

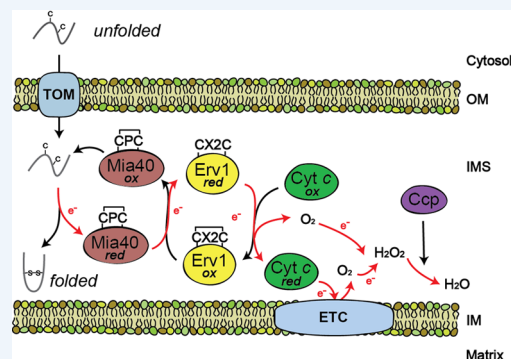
# The MIA Pathway: A Key Regulator of Mitochondrial Oxidative Protein Folding and Biogenesis

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**CONSPECTUS:** Mitochondria are fundamental intracellular organelles with key roles in important cellular processes like energy production, Fe/S cluster biogenesis, and homeostasis of lipids and inorganic ions. Mitochondrial dysfunction is consequently linked to many human pathologies (cancer, diabetes, neurodegeneration, stroke) and apoptosis. Mitochondrial biogenesis relies on protein import as most mitochondrial proteins (about 10–15% of the human proteome) are imported after their synthesis in the cytosol. Over the last several years many mitochondrial translocation pathways have been discovered. Among them, the import pathway that targets proteins to the intermembrane space (IMS) stands out as it is the only one that couples import to folding and oxidation and results in the covalent modification of the incoming precursor that adopt internal disulfide bonds in the process (the MIA pathway). The discovery of this pathway represented a significant paradigm shift as it challenged the prevailing dogma that the endoplasmic reticulum is the only compartment of eukaryotic cells where oxidative folding can occur.

The concept of the oxidative folding pathway was first proposed on the basis of folding and import data for the small Tim proteins that have conserved cysteine motifs and must adopt intramolecular disulfides after import so that they are retained in the organelle. The introduction of disulfides in the IMS is catalyzed by Mia40 that functions as a chaperone inducing their folding. The sulfhydryl oxidase Erv1 generates the disulfide pairs de novo using either molecular oxygen or, cytochrome *c* and other proteins as terminal electron acceptors that eventually link this folding process to respiration. The solution NMR structure of Mia40 (and supporting biochemical experiments) showed that Mia40 is a novel type of disulfide donor whose recognition capacity for its substrates relies on a hydrophobic binding cleft found adjacent to a thiol active CPC motif. Targeting of the substrates to this pathway is guided by a novel type of IMS targeting signal called ITS or MISS. This consists of only 9 amino acids, found upstream or downstream of a unique Cys that is primed for docking to Mia40 when the substrate is accommodated in the Mia40 binding cleft. Different routes exist to complete the folding of the substrates and their final maturation in the IMS. Identification of new Mia40 substrates (some even without the requirement of their cysteines) reveals an expanded chaperone-like activity of this protein in the IMS. New evidence on the targeting of redox active proteins like thioredoxin, glutaredoxin, and peroxiredoxin into the IMS suggests the presence of redox-dependent regulatory mechanisms of the protein folding and import process in mitochondria. Maintenance of redox balance in mitochondria is crucial for normal cell physiology and depends on the cross-talk between the various redox signaling processes and the mitochondrial oxidative folding pathway.



## 1. INTRODUCTION

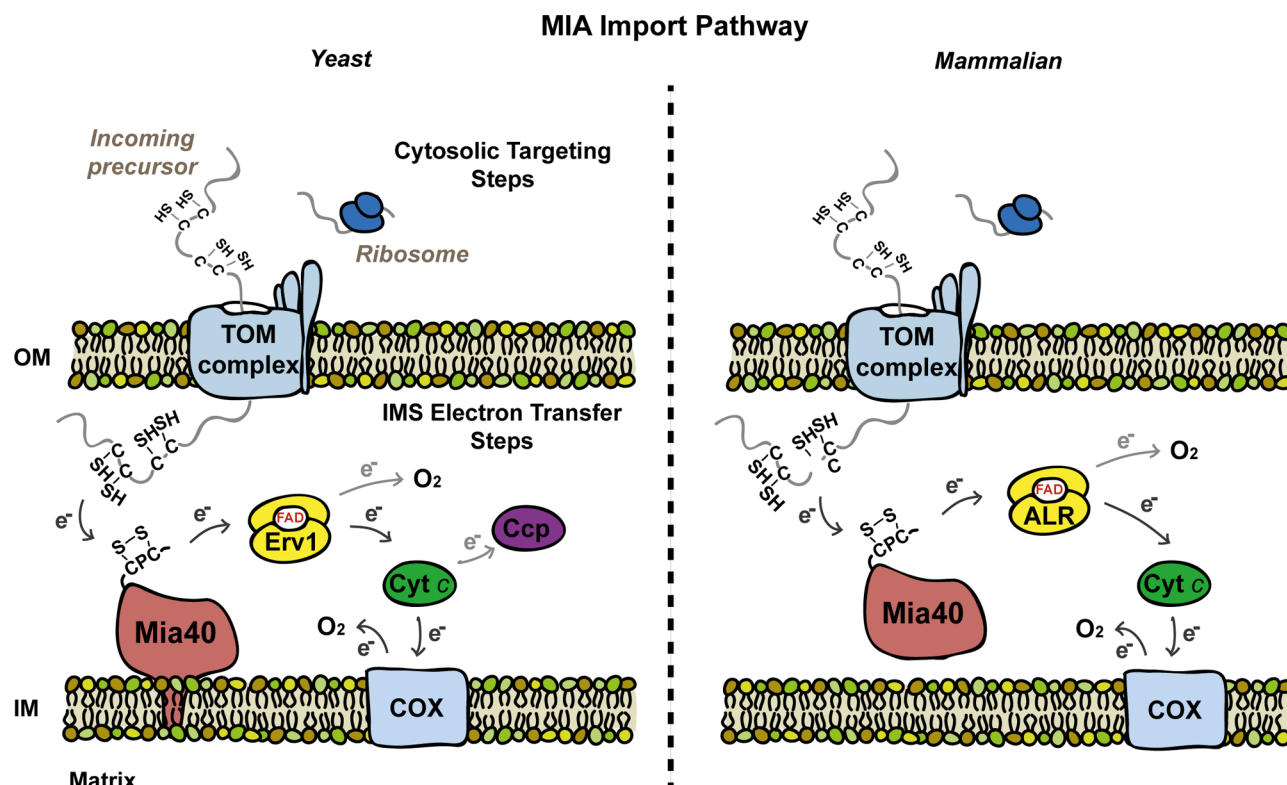
Mitochondria are multifunctional, endosymbiotic organelles. Their biogenesis depends on sophisticated import machineries to correctly target, sort, and fold around 99% of cytosolically synthesized mitochondrial proteins into their destined sub-compartments. The common entry point for incoming precursors is the translocase of the outer membrane (TOM complex). Then, depending on their target destination, they continue through one of many different types of machinery. For insertion into the outer membrane, precursors follow the sorting and assembly machinery (SAM). For insertion into the inner membrane, they enter via the TIM22 or TIM23 complex, while entry into the matrix requires the TIM23 complex. Alternatively, proteins of the intermembrane space (IMS) follow different routes depending on their target presequences. IMS proteins with cysteine residues follow the mitochondrial

IMS assembly (MIA) pathway for entrapment within the IMS by disulfide bond formation. IMS proteins without cysteines often contain bipartite targeting sequences that are cleaved after engaging with the TIM22 complex, resulting in their release into the IMS.<sup>1,2</sup>

The yeast mitochondrial proteome contains approximately 1000 proteins;<sup>3,4</sup> 5% of which reside in the smallest subcompartment, the IMS.<sup>5</sup> Typically, IMS proteins have a size around 6–22 kDa. A subset of them, like the small Tims, possess internal, noncleavable mitochondrial IMS-targeting/sorting signals (ITS/MISS) harboring conserved cysteine motifs.<sup>6,7</sup> The biogenesis of these proteins relies on Mia40, a redox-regulated IMS receptor that introduces disulfide bonds

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**Figure 1.** Electron transfer across the MIA pathway in the mitochondrial IMS. Precursors that have been synthesized on cytosolic ribosomes enter the mitochondria through the TOM complex, in a reduced and unfolded state. Those destined for detainment in the IMS by oxidative folding follow the MIA pathway. Electron flow begins from the reduced precursor to the redox active cysteine-proline-cysteine (CPC) motif of Mia40/MIA40, to the N-terminus of one Erv1/ALR subunit, to the Erv1/ALR core FAD domain of the C-terminus of the other Erv1/ALR subunit, to cytochrome *c* (Cyt *c*), to cytochrome *c* oxidase, and last to oxygen ( $O_2$ ). Alternatively, electrons can flow from Erv1/ALR directly to  $O_2$ , and in yeast from Cyt *c* to cytochrome *c* peroxidase (Ccp). Note that, in mammalian cells, MIA40 is soluble in the IMS.

via a series of electron transfer reactions, trapping them within the IMS. Examples are the twin  $CX_3C$  and twin  $CX_9C$  motif-containing proteins, such as the small Tims or members of the cytochrome *c* oxidase (COX) family, respectively.<sup>1,8</sup> In contrast to other mitochondrial proteins, IMS-targeted proteins do not require the inner membrane potential or matrix ATP hydrolysis to drive their import. This is the only mitochondrial import pathway that results in a covalent modification of the imported precursors.

The concept of oxidative folding in the IMS was first proposed in 2004<sup>9</sup> based on observations that (i) correct folding of the small Tims required disulfide bond formation after their import across the outer membrane and (ii) their oxidation *in vitro* occurred too slowly for the reaction to occur without protein-mediated catalysis.<sup>9</sup> These results expanded the first evidence of formation of disulfides in small Tims that was presented by Curran et al.<sup>10</sup> Subsequent studies identified the key components of this pathway, acknowledged as the MIA pathway/machinery, using *Saccharomyces cerevisiae*, a simple and highly amenable model organism. These components are the essential oxidoreductase Mia40 and the essential sulfhydryl oxidase Erv1. In this Account, we provide an overview of the MIA pathway from its original proposal 11 years ago to what is understood now, with a focus on the structural characterization, reconstitution, substrate specificity, and molecular recognition of substrates by Mia40. We will then discuss questions pertaining to the regulation of the pathway that remain unresolved and discuss the significance of the MIA pathway for human health and disease.

## 2. DISCOVERY OF THE KEY COMPONENTS OF THE MIA PATHWAY

The identification of Mia40 was reported almost simultaneously by three different groups.<sup>11–13</sup> Naoé et al.<sup>11</sup> carried out a reverse proteomic approach whereby essential proteins within the yeast proteomic database were systematically screened for localization to the mitochondria. Tim40 was identified (now denoted Mia40) residing on the inner membrane, facing the IMS. Analogous to this approach, Chacinska et al.<sup>12</sup> identified Mia40 by screening the yeast mitochondrial proteome for essential proteins containing predicted IMS-targeting signals. Mia40 was predicted to possess an N-terminal bipartite targeting signal and a highly conserved C-terminus. Depletion of Mia40 from yeast cells resulted in the defective import of small Tims to the IMS, but import of matrix, inner membrane and outer membrane proteins was unaffected.<sup>11–13</sup> Translocation intermediates were observed between Mia40 and the small Tims, before their assembly into larger complexes, obtained by analyzing radiolabeled Tim9 after import into mitochondria by BN-PAGE,<sup>11</sup> use of chemical cross-linkers,<sup>12</sup> or coimmunoprecipitation.<sup>13</sup> In addition, chemical cross-linking identified an interaction between Mia40 and Cox17/19 which are also resident IMS proteins.<sup>12</sup> Overall, these studies provided substantial evidence that Mia40 is a key mediator of the sorting and assembly of cysteine-containing IMS proteins.

After the discovery of Mia40, the next step was to determine its molecular mechanism in mediating the import of target IMS substrates. Less than a year later, the identification of Erv1 as a component of Mia40-mediated protein import was reported,

again, by three groups.<sup>14–16</sup> Erv1 has a long history in the literature and is a well-known FAD-dependent essential sulfhydryl oxidase in the yeast IMS. Erv1 was originally identified in 1992 and *erv1* conditional mutants of its gene displayed disrupted oxidative phosphorylation, a reduction in mitochondrial DNA transcripts, and severe growth defects, while its null mutant was inviable.<sup>17</sup> In 2005, Erv1 was suggested to play a role in Mia40-dependent import of IMS proteins as yeast *erv1* mutants resulted in a specific defect of import of small IMS proteins, and, a direct DTT-sensitive interaction with Mia40 was observed by coimmunoprecipitation.<sup>14–16</sup> Finally it was shown that Erv1 reoxidizes reduced Mia40.<sup>14–16</sup> Erv1 does not directly oxidize precursor proteins, as shown by thiol trapping assays.<sup>14</sup> Instead, it functions as a recycler of Mia40 by accepting electrons from reduced Mia40; in this mechanism Erv1 binds to Mia40 after Mia40 has accepted electrons from incoming precursor proteins via disulfide bond formation.<sup>15</sup> The electron acceptor of reduced Erv1 was found to be cytochrome *c* (cyt *c*),<sup>14</sup> which shuttles electrons through the respiratory chain via cytochrome *c* oxidase/complex IV,<sup>18</sup> therefore establishing a link between IMS protein import and mitochondrial respiration. Molecular oxygen was identified as an alternative electron acceptor from Erv1, and, as a final electron acceptor of cytochrome *c* along with cytochrome *c* peroxidase.<sup>18,19</sup> While it is not entirely known what specifies the electron acceptor that reduced Erv1 will transfer its electrons to in vivo, it is likely that the pathway changes under different physiological conditions (i.e., low vs high oxygen levels and respiratory chain activity). It is possible that there are further electron acceptors of reduced Erv1 and also of reduced Mia40 that have yet to be identified, especially those that are required in anaerobic conditions.

After the discovery of the key components, the focus was on characterizing the full MIA pathway by a combination of reconstitution experiments and detailed structural studies on Mia40 and Erv1. These studies provided initial information on the flow of electrons across the pathway, the interactions between these components, and how their domains guide their interactions.

### 3. INITIAL RECONSTITUTION OF THE MIA PATHWAY

In 2009, the full in vitro reconstituted MIA pathway was reported by the Koehler group using Tim13, as a twin CX<sub>3</sub>C substrate.<sup>20</sup> Oxygen consumption assays were used to determine the midpoint potentials ( $E_m$ ) of Mia40 and Tim13 throughout the reaction, which began by incubating reduced Tim13, Mia40, Erv1, and molecular oxygen. The resultant products were oxidized Tim13 and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), as expected. Importantly, the  $E_m$  values were more positive along the reaction (from Tim13 to Mia40 to Erv1 to oxygen), indicating that the electron transfer reaction was thermodynamically favorable.<sup>20</sup> Further support came from a study using Cox19 as a twin CX<sub>9</sub>C substrate and cytochrome *c* as the final electron acceptor<sup>21</sup> showing a complete oxidation of Cox19. In this work, it was also reported that Erv1 functions as a noncovalently bound homodimer. Electrons from reduced Mia40 are shuttled to the N-terminus of one subunit of Erv1 and then onto the FAD domain of the C-terminus of the second subunit. These reconstitution assays in combination with detailed structural studies of Mia40 and Erv1 began to reveal the molecular interactions that result in electron transfer. A schematic depiction of the flow of electrons across the MIA pathway is shown in Figure 1.

## 4. STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF THE IMS RECEPTOR Mia40

Yeast Mia40 is a 44 kDa, IMS protein anchored to the IM via its hydrophobic N-terminus, with its soluble C-terminal domain exposed to the IMS.<sup>13</sup> The N-terminal stretch is not essential for the function of Mia40 as its deletion in yeast is viable; however, it contains an amphipathic IM-targeting signal which directs Mia40 to the TIM23 complex for entry into the IM.<sup>11,12</sup> Mia40 homologues in higher eukaryotes (such as human MIA40) do not possess this signal sequence and are therefore found only as soluble IMS proteins, suggesting that activity of Mia40 lies within its conserved IMS domain. There are six highly conserved cysteine residues (CPC...CX<sub>9</sub>C...CX<sub>9</sub>C) present within the IMS domain, each found to have distinct roles based on reconstitution and mutagenesis experiments.<sup>11,12,23</sup> The first two cysteines make up a small redox active cysteine-proline-cysteine (CPC) motif of which the second cysteine is involved in catalyzing the formation of a mixed disulfide intermediate with incoming IMS precursors and is subsequently resolved by the first.<sup>23–25</sup> Mutation of the first two cysteines to serines (SPS) in yeast Mia40 resulted in lethality in vivo as presumably the SPS mutant could not oxidize any substrates (some of which are essential).<sup>11</sup> Neither one of the single cysteine mutants (SPC or CPS) nor the SPS mutant could oxidize Tim10 in vitro.<sup>23,24</sup> Mutations of the other cysteines (those that comprise the twin CX<sub>9</sub>C motif) to serines resulted in a loosely folded Mia40; both the SX<sub>9</sub>S–CX<sub>9</sub>C and CX<sub>9</sub>C–SX<sub>9</sub>S resulted in lethality in vivo.<sup>23</sup> This was suggestive of their involvement in the structural integrity of Mia40 via the formation of two intramolecular disulfide bonds between the two CX<sub>9</sub>C motifs, C4–C5 and C3–C6.<sup>23</sup>

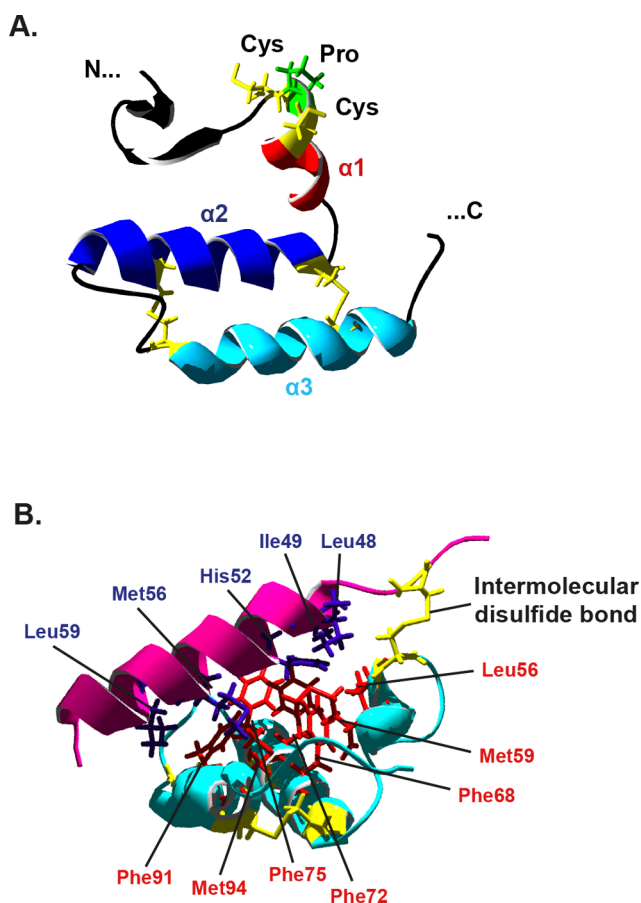
A comprehensive structural characterization of human MIA40 using NMR<sup>24</sup> clearly showed that the CPC motif functions as the catalytic domain and that structural intramolecular disulfides stabilize its structure. Similar results were reported by X-ray crystallography of the yeast Mia40 C-terminal domain.<sup>25</sup> Both studies agreed on the roles of the different cysteine residues in mediating intermolecular (via the first and second cysteines) and intramolecular disulfide bond formation (between the third and sixth, and the fourth and fifth).<sup>24,25</sup> These studies also highlighted that the CPC motif is part of a “flexible” helix functioning as a lid, adjacent to a stretch of hydrophobic amino acids making up a hydrophobic cleft that prominently exposes the CPC motif to the IMS.<sup>23,24</sup> The hydrophobic cleft was found to be essential for the recognition and binding of substrate proteins as mutations within this region were lethal in vivo and prevented binding of substrates in vitro<sup>24</sup> (Figure 2). These defects resulted from a combinatorial effect of several mutated hydrophobic residues (Leu56, Met59, Phe72, Phe75, Phe91, and Met94) to alanines.

## 5. PROTEIN FOLDING

### 5.1. Molecular Recognition of Substrates and Their Initial Oxidation by Mia40

The recognition of twin CX<sub>3</sub>C and CX<sub>9</sub>C substrates by Mia40 has been extensively characterized and has been found to occur via site-specific hydrophobic interactions which allow a two-step disulfide bond formation event between the active CPC site of Mia40 and their ITS/MISS (Figure 3).<sup>6,26–28</sup> The ITS/MISS is nine amino acids long and is located either upstream or downstream of the “docking” cysteine; its presence is adequate and essential for Mia40 recognition.<sup>6</sup> Grafting this peptide to a





**Figure 2.** Solution structures of human MIA40 alone and in complex with human COX17. (A) Solution NMR structure of human MIA40 (hMIA40) (PDB ID: 2K3J). The redox active cysteine-proline-cysteine (CPC, yellow, green, yellow) motif is located within a one and a half turn, flexible helical structure ( $\alpha 1$ , red). Two other helices are present within the core domain ( $\alpha 2$ , blue;  $\alpha 3$ , cyan), creating a hydrophobic cleft which is connected by two intramolecular disulfide bonds (yellow). (B) Solution NMR structure of core hMIA40 (cyan) in complex with the MIA40-induced  $\alpha$ -helix in hCOX17 (magenta) (PDB ID: 2L0Y). hCOX17 sits on top of the hydrophobic cleft of hMIA40 adjacent to the CPC motif. This interaction is covalent via an intermolecular disulfide bond but the initial recognition of hMIA40 and hCOX17 occurs via noncovalent interactions mediated by hydrophobic residues present in both hMIA40 (red) and hCOX17 (blue).

nonmitochondrial protein can target the protein to the IMS.<sup>6</sup> Substrates that contain a twin CX<sub>3</sub>C or CX<sub>9</sub>C motif require the formation of two disulfide bonds for their complete oxidation. Crucially, the import of the small Tims and Cox17 requires them to be in a reduced, unfolded state within the cytosol prior to their passage through the TOM complex.<sup>9,12</sup> Within milliseconds upon entry to the IMS, substrates engage in dynamic hydrophobic interactions with Mia40.<sup>29,6</sup> This rapid positioning allows the selection of the first of their N-terminal CX<sub>n</sub>C motif for “docking” in a mixed disulfide intermediate with the second cysteine of the CPC motif following a nucleophilic attack. This two-step mechanism was described as the sliding-docking model.<sup>6</sup> The precursor binding is coupled to the Mia40-induced folding of the first substrate helix, creating a partially folded ITS.<sup>28</sup> The substrate is then released from Mia40 as a result of a second nucleophilic attack, this time on the “docking” cysteine by the second resolving cysteine of

the substrate C-terminal CX<sub>3</sub>C motif.<sup>26–28</sup> After substrate release, the Mia40 CPC motif remains reduced and gets reoxidized by Erv1 back to its functional state as an IMS receptor.

## 5.2. Oxidation of the Second Disulfide Bond

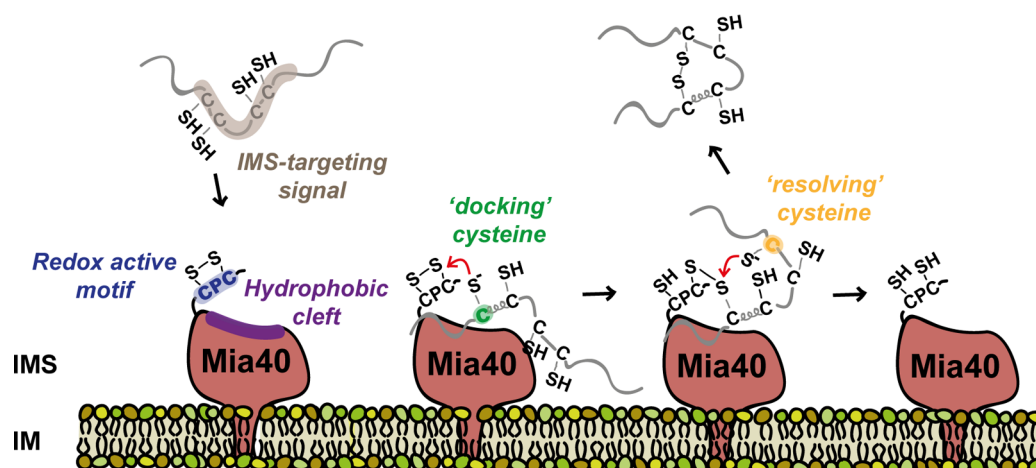
Various mechanisms have been proposed for the formation of the second intramolecular disulfide bond in twin CX<sub>3</sub>C and CX<sub>9</sub>C substrates. One proposal is that the release of substrates from Mia40 with their partially folded ITS/MISS induces folding of the second coiled-coil helix and is coupled to the formation of an intramolecular disulfide bond between the inner two cysteine residues; catalyzed either by molecular oxygen, glutathione, or a yet to be identified oxidant<sup>26,28</sup> (Figure 4A). Further evidence for this mechanism of induced folding coupled to oxidation was recently reported,<sup>30</sup> as chemical induction of a structure resembling the coiled-coil helix was sufficient to accelerate oxidative folding in the absence of Mia40. The earlier reconstitution assays by Bien et al.<sup>21</sup> support a similar mechanism, as the oxidation of both disulfides in Cox19 required only excess of oxidized Mia40 (bypassing the need for reoxidation of Mia40). This could either occur via the mechanism mentioned above (Figure 4A) or via repeat substrate binding to Mia40 (Figure 4B). However, in addition to the presence of completely oxidized Cox19, the reaction also produced long-lived mixed disulfide intermediates. Therefore, although complete oxidation can occur solely by Mia40, it may not represent the most efficient oxidation mechanism. A third possibility is that a ternary complex between Mia40, Erv1 and the substrate forms, thereby allowing Mia40 to introduce both disulfide bonds before substrate release<sup>31</sup> (Figure 4C). Such a ternary complex has been observed both in organello<sup>31,32</sup> and in vivo,<sup>32</sup> most likely via noncovalent interactions between Mia40 and Erv1, but it is not known how electron shuttling through this complex occurs.

During the reviewing process of this manuscript, a new study from the Koehler lab suggests that Mia40 can act as an “electron sink” by accepting up to six electrons from substrates. This was based on reconstitution and gel shift assays indicating that Mia40 can be with all three of its cysteine pairs completely reduced—both in vitro and in vivo.<sup>33</sup> As two electrons must be accepted by Mia40 per disulfide bond formed, Mia40 in this scenario can insert up to three disulfide bonds into substrates.

## 5.3. The Formation of Long-lived Intermediates Facilitates Proofreading of Non-Native Disulfides

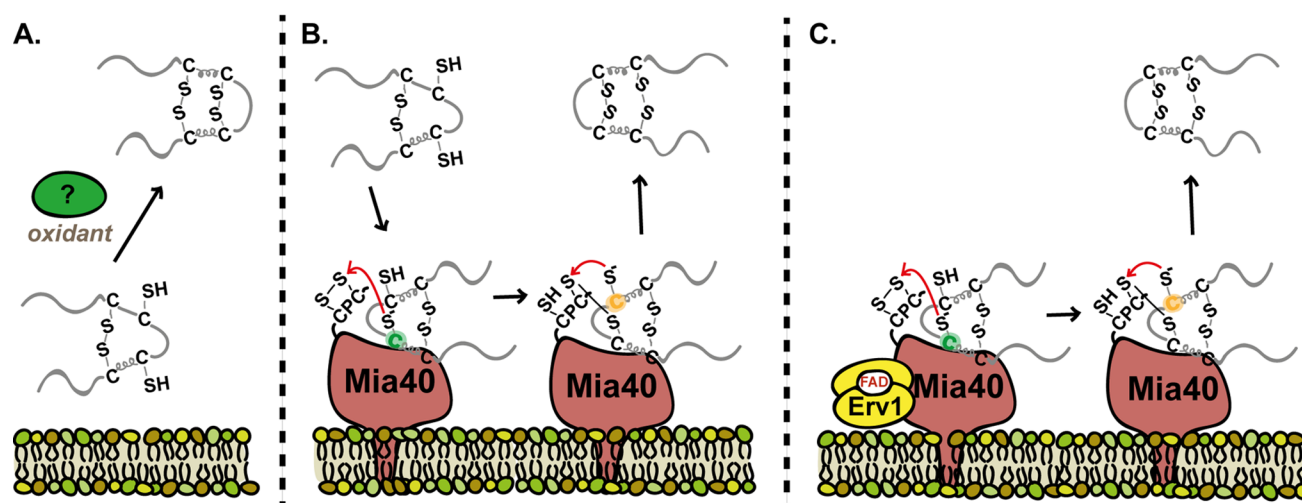
How does Mia40 recognize that it has correctly introduced the native disulfide before releasing the substrate? A proofreading role by reduced glutathione (GSH) was suggested,<sup>21</sup> as its addition to the in vitro assay (with oxidized Mia40 and Cox19) prevented the formation of long-lived intermediates and accelerated the oxidation of Cox19. However, as this study could not characterize which intermediates were present it is unclear whether GSH directly reduces wrong disulfides and a proofreading role of GSH in vivo is still unclear. A somewhat different scenario has recently been suggested proposing that Mia40 itself is involved in directing the native folding pathway and in the reshuffling of non-native disulfides acting concurrently as a disulfide isomerase.<sup>34,35</sup> However, an isomerase function for Mia40 (or indeed any other protein of the IMS) in vivo is still unclear. Although the twin CX<sub>3</sub>C and CX<sub>9</sub>C substrates, used in all of the reconstitution studies to date, only require two disulfide bonds, other Mia40 substrates require many more. The folding pathway of more complex

## Recognition of Substrates by Mia40 and Their Initial Oxidation in the IMS



**Figure 3.** Molecular recognition of substrates containing twin CX<sub>3</sub>C or CX<sub>9</sub>C motifs by Mia40. Precursors containing IMS-targeting signals (ITS/MISS) “slide” onto the hydrophobic binding cleft of Mia40 via hydrophobic interactions. This allows docking of its active cysteine to the CPC motif of Mia40 intermolecularly. The substrate is released when the “resolving” cysteine forms an intramolecular disulfide with the “docking” cysteine.

## Completion of Substrate Oxidation



**Figure 4.** Possible mechanisms for completion of substrate oxidation. Sliding of the substrate onto Mia40 via hydrophobic interactions results in the nucleophilic attack of the “docking” cysteine of the substrate on the first cysteine of the CPC motif of Mia40 (red line). This forms a mixed, covalent intermediate which is coupled to folding of the substrate's first coiled-coil helix. After this, the substrate is thought to follow one of three possible scenarios. (A) Substrate release after initial oxidation is coupled to the folding of the second coiled-coil helix which induces formation of the second disulfide bond by an unknown oxidant. Or (B) the partially oxidized released substrate slides onto another oxidized Mia40 nearby and the reaction occurs again, releasing the fully oxidized substrate. Or (C) the full reaction occurs sequentially at the same site in a ternary complex between Mia40, Erv1, and the substrate due to the reoxidation of Mia40 by Erv1.

cysteine-containing substrates has yet to be completely characterized; it is conceivable that Mia40 can bind to more than one location along the substrate engaging in multiple rounds of substrate oxidation. Exactly how this might occur *in vivo* has yet to be determined.

## 6. THE EXPANDING REPERTOIRE OF TARGET SUBSTRATES

### 6.1. Twin CX<sub>3</sub>C and CX<sub>9</sub>C Substrates

The first recognized substrates of Mia40 contained twin CX<sub>3</sub>C (i.e., the small Tims) or twin CX<sub>9</sub>C motifs (i.e., Cox17/19) that

share a coiled coil–helix1–coiled coil–helix 2 (CHCH) fold.<sup>12,14–16</sup> The small Tims function as chaperones of the IMS<sup>36</sup> and all possess highly conserved twin CX<sub>3</sub>C motifs. Systematic studies were carried out in yeast to compile a comprehensive list of proteins containing (or predicted to contain) twin CX<sub>9</sub>C motifs.<sup>8,37,38</sup> Some of these were confirmed experimentally to be substrates of Mia40 (including Mdm35, Mic14/17, and Cmc2/3/4, among others<sup>8,37</sup>). A genome-wide analysis revealed that most of these proteins elicit only a few different functional roles, mainly structural, within mitochondria.<sup>38</sup>

Table 1. Known Substrates of Yeast Mia40

protein (yeast)	size (kDa)	cysteine motifs	function	ref
small Tims (Tim8, Tim9, Tim10, Tim12, Tim13)	10, 10, 10, 12, 11	...CX <sub>3</sub> C...CX <sub>3</sub> C...	chaperones of mitochondrial protein import	11–13
Cox family (Cox17, Cox19)	8,11	...CX <sub>3</sub> C...CX <sub>3</sub> C...	copper chaperones	12, 13
Mia40	44	...CX <sub>9</sub> C...CX <sub>9</sub> C...	mitochondrial oxidative folding; IMS receptor	42, 43
Erv1	22	...CX <sub>2</sub> C...CX <sub>2</sub> C...CX <sub>16</sub> C...	Fe/S cluster biogenesis and mitochondrial oxidative folding; reoxidises Mia40	8, 40, 41
Dre2	39	...CX <sub>2</sub> C...CX <sub>2</sub> C...	Fe/S cluster biogenesis	44
Sod1	16	...CX <sub>88</sub> C...	superoxide dismutase	39
Ccs1	27	...CX <sub>2</sub> CX <sub>6</sub> CX <sub>36</sub> CX <sub>94</sub> C...CXC...	copper chaperone for Sod1	39
Cmc2, Cmc3, Cmc4	13, 17, 8	...CX <sub>9</sub> C...CX <sub>9</sub> C...	respiratory chain assembly	37
Mix14, Mix17	14, 17	...CX <sub>9</sub> C...CX <sub>9</sub> C...	mitochondrial respiration	8
Mdm35	10	...CX <sub>9</sub> C...CX <sub>9</sub> C...	mitochondrial distribution and morphology	8
Tim22	22	...CX <sub>98</sub> C...	key component of the tim22 complex	47
Mrp10	11	...CX <sub>9</sub> C...CX <sub>9</sub> C...	mitochondrial ribosomal protein	48
Atp23	32	...CX <sub>2</sub> C...CX <sub>2</sub> C...CX <sub>38</sub> C...CX <sub>24</sub> C...CX <sub>16</sub> C...	putative metalloprotease	45

## 6.2. Substrates with Alternative Cysteine Motifs

Over time it became evident that Mia40 can mediate the import of a much wider subset of IMS proteins, including some with unconventional cysteine motifs that do not contain a CHCH domain and are larger. Examples are the 27 kDa copper chaperone Ccs1 which contains a CX<sub>2</sub>CX<sub>6</sub>CX<sub>36</sub>CX<sub>n</sub>C motif,<sup>39</sup> Erv1 which contains a CX<sub>15</sub>C motif and twin CX<sub>2</sub>C motifs,<sup>8,40–43</sup> and the Fe/S cluster protein human Anamorsin/yeast Dre2.<sup>44</sup> The exact molecular recognition of these substrates is not yet well understood. The list of MIA-substrates continues to grow in unexpected ways revealing a much more extended role of Mia40 in the import and folding even of mitochondrial proteins outside the IMS.<sup>45–48</sup>

## 6.3. Novel Substrates Reveal Expanded Chaperone-like Activities of Mia40

Mia40 was first suggested to function as a molecular chaperone by Banci et al.<sup>28</sup> based on the observation that binding to Mia40 induces helical folding of their CHCH domains. This chaperone activity of Mia40 is largely dictated by the hydrophobic cleft of Mia40 that can accommodate unfolded segments of the substrates as these frequently exhibit hydrophobic patches. Novel substrates that were identified to rely on the chaperone activity of Mia40 were Atp23, Tim22, human ChChd3, and Mrp10.<sup>45–48</sup> The IMS protease Atp23 requires formation of five disulfide bonds which are thought to accumulate during multiple rounds of Mia40 binding.<sup>45</sup> Interestingly, import of Atp23 can occur independently of oxidation as a cysteine-free Atp23 mutant still gets imported in a Mia40-dependent manner. In this case, the hydrophobic cleft of Mia40, and not its redox active CPC motif, was the critical factor for Atp23 import and prevention of aggregates. Thus, it was concluded that Mia40 exerts a general folding role within the IMS reminiscent of chaperone proteins,<sup>45</sup> in agreement with the prior studies on CX<sub>3</sub>C substrates.<sup>28</sup> Moreover, this chaperone-like activity is not restricted to IMS proteins, since Mia40 promotes the integration of Tim22<sup>47</sup> (a polytopic IM protein) and human ChChd3<sup>46</sup> (an IM-anchored protein), and Mrp10<sup>48</sup> (a matrix-targeted ribosomal subunit Mrp10). Mrp10 contains a twin CX<sub>9</sub>C motif that is oxidized by Mia40 in transit via the TIM23 complex into the matrix. However, its oxidation is not essential for import as (i) Mrp10 can still be imported in the absence of Mia40 and (ii) a cysteine-free variant of Mrp10 can be imported.<sup>48</sup> Instead, Mrp10 oxidation by Mia40 stabilizes the protein and prevents its degradation in the

matrix. It is therefore plausible that Mia40 plays a regulatory role in the expression of mitochondrially encoded proteins by controlling the stability of ribosomal proteins. In addition, APE1, a human matrix protein involved in repairing damaged nuclear and mtDNA has now been identified as a substrate of MIA40.<sup>49</sup>

Overall, these studies are rapidly revealing additional chaperone-like roles of Mia40 for substrates that simply pass through the IMS and that do not contain the typical ITS/MISS sequences. Mia40 likely acts as a chaperone for a subset of substrates that reside in multiple mitochondrial subcompartments upon their initial entry through the TOM complex. In future years, the full repertoire of yeast Mia40 (and mammalian MIA40) substrates and its functional capabilities will be characterized; yeast substrates that have been characterized to date are listed in Table 1.

## 7. REGULATION OF THE OXIDATIVE FOLDING PATHWAY

Redox homeostasis is crucial for the diverse functions of mitochondria and so it is expected that the MIA40 pathway is regulated to adapt to redox changes. The IMS proteome from yeast mitochondria<sup>5</sup> led to the discovery that key antioxidant enzymes involved in reductive reactions like thioredoxin 1 (Trx1) and thioredoxin reductase (Trr1) as well as peroxiredoxin (Hyr1/Gpx3) reside in the IMS. Additionally, a glutaredoxin 2 (Grx2) activity that is thought to control the levels of GSH has also been reported for the IMS.<sup>50,51</sup> The extent of the interactions of these redox balancing systems with the MIA machinery remain to be discovered and they may involve not just protein–protein interactions but also small molecule oxidants (H<sub>2</sub>O<sub>2</sub>, superoxide anion), and reductants (NADPH). The ramifications of these interactions are important for a full understanding of the regulation of the mitochondrial biogenesis process and also for the links between mitochondrial dysfunction and the cellular redox signaling pathways.

## 8. CONCLUDING REMARKS

The discovery of the mitochondrial oxidative folding pathway brought cellular redox chemistry mechanisms to the heart of mitochondrial protein biogenesis processes. The major components of the MIA pathway, their structures at atomic resolution, and the basic features of the underpinning



mechanism of oxidative folding are known to a good degree. However, future studies will have to address the mechanistic details of the key molecular interactions for an ever increasing spectrum of different substrates using biophysical and high-resolution structural techniques. There is increasing awareness of the important relevance of the oxidative folding MIA pathway for human pathology and biomedical conditions. These include hypoxic signaling in cancer where Mia40 can interact with p53<sup>52</sup> and affects the stabilization of HIF1 $\alpha$ ,<sup>53</sup> Huntington's disease where a mouse model for the disease displayed defects in mitochondrial oxidative folding,<sup>54</sup> and amyotrophic lateral sclerosis where folding mutants of superoxide dismutase 1 (SOD1) linked to ALS reside in the IMS subject to interaction with the oxidative folding machinery.<sup>55</sup> These conditions may represent different cellular responses to a variety of stress stimuli that affect the protein homeostasis process in the cell, and as a consequence normal cell physiology. Understanding the plasticity and dynamics of mitochondrial protein biogenesis in response to stress in molecular terms will be an exciting challenge for the future.

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### Notes

The authors declare no competing financial interest.

### Biographies

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**Kostas Tokatlidis** was born in Veria, Greece in 1966. He received his undergraduate degree from the University of Thessaloniki and his PhD from the University of Delaware jointly with the Institut Pasteur in 1993. After a 5 year postdoc with Jeff Schatz at the Biozentrum in Basel he set up his group in 1998 at Manchester University. He moved to IMBB-Crete in 2003 where he was promoted to Professor in 2011. In 2013, he was elected EMBO member, Wolfson-Royal Society research merit Fellow and holds the Cathcart Chair of Biochemistry at the University of Glasgow.

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