, a key mechanistic component of mitochondrial biogenesis is the import of these proteins after their synthesis in the cytosol. These proteins are recognised and imported by the diverse transport machineries of mitochondria (Fig. 1) [5,6].

The first transport machine to engage most precursor proteins is the TOM (translocase of the outer membrane)[1] complex [7] (Fig. 1, brown). This multi-subunit complex includes the Tom20 [8] and Tom22 [9] receptors that recognise a range of TOM complex substrates. Some α-helical outer membrane (OM) proteins are integrated directly into the membrane by the mitochondrial import complex (MIM) [10], but most proteins are imported through the TOM complex before the import pathways to the different mitochondrial compartments diverge (Fig. 1). Proteins forming β-barrels in the OM are inserted by the SAM (sorting and assembly machinery) complex [11], recently shown to be directly coupled to the TOM complex [12]. SAM complex substrates in the intermembrane space (IMS) are also bound by the small TIM (translocase of the inner membrane) chaperones [13]. Small TIM chaperones also escort inner membrane (IM) proteins to the TIM22 complex

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[1] Abbreviations: translocase of the outer membrane (TOM), outer membrane (OM), mitochondrial import complex (MIM), sorting and assembly machinery (SAM), intermembrane space (IMS), translocase of the inner membrane (TIM), inner membrane (IM), presequence translocase-associated import motor (PAM), oxidase assembly (OXA), mitochondrial intermembrane space transport and assembly (MIA), mitochondrial processing peptidase (MPP), inner membrane protease (IMP), mitochondrial inner membrane organizing system (MINOS).

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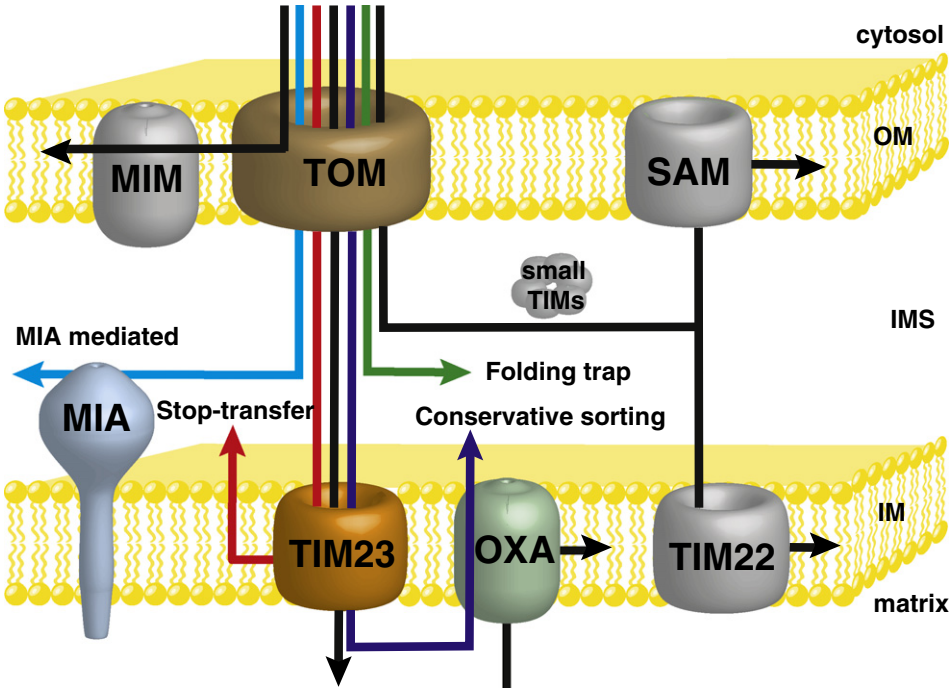

Fig. 1. Overview of mitochondrial protein import pathways. Pathways into the intermembrane space (IMS) are shown in colour and discussed in more detail in the text. Import machinery not discussed in depth in this review is shown in grey. Pathways diverge at the outer membrane (OM) after recognition by the TOM (translocase of the outer membrane) complex (brown). Some proteins are inserted directly into the OM by the mitochondrial import machinery (MIM), but most pass through the OM through the TOM complex. Proteins imported by the MIA (mitochondrial intermembrane space transport and assembly) pathway (bright blue), or folding trap pathway (green), remain in the IMS. Small TIM (translocase of the inner membrane) proteins escort proteins to the SAM (sorting and assembly machinery) complex, or TIM22 machinery to be assembled into the OM or inner membrane (IM) respectively. Proteins imported by a stop-transfer pathway (red) or conservative sorting pathway (dark blue) also use the TIM23 complex (orange) to traverse the IM. Proteins with domains that then pass back through the IM sometimes use the OXA (oxidase assembly) machinery, which also transports proteins translated in the matrix.

for assembly into the inner membrane [14,15]. In contrast, the TOM complex interacts directly with the TIM23 complex (Fig. 1, orange) to transport matrix-destined proteins through both membranes [16,17]. This movement is a membrane potential dependent process driven by the PAM (presequence translocase associated import) motor module of the TIM23 complex. In some cases this transport process is interrupted and the precursors are laterally released from TIM23 into the inner membrane. In a few cases insertion of inner membrane proteins from the matrix side has been found to require the oxidase assembly (OXA) translocation machinery (Fig. 1, green) [18], which is primarily involved in the insertion of proteins synthesised within the mitochondria into the IM [19,20]. The most recently discovered transport machinery is the MIA (mitochondrial intermembrane space transport and assembly) machinery (Fig. 1, blue), which ensures proper import and folding of a number of IMS proteins [21].

Recent work has identified regulatory controls on mitochondrial protein import, which modulate mitochondrial function and biogenesis in line with the metabolic needs of the cell [22,23]. However in addition to these acute regulatory responses to environmental stimuli, a much more drastic, long-term modulation of the import pathways seems to be at work. The import pathways for mitochondrial proteins appear to have been rewired over evolutionary time to accommodate the distinct metabolic circumstances of a given biological species in its environmental niche. This idea has received support from a recent study done in our laboratories comparing protein import into the IMS in distantly related fungal species [24], as well as from previous work on the IMS protein Mia40 in yeast and mammals [25]. Prompted by these discoveries, we use this review as an opportunity to provide an up-dated account of the different pathways used for protein import into the mitochondrial IMS and discuss the concept of evolutionary rewiring of these import pathways.

2. Unexpected journeys into the IMS

Proteins are targeted to the IMS by diverse mechanisms, perhaps reflecting the different roles of the proteins found in this compartment. Specific machinery for import through the IM or assembly in the IMS recognise further signal elements within precursor proteins after their import through the TOM complex. The IMS proteins discussed in this review include both soluble IMS proteins and IMS-exposed proteins with anchors in the inner membrane. We discuss known features of the stop-transfer, conservative sorting, folding trap and MIA pathways. Many of the mechanisms of import into the IMS are still being elucidated and new examples of interactions between these mechanisms and other import processes are still being identified.

2.1. Arrest at the inner membrane: the stop-transfer pathway

Some IMS proteins are synthesised with a cleavable N-terminal presequence, containing matrix-targeting information [26]. In proteins destined for the mitochondrial matrix this targeting information leads them to and through the TOM complex and TIM23 complex [27]. A positively charged presequence at the N-terminus is recognised by the receptors Tom20 and Tom22 and facilitates import through the outer membrane via the TOM complex [28] (Fig. 2A). The presequence also enables the protein to engage with Tim50 and Tim23 resulting in membrane potential-dependent insertion of the precursor into the Tim23 channel in the TIM23 complex [29] (Fig. 2A). However, in IMS-destined proteins, this charged sequence is followed by a hydrophobic sorting signal that is recognised by the TIM23 complex, resulting in a lateral transfer of the sequence into the IM thereby preventing translocation into the matrix [26,30,31] (Fig. 2B). This process may require rearrangement of the TIM23 complex [32], but unlike translocation into

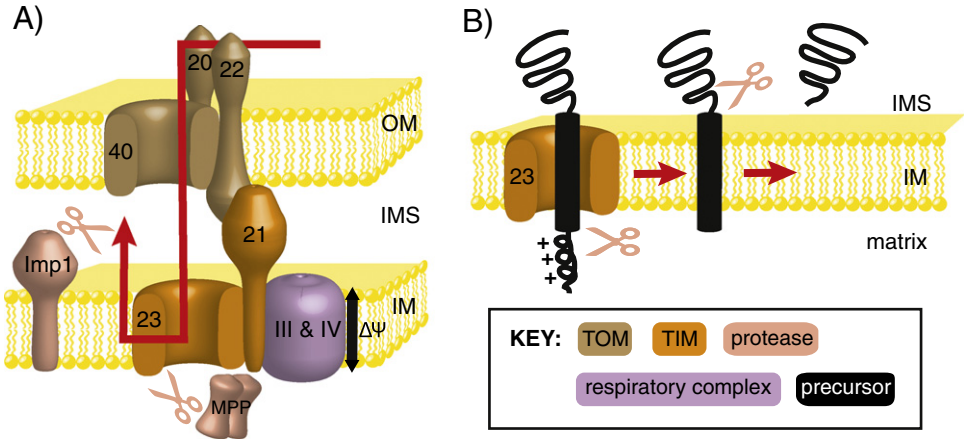

Fig. 2. General features of the stop-transfer pathway. (A) The components and interactions of the stop-transfer pathway are shown. The red arrow shows the path of a precursor through the TOM complex (brown) and on into the TIM23 complex (orange). This is followed by the lateral release of the precursor into the inner membrane (IM) and, in some cases, proteolysis to release the precursor into the intermembrane space (IMS). The locations of proteases (pink) and the coupling of the respiratory complexes (purple) to the TIM23 complex are also shown. The black arrow indicates that this pathway requires a membrane potential ($\Delta\Psi$) across the IM. (B) Processing of the bipartite signal sequence of the precursor (black) to produce membrane anchored or soluble IMS proteins.

the matrix, it is not dependent on the activity of the PAM module of the complex [33,34].

In the stop-transfer pathway, the positively charged presequence protruding into the matrix through the pore of the TIM23 complex is cleaved by a matrix protease, in most cases, the mitochondrial processing peptidase (MPP) (Fig. 2, pink) (reviewed in Refs. [35,36]). The now terminal hydrophobic sorting signal crossing the inner membrane acts as a brake in the pathway, preventing the translocation of the precursor into the matrix [37]. Hydrophobic and proline residues in the transmembrane domain [38], and charged residues following this hydrophobic region [39], have all been shown to play a role in directing precursor proteins into the stop-transfer pathway, but the mechanistic basis behind these observations is still unclear [31].

After the stop signal is recognised, proteins are laterally released from the TIM23 machinery (Fig. 2B). The mechanism of release from TIM23 is unclear, but the pathway is impaired in mutants of Tim17 (a TIM23 complex subunit), or if the interaction between the TOM complex and the TIM23 complexes via the Tom22 and Tim21 proteins is disrupted [40]. This membrane potential-dependent step is also stimulated by coupling of the TIM23 complex to the respiratory chain complexes III and IV via Tim21 [41] (Fig. 2A, purple). Proteins that do not undergo any further processing remain anchored by their N-terminal transmembrane domain in the inner membrane [5].

Further cleavage of the stop-transfer sequence by specific proteases can release soluble proteins into the IMS (Fig. 2B). Most commonly the hydrophobic sequence is cleaved by the inner membrane protease (IMP) (Fig. 2, pink) (reviewed in Refs. [5,42]). The key features of this pathway were identified through work on the *Saccharomyces cerevisiae* protein cytochrome b_2 (Cyb2) [30], but this protein has an additional requirement for Ssc1 (mitochondrial Hsp70) for proper folding of its heme-binding domain [43]. Mcr1 is another substrate of this pathway with an interesting variation [44]; the hydrophobic sequence can also be inserted in the OM, resulting in both OM-anchored and IMS forms of this protein [45].

A similar two-step, stop-transfer process produces the soluble IMS form of Mgm1, a dynamin-related GTPase with both soluble and membrane associated isoforms [46]. In this instance however, the MPP cleavage step is followed by the action of a different protease, Pcp1 [47]. The position of the transmembrane domain in this process is critical for the cleavage step to occur and is dependent on ATP levels within the mitochondria [48]. Thus the metabolic status of mitochondria regulates the import process and controls the relative amounts of the cleaved soluble forms and the laterally released membrane-anchored form of Mgm1. The second processing step of cytochrome c peroxidase (Ccp1) is also mediated by Pcp1 cleavage, in this case after an AAA protease (an ATPase associated with various cellular activities [49]) removes the first segment of the targeting signal [50]. The specific proteolytic processing of precursors has recently been recognised as a mechanism for the regulation of protein stability [51].

A final variation on the stop-transfer pathway anchors proteins in the inner membrane via their C-termini. In the case of cytochrome c_1 (Cyt1), cleavage of the N-terminal signal by the IMP occurs only after the insertion of the C-terminus into the inner membrane [52]. The sequences and predicted secondary structure of Shy1 [53] suggests that it may be imported via a similar pathway [52]. While Shy1 remains anchored to the IM via its C-terminus, its major functional domain is exposed to the IMS [54]. Shy1 is the yeast homologue of human Surf1, which is mutated in the mitochondrial disease, Leigh Syndrome. In contrast to findings in yeast, both hydrophobic targeting sequences of the human protein are important for its proper function [55], raising the possibility that defective import could play a role in the disease mechanism. A similar C-terminal transmembrane domain followed by an internal presequence-like signal is required for the IM insertion of Bcs1 [56]. This mechanism may further extend to other IMS-exposed substrates such as the J-proteins Tim14 [57], Mdj2 [58] and Jid1 [59].

2.2. There and back again: the conservative sorting pathway

In the conservative sorting pathway, proteins are firstly imported into the matrix via the TIM23 complex requiring ATP-dependent action of the PAM module [27] (Fig. 3) and stabilisation by the matrix-located isoform of Hsp60 [60]. This is followed by a further export-like step to relocate the protein back into the inner membrane [5,61]. This pathway was initially characterised through studies of the IMS-exposed Rieske iron sulfur protein (Rip1). Rip1 has a single N-terminal transmembrane domain anchoring the protein in the membrane, and a large C-terminal domain that is exposed to the IMS [61]. The protein is imported into the matrix, and the C-terminal domain is translocated back across the IM by the AAA-protease Bcs1 [62]. Other proteins that use the conservative sorting mechanism to translocate their N-termini back across the IM into an IMS-exposed location are subunit 9 of the *Neurospora crassa* F_1F_0-ATPase [63] and the polytopic inner membrane protein Oxa1 (discussed in more detail below) [64].

For many proteins this final step towards the IMS is mediated by Oxa1 [19], which is also part of the OXA machinery that assembles inner membrane proteins encoded on the mitochondrial genome into

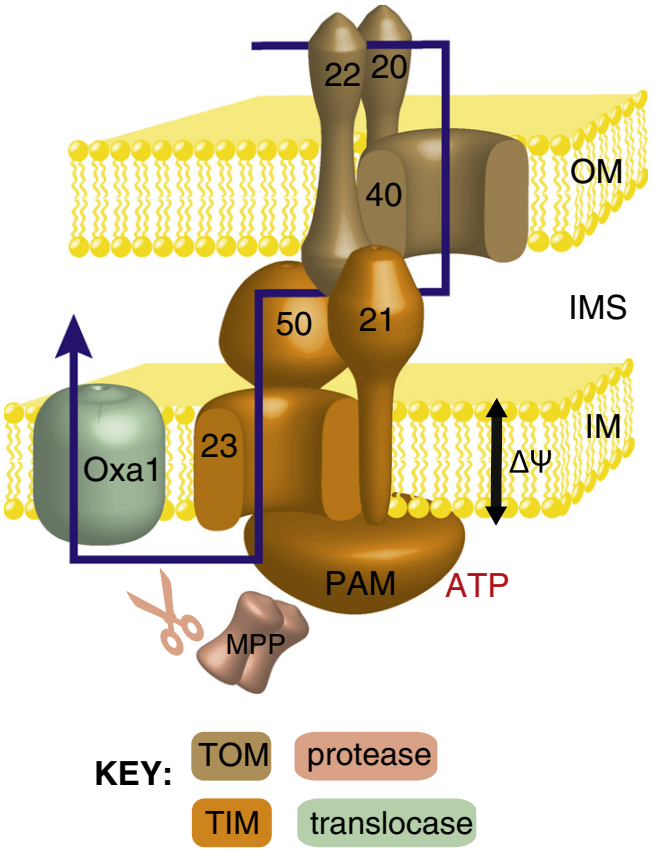

Fig. 3. General features of the conservative sorting pathway. The components and interactions of the conservative sorting pathway are shown. The blue arrow shows the path of a precursor through the TOM complex (brown) in the outer membrane (OM) and on into the TIM23 complex (orange). The precursor is imported into the matrix in a PAM (presequence translocase-associated import motor) dependent process that requires ATP and a membrane potential ($\Delta\Psi$) across the inner membrane (IM). Processing by a protease (pink), in most cases MPP (mitochondrial processing peptidase) precedes translocation back across in the IM by translocation machines (green), such as the Oxa1 machinery.

the IM [5,65]. This pathway may be particularly important for multi-topic membrane proteins originating from the bacterial endosymbiont [66], but it is not restricted to these substrates. Dependence on this endosymbiont-derived export machinery shows that there are limits to the more recently evolved systems in their ability to insert inner membrane proteins directly from the IMS [67]. Also implicated in the insertion of proteins into the IM from the matrix side are Mba1 [68], Cox18 (Oxa2) [69] and Bcs1 [56], but the general principles governing this step are still to be determined [70].

Recent work has revealed that pathways can cooperate to import proteins with multiple transmembrane domains. Containing six transmembrane domains and loops exposed to both the IMS and matrix, Mdl1 was the first protein shown to require both the stop-transfer and conservative sorting pathways [71]. Cleavage of the presequence and insertion of the first two transmembrane domains has the hallmarks of the stop-transfer process, whereas the insertion of the less hydrophobic transmembrane domains within the polypeptide requires both Ssc1 and Oxa1. Similarly, the two moderately hydrophobic transmembrane segments at the N-terminus of Sdh4 are imported into the matrix from where the transmembrane segments are inserted into the membrane, and the N-terminus passes back across the membrane making it IMS-exposed in the mature protein. The third transmembrane domain of Sdh4 is sufficiently hydrophobic to follow the stop-transfer pathway [70]. These are the first examples showing that the stop-transfer mechanism is able to process sufficiently hydrophobic transmembrane domains, but that mitochondria still require additional translocation machinery for the insertion of less hydrophobic transmembrane segments unable to be recognised for lateral sorting [72]. It is likely there are more examples and variations of cooperation between import pathways that remain to be discovered.

Some organisms retain other ancient components of their ancestral bacterial protein export machinery, namely components of the SecYEG translocon and the Tat machinery [73,74]. Proteins in these organisms could be assembled into the IM by the translocon or exported into the IMS by the Tat machinery, but this has not yet been demonstrated.

2.3. Thou shalt not pass: no escape from the folding trap

As well as travelling to the correct compartment, proteins must be properly folded and assembled with co-factors, enzymes or partner proteins in order to function. For example a Cyt1-specific heme lyase is found in the IMS and is needed to incorporate heme into Cyt1 [75]. It is not clear how important the protein folding reaction is in driving the translocation of such precursors through the TOM complex, but this probably varies depending on the substrate. Once folded, the protein is unable to escape from the IMS, at least as long as the outer membrane remains intact.

Another example of this folding trap mechanism is the retention of cytochrome c (Cyc1) in the IMS, which requires binding to cytochrome c heme lyase (Cyc3/CCHL). Cyc3 is found in the IMS associated with the inner membrane [76], and catalyses the attachment of a heme group to Cyc1. The superoxide dismutase, Sod1, is another metal-binding protein that is imported via a folding trap [77]. This protein reduces oxidative stress by reacting with superoxides, and overexpression of Sod1 has recently been shown to be protective in diabetes-prone mice [78]. Sod1 precursors interact with the copper-binding chaperone Ccs1, which helps assemble the apoprotein with zinc and copper co-factors [79]. Ccs1 and Sod1 may also have roles in assembly, import or folding of other metal-binding IMS proteins [80].

Unexpected flexibility in import pathways has been revealed by the recent publication of Ccs1-independent import of Sod1 [81]. Varabyova and colleagues show reduced and mutant forms of Sod1 can be imported into the IMS by the MIA pathway in conjunction with the mitochondrial inner membrane organising system (MINOS) [82]. The mutant forms of Sod1 are associated with amyotrophic lateral sclerosis in humans, presenting the intriguing possibility of connections between regulation of mitochondrial import and this disease [81].

2.4. More than just disulfide bonds: the MIA pathway

In 2004, Mia40 was discovered and named for its role in mitochondrial intermembrane space transport and assembly [21]. Initially, substrates were thought to be restricted to cysteine rich proteins containing conserved CX_3C or CX_9C disulfide bonded motifs [83,84]. The interaction of Mia40 with these canonical substrates ensures the proper formation of disulfide bonds before the oxidised substrate is released into the IMS ([21,85] and reviewed in [86–88]). Substrates with more diverse cysteine organisations have since been identified [42,84,89–91].

The importance of hydrophobic residues in the interaction of Mia40 with small IMS substrates [92,93] suggested an even broader range of substrates might use this pathway. This was confirmed with the recent discovery that import of Atp23 does not require Mia40-mediated disulfide formation [89]. Instead the hydrophobic interactions between Mia40 and Atp23 were shown to be more important for import. Hydrophobic interactions are also important for the role of Mia40 in the import of the IM protein Tim22 [91].

Mia40 works in conjunction with a number of other proteins in the IMS and IM (Fig. 4). The roles of these partners are described briefly below, as they have recently been reviewed in depth elsewhere [88]. The sulfhydryl oxidase Erv1 (ALR in mammals [94]) helps control the oxidative folding of disulfide-containing proteins in the IMS [85].

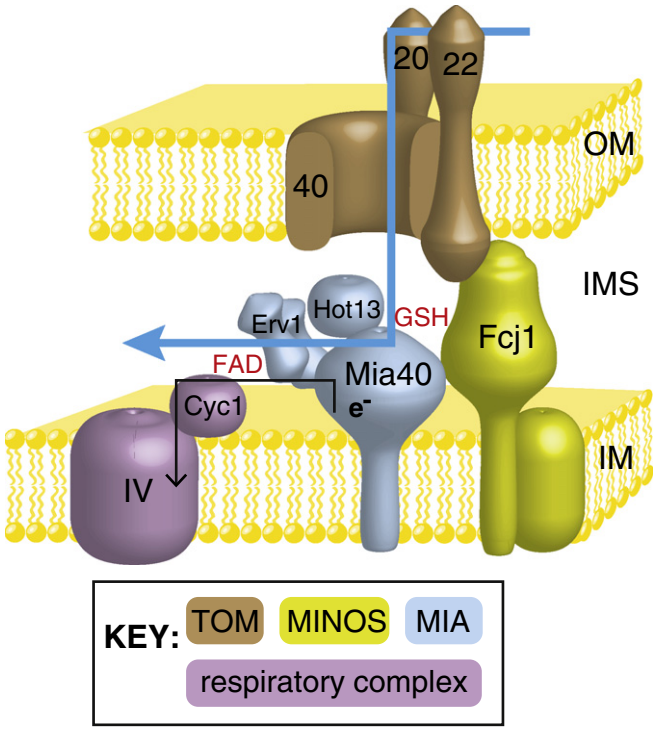

Fig. 4. General components of Mia40 mediated import. The import path of a precursor protein and its interactions with proteins associated with MIA import is shown by the blue arrow. The FAD-dependent transfer of proteins from Mia40 to the respiratory complexes (purple) via Erv1 (blue) is shown by the black arrow. Glutathione (GSH) is important in maintaining the redox states of Mia40 and Hot13 (blue) in the intermembrane space (IMS). The interaction of the Fcj1 (yellow) component of MINOS (mitochondrial inner membrane organising system) with the TOM complex (brown) in the outer membrane (OM), and Mia40 (blue) in the inner membrane (IM), links these three complexes and mitochondrial compartments.

Electron transfer to the respiratory chain via cytochrome c (Cyc1) re-oxidises Erv1 in an FAD-dependent process [80,95]. Hot13 has also been shown to contribute to the import of disulfide-containing IMS proteins [96], possibly by helping to maintain proteins in appropriate redox states during redox active MIA import [97,98]. Mia40 has also been found to associate with small amounts of the inner membrane protein Fcj1 (a component of MINOS), and the deletion of this component produces defects in IMS import [82]. The interaction of Fcj1 with both Mia40 and the TOM complex may help localise Mia40 near precursors as they exit the TOM complex [82], and may be even more important to localise the mammalian Mia40 as it lacks the transmembrane domain that anchors the yeast form of Mia40 in the IM [99].

The coupling of import and oxidative folding via the interaction between Mia40 and the TOM complex may help increase import rates via the MIA system [100]. The import rate is also increased by the presence of glutathione [85], which releases Mia40 from unproductive substrate intermediates both *in vitro* and *in vivo* [98,100]. The latter study by Fischer et al. also shows that in mammalian mitochondria, Cox19 import is greatly reduced when the membrane potential is disrupted. This contrasts with findings in yeast, where import into the IMS via the MIA pathway is independent of the membrane potential [101]. This may provide a mechanism to ensure only healthy mitochondria import some substrates, hinting at an import regulated quality control process that may only be functioning in multicellular organisms.

3. Many paths to tread: rewiring import pathways to the IMS

Studies of mitochondrial protein import in more diverse organisms have produced the surprising revelation that while many organisms share homologues of IMS proteins, these homologues may be imported by different mechanisms in different organisms. In a few cases the alternative mechanism has been identified. For example, *S. cerevisiae* Mia40 is imported by the stop-transfer pathway, but human Mia40 lacks the transmembrane sorting signal [102], so is imported by the MIA pathway [99]. Since other eukaryotes also lack the N-terminal signal, it is likely that interactions with other proteins, such as the Mia40-Fcj1 interaction, could compensate for or perhaps be even more effective in localising Mia40 in an appropriate position. Once able to engage with an alternative import pathway the Mia40 precursor would no longer have a need for a transmembrane sorting signal. Recent work also suggests that the import rate may be influenced by the folding state of the cytosolic form of Mia40, and consequently the available cofactors and chaperones which differ considerably between yeast and humans [103].

In most cases we have evidence alternative mechanisms must be functioning, but we have yet to identify which ones. In *S. cerevisiae* the F_1F_0-ATPase is encoded in the mitochondrial genome, but in *N. crassa* the nuclear encoded protein is imported via the conservative sorting pathway [63], where the final translocation step back through the inner membrane is yet to be elucidated. In *S. cerevisiae*, Cyt1 and Cyb2 use variations of the stop-transfer pathway to reach the IMS [30]. However, the lack of stop-transfer sequence in Cyt1 from *Trypanosoma brucei* [104], and the lack of bipartite targeting signal in the *Candida albicans* Cyb2 homologue both suggest that alternative import routes must be used [24]. In other words, it is likely that evolutionary rewiring of import pathways has occurred between these organisms.

Why rewire the mitochondrial protein import routes of a given protein between biological species? The answer might lie in the metabolic constraints that are imposed by the environmental niches inhabited by organisms. *S. cerevisiae* and *C. albicans* are two yeast species that diverged 100–300 million years ago [105]. As a commensal of humans, the natural niche of *C. albicans* is the hypoxic environment of the gastrointestinal tract [106]. These conditions have been shown to alter the redox state of proteins in the IMS [107] and impair the generation of a healthy mitochondrial membrane potential [108–110]. The stop-transfer pathway, which *S. cerevisiae* uses to import Cyb2 into the IMS, critically depends on the mitochondrial membrane potential [111]; however this pathway may not function as efficiently in the hypoxic niche of *C. albicans*. This may have given organisms with mutant proteins, able to engage with other pathways, a selective advantage and eventually resulted in the loss of the signal sequence. Whether the MIA pathway is indeed involved in the import of *C. albicans* Cyb2 into the IMS needs to be determined. A better understanding of the changes in oxygen levels, ATP availability, redox states of translocase components, and membrane potential for cells growing in environments, such as host niches or multicellular biofilms, is needed to suggest which factors or pathways might be most mutually compatible.

The complexities of the import machineries suggest that the pressure to evolve a more efficient import process in response to environmental stimuli must be high. Cyb2 encodes L-lactate cytochrome-c oxidoreductase, an enzyme essential for the utilisation of L-lactate [112]. In the human host, the niches of *C. albicans* are very poor in glucose, while lactate is available as a carbon source. Therefore, there would be a significant growth advantage to *C. albicans* able to efficiently import Cyb2 into the IMS and make efficient use of lactate. The function of Cyb2 in *C. albicans* biology and pathogenesis has not been studied yet. However, in another pathogenic yeast species *Candida glabrata*, which is also a human commensal and occupies similar niches to *C. albicans*, Cyb2 is essential for utilisation of lactate. The use of this carbon source is thought to be involved in the ability of *C. glabrata* to grow in the gastrointestinal tract of its host, as determined in the murine infection model using wild type and *cyb2* mutants [113]. Importantly, *C. glabrata* and *C. albicans* are better at utilising lactate than *S. cerevisiae* when grown in low oxygen conditions *in vitro* [113]. This mimics the hypoxia present in the host niches and fits with our model that *Candida* can import Cyb2 in the IMS in hypoxic conditions, likely utilising import

pathways different to the stop-transfer pathway. As we identify more examples and characterise this rewiring process we will gain insights into the evolutionary pressures on these pathways. This will also reveal the significance of these variations for the regulation of protein import and of mitochondrial metabolism and the ability of organisms to thrive in their environmental niches.

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