Development of the Mitochondrial Intermembrane Space Disulfide Relay Represents a Critical Step in Eukaryotic Evolution

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Abstract

The mitochondrial intermembrane space evolved from the bacterial periplasm. Presumably as a consequence of their common origin, most proteins of these compartments are stabilized by structural disulfide bonds. The molecular machineries that mediate oxidative protein folding in bacteria and mitochondria, however, appear to share no common ancestry. Here we tested whether the enzymes Erv1 and Mia40 of the yeast mitochondrial disulfide relay could be functionally replaced by corresponding components of other compartments. We found that the sulfhydryl oxidase Erv1 could be replaced by the Ero1 oxidase or the protein disulfide isomerase from the endoplasmic reticulum, however at the cost of respiration deficiency. In contrast to Erv1, the mitochondrial oxidoreductase Mia40 proved to be indispensable and could not be replaced by thioredoxin-like enzymes, including the cytoplasmic reductase thioredoxin, the periplasmic dithiol oxidase DsbA, and Pdi1. From our studies we conclude that the profound inertness against glutathione, its slow oxidation kinetics and its high affinity to substrates renders Mia40 a unique and essential component of mitochondrial biogenesis. Evidently, the development of a specific mitochondrial disulfide relay system represented a crucial step in the evolution of the eukaryotic cell.

Key words: **evolution, eukaryotic cells, Mia40, mitochondria, oxidative protein folding, Pdi1, protein import.**

Article

Introduction

Protein structures can be stabilized by covalent interactions between cysteine residues. The presence of disulfide bonds in proteins is largely confined to three specific cellular compartments in which proteins reach their 3D state in a process referred to as oxidative protein folding (Banci et al. 2010; Peleh et al. 2014; Arts et al. 2016; Ponsero et al. 2017; Ellgaard et al. 2018; Kritsiligkou et al. 2018). These compartments are 1) the periplasm of gram-negative bacteria, 2) the endoplasmic reticulum (ER), and 3) the intermembrane space (IMS) of mitochondria (fig. 1*A*). In other cellular compartments, in particular in the cytosol, most cysteine residues are present in the reduced state and disulfide bonds are largely restricted to redox enzymes or proteins that contain thiol switches for regulatory purposes (Buchanan and Balmer 2005; Hillion and Antelmann 2015; Leichert and Dick 2015; Riemer et al. 2015; Topf et al. 2018). Oxidative protein folding is mediated by specific oxidoreductases, which are called DsbA in the periplasm, protein disulfide isomerase (PDI) in the ER, and Mia40 in the IMS (fig. 1*B* and *C*). DsbA and PDI belong to a large protein family of thioredoxins (Lu and Holmgren 2014). They are characterized by structures of four beta sheets sandwiched between three alpha helices that contain a redox-active CXXC motif. Members of the thioredoxin superfamily are found in all three kingdoms of life and in almost every cellular compartment. Depending on the structure around the redox-active cysteine pair, the redox potential of thioredoxins can differ considerably. Some family members keep their substrate proteins mainly reduced, cytosolic thioredoxins for instance, whereas others predominantly oxidize substrate proteins (Mossner et al. 2000). By mutagenesis of two variant residues in their CXXC motif, the redox potential of thioredoxins and DsbA can be considerably changed, modulating their properties rather freely between reducing and oxidizing activities (Huber-Wunderlich and Glockshuber 1998; Mossner et al. 1998; Jonda et al. 1999; Mossner et al. 1999; Maskos et al. 2003).

The protein oxidation machinery in the IMS of mitochondria consists of two essential proteins, Mia40 and Erv1 (Chacinska et al. 2004; Naoe et al. 2004; Allen et al. 2005; Mesecke et al. 2005). They are present in many eukaryotes (fig. 1; Basu et al. 2013) but are not present in bacteria. The flavoprotein Erv1 is a sulfhydryl oxidase that forms disulfide bonds in Mia40 thereby transferring electrons into the respiratory chain of the inner membrane (Farrell and Thorpe 2005; Bihlmaier et al. 2007; Dabir et al. 2007). Mia40 is a simply

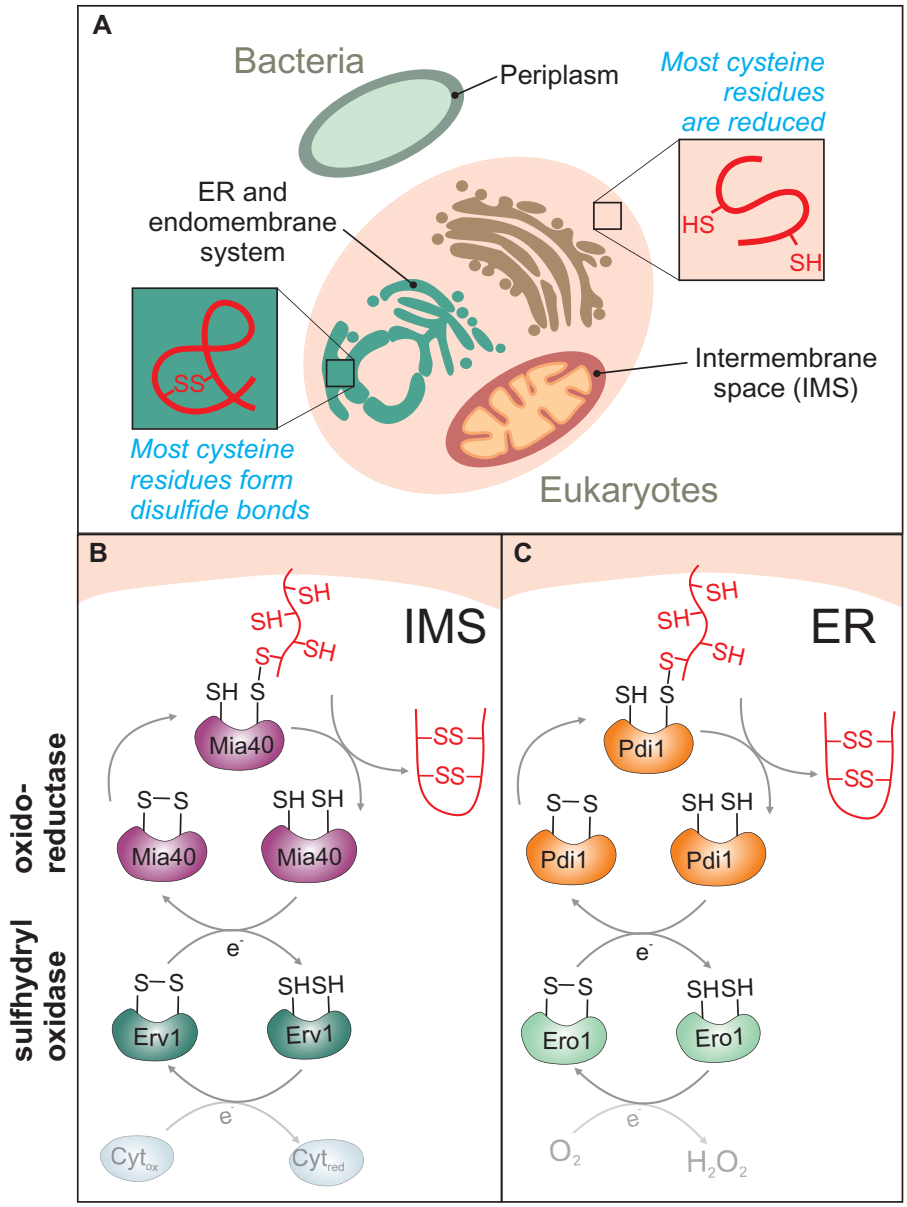

Fig. 1. Cells employ structurally different, unrelated oxidoreductases for oxidative protein folding. (*A*) In the periplasm, the IMS and the ER, most protein thiols are oxidized whereas structural disulfides are largely absent from cytosolic proteins. (*B*, *C*) Schematic representation of the disulfide relays operating in the IMS of mitochondria and the ER. Although the oxidoreductases and sulfhydryl oxidases catalyze the analogous biochemical reactions, the two systems apparently originated from different ancestors.

structured helix-loop-helix protein with a redox-active CPC motif. It is not related to the thioredoxin superfamily and its structure is entirely different. Nevertheless, its functional properties are analogous to that of some members of the thioredoxin family. Just like thioredoxins, Mia40 interacts with its substrates via a hydrophobic binding groove that is in close proximity to a redox-active cysteine pair (Banci et al. 2009; Kawano et al. 2009; Backes and Herrmann 2017). Why the IMS employs such a unique and unconventional oxidoreductase instead of an IMS-specific member of the thioredoxin family is not known. The IMS of mitochondria evolved from the periplasm of bacteria and, hence, from a compartment where disulfide bonds were formed, likely by a member of the DsbA-type thioredoxin family. The origin of the eukaryotic cell was radical and characterized by the emergence of elaborate cell compartmentalization (Gould et al. 2016) and thousands of new gene families (Koonin 2015). It also included the substitution of the thioredoxin-mediated oxidation machinery of the mitochondrial IMS through the Mia40 system, but for reasons that remain obscure.

Despite its analogous function as an oxidoreductase, Mia40 shows a few critical differences to thioredoxins. Whereas thioredoxins rapidly react with their substrates (milliseconds time range), Mia40 traps its substrates for several minutes, presumably to facilitate the translocation of reaction intermediates across the outer membrane (Naoe et al. 2004; Rissler et al. 2005; Bien et al. 2010; Fischer et al. 2013; Koch and Schmid 2014b). In contrast to thioredoxins, Mia40 shows a narrow substrate specificity; its hydrophobic binding pocket selectively interacts with signatures in helical

structures of its substrates, called mitochondrial IMS sorting sequence or IMS targeting signal sequences (Milenkovic et al. 2009; Sideris et al. 2009; Topf et al. 2018). This differs from thioredoxins, whose cysteine pairs readily equilibrates with exposed thiols of many proteins and to some extent even with nonprotein thiols such as glutathione (GSH) (Lundstrom and Holmgren 1993; Kojer et al. 2015). Finally, the direction of the redox reaction of thioredoxins and their substrates simply depends on their redox potentials; in contrast, it is not clear whether Mia40 also reduces substrate proteins in the IMS, albeit recent in vitro studies suggest that Mia40 can exhibit isomerase activity to rescue nonnative substrates (Koch and Schmid 2014a, 2014b; Hudson and Thorpe 2015).

In order to better understand the specific properties of the mitochondrial protein oxidation machineries, we tested whether the mitochondrial redox relay of yeast can be replaced by components of the oxidation machinery of the yeast ER or the periplasm of *Escherichia coli*. To this end, we generated IMS-targeted variants of yeast PDI (*ims*-Pdi1), its oxidase Ero1 (*ims*-Ero1), thioredoxin and DsbA (*ims*-Trx or *ims*-DsbA). None of these factors could functionally replace Mia40 in vivo. We found that in the IMS, Pdi1 is fully reduced unless the cellular GSH pool is chemically oxidized, indicating that its interaction with GSH prevents its oxidase activity in vivo. Upon depletion of reduced GSH, *ims*-Pdi1 becomes partially oxidized but, presumably due to its poor trapping properties, still fails at mediating the import of Mia40 substrates. Surprisingly, however, the expression of *ims*-Pdi1 allowed yeast cells to lose the otherwise essential Erv1 oxidase, but these Erv1-deletion mutants showed severe mitochondrial dysfunctions. Our observations suggest that the replacement of the bacterial thioredoxin-like oxidoreductase was a necessary step in eukaryogenesis to mediate disulfide bond formation in the inter membrane space of the evolving mitochondrion and maybe associated with the need to develop a machinery for the import of hundreds of proteins from the cytosol.

Results

Eukaryotes are the product of endosymbiosis and the integration of a proteobacterium into the cytosol of an archaeal host. Due to the unique nature of eukaryogenesis and it having occurred some 1.8 billion years ago (Betts et al. 2018), it is no easy task to trace back the origin of all eukaryotic gene families. We screened the genomes of 150 eukaryotes (fig. 2; supplementary fig. 1, Supplementary Material online) to analyze the distribution of the relevant genes in question (Erv1, Ero1, Pdi1, and Mia40) in more detail, using the yeast genes as queries as they are in the focus of this experimental study. As a consequence, we find the most conserved set of genes among the opisthokonts (fig. 2, supplementary fig. 1, Supplementary Material online, supplementary tables 1 and 2, Supplementary Material online). Erv1, functional partner of Mia40, is the most conserved and present in all eukaryotic groups. The distribution of the other genes is patchier, in particular those expressing Ero1 and Mia40, but largely consistent with what was observed for a smaller set of eukaryotes (Basu et al. 2013). It is likely that our search did not identify all Ero1 and Mia40 homologs. Using a different approach (e.g., a HMM-based search), other databases, or less stringent cutoffs leads to the identification of additional homologs such as that of *Arabidopsis*, which considerably differs functionally and structurally from that of baker's yeast (Peleh et al. 2016, 2017). But in any case, Mia40 is found encoded across the animal and plant divide, providing evidence for its ancient origin. Addition of remote homologs in searching strategies would only strengthen the case made here.

An IMS-Targeted Version of Ero1 Renders Erv1 Dispensable

Though not related by common ancestry, Ero1 and Erv1 are both flavine adenine dinucleotide (FAD)-binding oxidoreductases of analogous structure (Gross et al. 2004; Kawano et al. 2009). Members of the Ero1 family oxidize proteins in the ER. The Erv1 family is more heterogeneous and members are employed by very different organisms and viruses to form disulfide bonds in the IMS (Erv1), the ER (Erv2), the late secretory pathway, and the extracellular space Quiescin Sulfhydryl (QSOX) or the cytosol (viral Erv1 homologs). In order to test whether Erv1 can be functionally replaced by Ero1, we constructed a fusion protein (*ims*-Ero1) consisting of the IMS-targeting region of Mia40 (residues 1–70) fused to the mature part of Ero1 (residues 56–424 that lack the ER signal peptide) and a hemagglutinin (HA) tag. We expressed this protein under control of the *MIA40* promoter in a shuffle strain that contained an Erv1-expression *URA3* plasmid in a Δ*erv1* background (Peleh et al. 2016, 2017). Erv1 is an essential protein and yeast cells without Erv1 are inviable. The expression of Erv1 from the *URA3* plasmid allowed this strain to grow (fig. 3A). To test whether the expression of *ims*-Ero1 likewise rescues the Δ*erv1* mutant, we grew these cells on 5-fluoroorotic acid (5-FOA). This compound is converted into the toxic nucleotide analog 5-fluoro uracil by URA3. Thus, in the presence of 5-FOA only cells that lost the *URA3*-containing plasmid can survive (fig. 3A). Some colonies of this strain were able to grow on 5-FOA, indicating the loss of the *URA3*-containing Erv1 expression plasmid (fig. 3B, sector 1). However, many colonies retained the Erv1-encoding plasmid and thus were unable to grow on 5-FOA, suggesting that even in the presence of *ims*-Ero1, the presence of Erv1 is of considerable advantage for the cells.

By Western blotting with Erv1-specific antibodies, we confirmed that the 5-FOA-resistant strain had lost the *ERV1* gene (fig. 3C). This mutant's growth on fermentable carbon sources was strongly impaired and inhibited on nonfermentable carbon sources (fig. 3D). Thus, expression of *ims*-Ero1 rendered the presence of the otherwise essential protein Erv1 dispensable. However, the pronounced growth phenotype of the resulting mutant suggests that Erv1 carries out critical activities in the IMS that cannot be fully complemented by Ero1.

The Essential Oxidoreductase Domain of Pdi1 Can Be Expressed in the IMS of Mitochondria

Next, we tested whether expression of the ER oxidoreductase PDI (called Pdi1 in yeast) can suppress the lethal

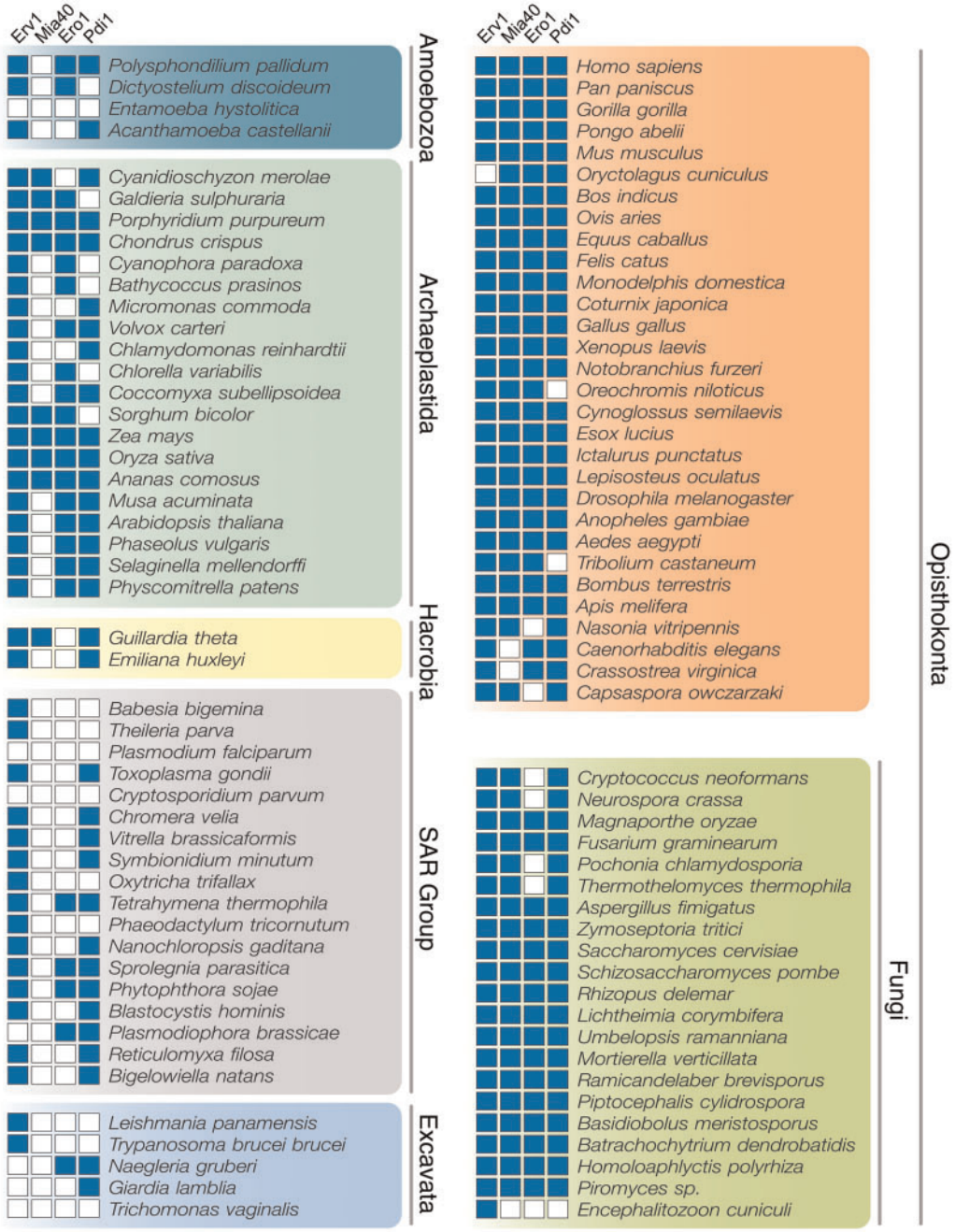

Fig. 2. Presence absence pattern (PAP) of homologs of *S. cerevisiae* Erv1, Mia40, Ero1, and Pdi1 across representatives of all major eukaryotic supergroups. The distribution of Mia40, for example, across the animal/fungi and plant divide (i.e., opisthokonts and archaeplastids) suggests the protein was present in the last eukaryotic common ancestor, last eukaryotic common ancestor (LECA). Its absence from others such as the diverse SAR supergroup could indicate early divergence (and low sequence conservation) or differential and early loss. Blue squares correspond to a reciprocal best BLAST hit to the yeast sequence in the respective genomes (30% sequence identity; E-value $1e^{-10}$). For a list of 150 sequenced eukaryotes and the detailed BLAST hits, please refer to supplementary material, Supplementary Material online, which include supplementary figure 1, Supplementary Material online, supplementary tables 1 and 2, Supplementary Material online. Hap., Haptophytes; Cryp., Cryptists.

consequences of an *ERV1* or *MIA40* deletion. We constructed a fusion protein consisting of the IMS-targeting region of Mia40 (residue 1–70) and an HA-tagged version of the a' domain of yeast Pdi1 (residues 372–492), which exhibits the essential PDI activity of the ER (Solovyov et al. 2004). This fusion protein (*ims*-Pdi1) was expressed in *ERV1* or *MIA40* shuffle strains and was detectable by Western blotting (Fig. 4A).

In order to test whether *ims*-Pdi1 can functionally replace Mia40 or Erv1, we followed again a plasmid shuffling strategy. Upon growth in the presence of 5-FOA, no viable colonies were obtained with the *MIA40* shuffle strain, however, the expression of the *ims*-Pdi1 fusion protein allowed the loss of *ERV1* (fig. 4B and C).

This strain grew extremely slow early on, unless low concentrations of L-buthionine sulfoximine (BSO, an inhibitor of

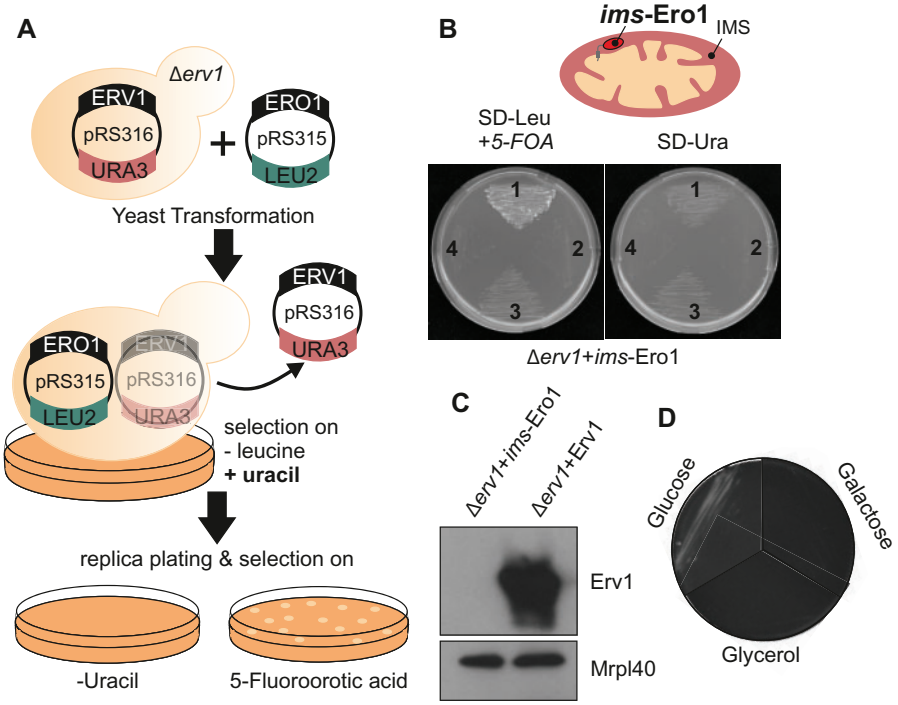

Fig. 3. The yeast Erv1 protein can be replaced by Ero1. (*A*) Schematic representation of the plasmid shuffling strategy used in this study. (*B*) An IMS-targeted version of Ero1 was expressed in Δ*erv1* yeast cells that contained an *ERV1* gene on an *URA3* plasmid. Counterselection against *URA3* on 5-FOA yielded cells that lacked the Erv1-encoding plasmid (sector 1). However, despite prolonged growth on uracil-containing media, the URA3 plasmid was maintained in most colonies, suggesting that the presence of Erv1 is still of considerable advantage even if *ims*-Ero1 is expressed (sectors 2–4). (*C*) Western blotting of cell extracts to confirm the absence of Erv1 after plasmid shuffling. Signals obtained with an antibody against the mitochondrial protein Mrpl40 were used as loading control. (*D*) The Δ*erv1* strain expressing IMS-targeted Ero1 was able to grow on the fermentable carbon source glucose but unable to grow on galactose or glycerol.

GSH synthesis) were added (fig. 4*D*). GSH diffuses from the cytosol into the IMS through porins of the outer mitochondrial membrane (Kojer et al. 2012, 2015). We reasoned that upon depletion of a reduced GSH pool, *ims*-Pdi1 might promote some protein oxidation in the IMS, either via Mia40 or via direct interaction with IMS proteins.

ims-Pdi1 Is Largely Present in Its Reduced State

After cycling through a few generations, the shuffled Δ*erv1* *ims*-Pdi1 strain improved its growth rate on glucose and became independent of BSO addition. Actually, addition of BSO reduced instead of increased the growth rate of this mutant (fig. 5*A*). Presumably this mutant adapted its redox conditions in some way so that the replacement of Erv1 with Pdi1 resulted in cells that were able to grow under fermentable conditions. This mutant, however, remained unable to respire and to grow on nonfermentable carbon sources (fig. 5*B*). Thus, we conclude that Erv1 but not Mia40 can be replaced by components of other disulfide relays, such as Ero1 or Pdi1.

In order to assess the redox state of *ims*-Pdi1, we isolated mitochondria from the mutant and treated these with increasing concentrations of the chemical oxidizer diamide. Mitochondrial proteins were subsequently precipitated with trichloroacetic acid to "freeze" the redox state of the thiols and before they were denatured in sodium dodecyl sulfate (SDS) and incubated with the small alkylating agent N-ethylmaleimide (NEM), the reductant tris carboxyethyl phosphine (TCEP) or/and the large alkylating compound methylpolyethylene glycol-24 maleimide ($mmPEG_{24}$). As shown in figure 5*C*, after reduction with TCEP the treatment with $mmPEG_{24}$ leads to a considerable size shift due to alkylation of the two cysteine residues of *ims*-Pdi1 (fig. 5*C*, maximum shift). An inverse shift experiment, in which reduced thiols were blocked by NEM before cysteines engaged in disulfide bonds were reduced with TCEP and modified with $mmPEG_{24}$ confirmed this result and showed that only after diamide treatment, oxidized *ims*-Pdi1 is detected in mitochondria.

To exclude that the reduced state of *ims*-Pdi1 is the result of its mis-localization to a "reducing compartment" such as the cytosol or the matrix, we isolated mitochondria from wild type and Δ*erv1* *ims*-Pdi1 cells. In mitochondria from the latter strain, *ims*-Pdi1 was detectable in Western blotting with HA-specific antibodies (fig. 5*D*). When the outer membrane of the mitochondria were ruptured by hypotonic swelling and the resulting mitoplasts were treated with proteinase K, *ims*-Pdi1 was degraded, indicating that it is present in the IMS of mitochondria. In contrast, matrix proteins such as Mrpl36 remained inaccessible to proteinase K. Thus, we conclude that *ims*-Pdi1 is indeed located in the IMS of mitochondria and resides there in the reduced form.

The observation that an oxidoreductase like Pdi1 can replace the sulfhydryl oxidase Erv1 was surprising and suggests that Pdi1 can to some degree promote the oxidative folding in the IMS; only to a very limited degree, however, as these

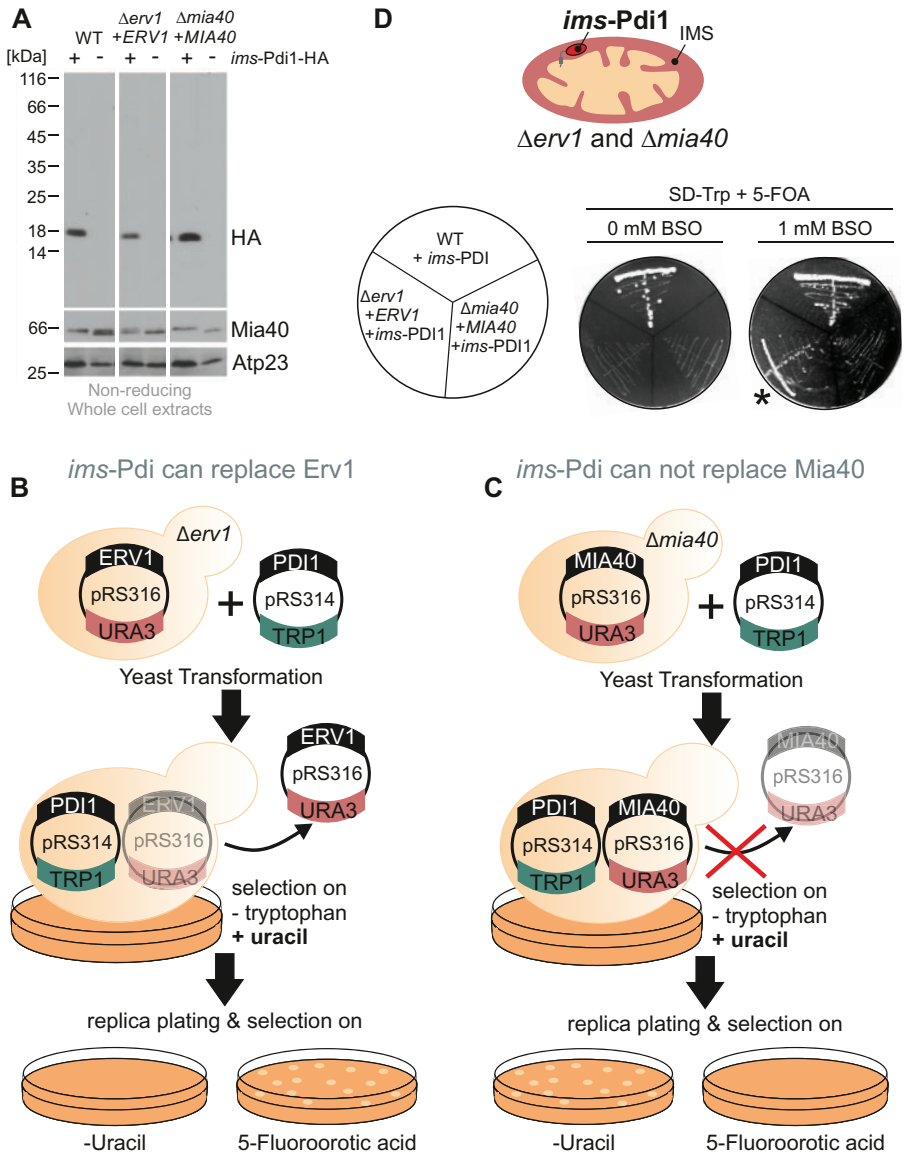

Fig. 4. Erv1 but not Mia40 can be replaced by IMS-targeted Pdi1. (*A*) An HA-tagged variant of the redox-active domain of Pdi1 (a' domain of Pdi1) was expressed from a pRS315 plasmid in the IMS of wild type cells or in *ERV1* and *MIA40* shuffle strains. HA-tagged *ims*-Pdi1 was detectable by Western blot in the indicated strains. Signals obtained with Mia40- and Atp23-specific antibodies were used as loading control. (*B, C*) Schematic representation of the plasmid shuffling strategy used here. (*D*) Cells were grown on tryptophan-deficient, uracil-containing media for 4 days and then transferred to 5-FOA plates that contained 0 or 1 mM BSO to inhibit GSH synthesis, respectively. Viable cells were obtained from the BSO-containing plate for the *ERV1* (asterisk) but not for the *MIA40* shuffle strain. The Δ*ura3* wild type strain served as positive control on the 5-FOA plates.

cells are not healthy. Since *ims*-Pdi1 is reduced, it is unlikely that it promotes the oxidation of Mia40 directly (fig. 5*E*).

The Erv1 Deletion Mutants Show Severe Problems in Respiration

Next, we assessed the levels of mitochondrial proteins in cells of the shuffle mutants before and after counterselection against the *ERV1* plasmid (fig. 6*A* and *B*). We observed that substrates of the mitochondrial disulfide relay, such as Cmc1 or Tim10, were considerably reduced and almost absent in the Δ*erv1* strains. This was also obvious by analysis of mitochondria isolated from these strains (fig. 6*C*). Thus, even in the presence of *ims*-Pdi1 and *ims*-Ero1, the deletion of Erv1 was detrimental and significantly reduced the levels of substrates of the disulfide relay in mitochondria. These extremely low levels of IMS proteins suggests severe problems in mitochondrial functionality. Indeed, by measuring the oxygen consumption rates upon Nicotinamide adenine dinucleotide (NADH) addition to isolated mitochondria, we observed that both shuffle mutants were unable to respire; their traces were indistinguishable from Δ*cox6* mutants, which completely lack any cytochrome *c* oxidase activity (fig. 6*D*).

Erv1 plays a critical role in the disulfide bond formation of the essential inner membrane protein Tim17 (Mokranjac 2016; Ramesh et al. 2016; Wrobel et al. 2016). Therefore, we tested the redox state in Tim17 in the shuffle mutants. Tim17 contains four cysteine residues. Alkylation of the four cysteine residues induces a size shift of about 8 kDa, which is only

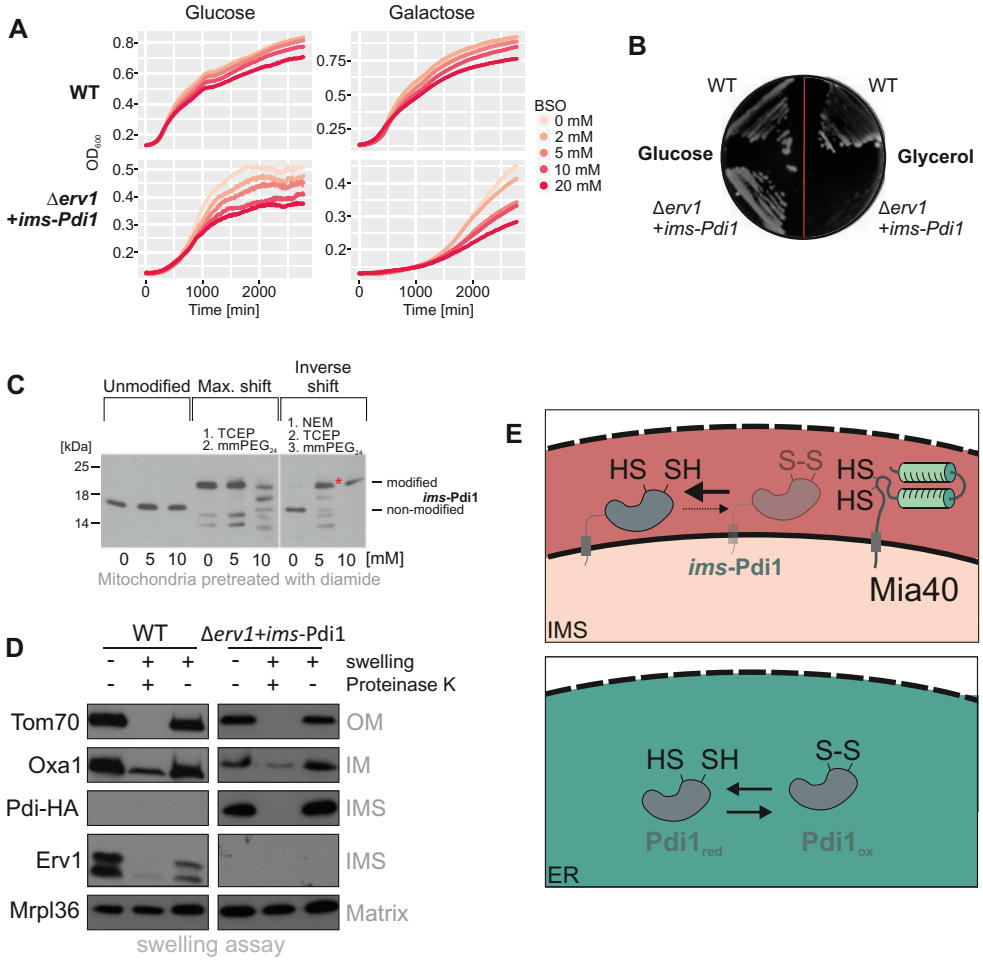

Fig. 5. *ims*-Pdi1 remains reduced in the IMS. (*A*) Growth curves in glucose and galactose medium of WT and *ims*-Pdi1-expressing Δ*erv1* cells in the presence of increasing concentrations of BSO. (*B*) WT and *ims*-Pdi1-expressing Δ*erv1* cells were grown on glucose or glycerol plates. *ims*-Pdi1-expressing Δ*erv1* cells were unable to grow on the nonfermentable carbon source glycerol. (*C*) Mitochondria were isolated from wild type cells expressing *ims*-Pdi1 and treated with 0, 5, or 10 mM of the chemical oxidizer diamide. Proteins were either directly resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) ("unmodified"), treated with TCEP to reduce dithiols before reduced thiols were alkylated with $mmPEG_{24}$ ("maximum shift") or reduced thiols were blocked with NEM, before oxidized thiols were reduced with TCEP and modified with $mmPEG_{24}$ ("inverse shift"). Unless treated with diamide, the thiol residues in *ims*-Pdi1 were fully accessible to NEM, indicating that the IMS-expressed Pdi1 is fully reduced as obvious from the band indicated with the red asterisk. (*D*) Mitochondria were incubated in iso-osmotic or hypo-osmotic "swelling" buffer to either retain the outer membrane intact or to open it by hypotonic swelling, respectively. Proteinase K (PK) was added when indicated. Protease treatment was stopped by phenylmethane sulfonyl fluoride (PMSF), mitochondria were reisolated, washed and analyzed by Western blotting using the indicated antisera. The Mrpl36 signal was used to verify equal loading. IM, inner membrane; OM, outer membrane. (*E*) Schematic representation of the IMS-expressed Pdi1. The protein is stable in the IMS but present in the reduced form and thus cannot efficiently promote the oxidation of Mia40.

observed if Tim17 is fully reduced by TCEP prior to alkylation (fig. 6*E*). Without reduction, Tim17 shifted half way by about 4 kDa. Since one $mmPEG_{24}$ moiety leads to a size shift of 2 kDa, this indicates that two of the four cysteine residues in Tim17 are reduced and two part of a disulfide bond. Hence, this disulfide bond in the essential component of the TIM23 translocase can be formed in the strains that lack Erv1, but express *ims*-Ero1 or *ims*-Pdi1 instead.

ims-Pdi1 Exacerbates Rather Than Rescues an Oxidation-Deficient Mia40 Mutant

Mia40 exhibits two distinct biochemical activities, which can be separated experimentally (Peleh et al. 2016): 1) it serves as an oxidoreductase that inserts disulfide bonds into its substrates (Naoe et al. 2004; Mesecke et al. 2005; Rissler et al. 2005; Sideris and Tokatlidis 2007; Fischer et al. 2013; Koch and Schmid 2014a), and 2) serves as a receptor in the IMS that traps translocation intermediates and supports their transport through the TOM complex via hydrophobic interactions (Banci et al. 2009, 2011; Kawano et al. 2009). It was previously demonstrated that a Mia40 mutant that lacks its catalytic cysteine residues (fig. 7*A*, "SPS mutant") is able to import proteins, but unable to mediate their oxidative folding in the IMS (Peleh et al. 2016, 2017).

We expressed *ims*-Pdi1 in addition to Mia40-SPS in the background of the temperature-sensitive Mia40 mutants (Chacinska et al. 2004) *mia40-3* and *mia40-4* (fig. 7*B*). Cells were grown to log phase and tenfold serial dilutions were

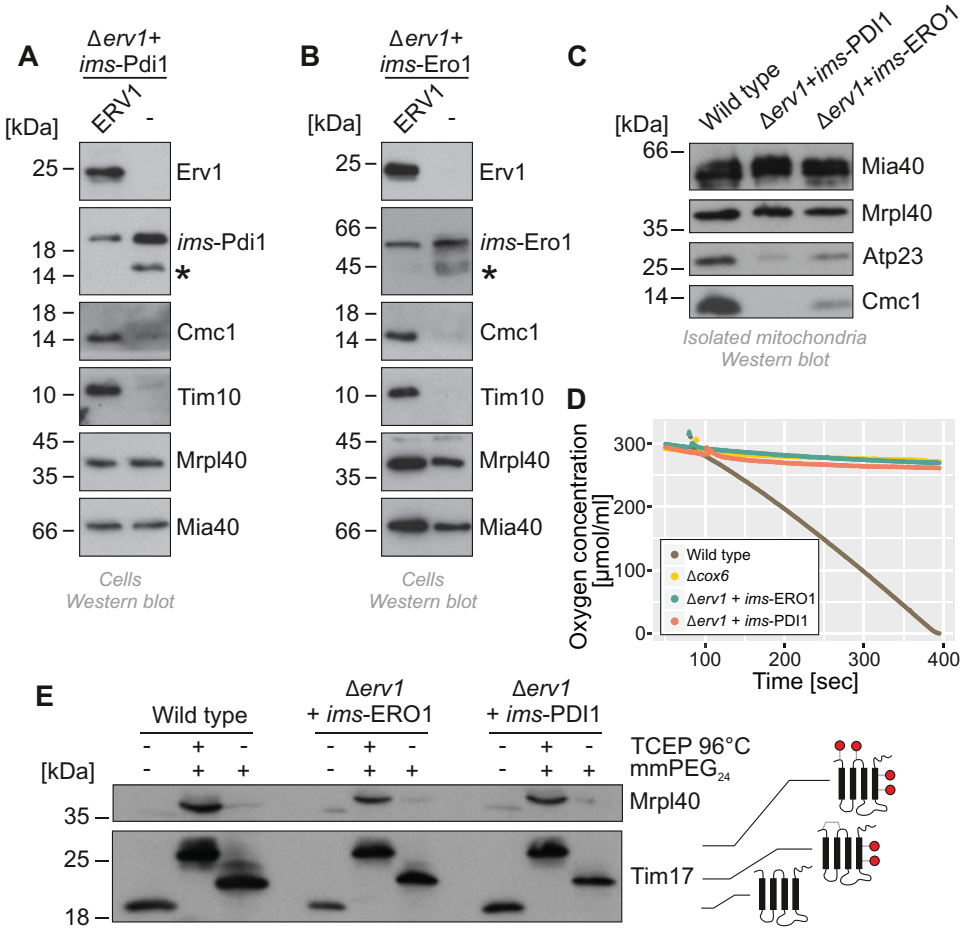

Fig. 6. Mitochondria of the Ero1- and Pdi1-expressing Δ*erv1* cells show severe defects. (*A*–*C*) Mitochondrial proteins of *ims*-Ero1- and -Pdi1-expressing Δ*erv1* cells (as well as the unshuffled Erv1-containing strains for control) were detected by Western blotting of whole cell extracts and isolated mitochondria. IMS proteins that rely on the mitochondrial disulfide relay (Cmc1, Tim9, and Atp23) were strongly reduced in the Δ*erv1* mutants. Due to the presence of HA-tags, *ims*-Pdi1, and *ims*-Ero1 were detected by HA-specific antibodies. *degradation products of the HA-tagged proteins. (*D*) The oxygen consumption of isolated mitochondria upon addition of 2 mM NADH was measured. Mitochondria from a respiration-incompetent Δ*cox6* mutant were used for control. (*E*) The redox state of Tim17 was analyzed in mitochondria of the indicated strains. Please note that in the absence of TCEP, two $mmPEG_{24}$ moieties were added to endogenous Tim17 of all mutants indicating the presence of the disulfide bond in Tim17 in these strains (Ramesh et al. 2016). The matrix protein Mrpl40 served as loading control.

dropped on plates containing the fermentable carbon source glucose or the nonfermentable carbon source glycerol. The expression of Mia40-SPS partially suppressed the growth phenotype of theses mutants, which was particularly obvious on glycerol. Interestingly, the expression of *ims*-Pdi1 did not improve growth but rather prevented it, suggesting that *ims*-Pdi1 does not facilitate the Mia40-independent oxidation of IMS proteins. This is also supported by the observation that *ims*-Pdi1 did not increase the minute levels of Mia40 substrates that accumulate at steady state levels in the IMS of *mia40-3* mitochondria (fig. 7*C*). Expression of *ims*-Pdi1 did not influence the import of Mia40 substrates into *mia40-3* mitochondria. Radiolabeled Cmc1 and Tim9 were imported with low efficiency into the IMS of *mia40-3* mitochondria regardless of whether *ims*-Pdi1 was expressed in these strains or not (fig. 7*D* and *E*).

The protein Atp23 10CS, a mutant of Atp23 in which the 10 native cysteine residues of the protein were replaced by serines, is a model substrate of the Mia40 import pathway whose import is independent of disulfide bond formation (Weckbecker et al. 2012). This protein is strictly imported in a Mia40-dependent, though oxidation independent manner (Fig. 7*F*). The import of Atp23 10CS was efficient in the presence of Mia40-SPS, and again, the presence of *ims*-Pdi1 did not influence the import efficiency (fig. 7*G*).

In summary, *ims*-Pdi1 appears to leave the receptor activity of Mia40 unaffected but is apparently unable to mediate the postimport oxidation and folding of IMS proteins. The exacerbated growth of the Mia40-SPS expressing mutants rather suggest that *ims*-Pdi1 counteracts the oxidative folding of Mia40 substrates (fig. 7*H*).

Thioredoxins Cannot Replace Mia40 Regardless of Their Redox Potential

Apparently, the oxidase activity of Erv1 is much easier to replace than the function of Mia40. Pdi1 serves as an oxidase and isomerase, whereas the DsbA of *E. coli* is a specialized

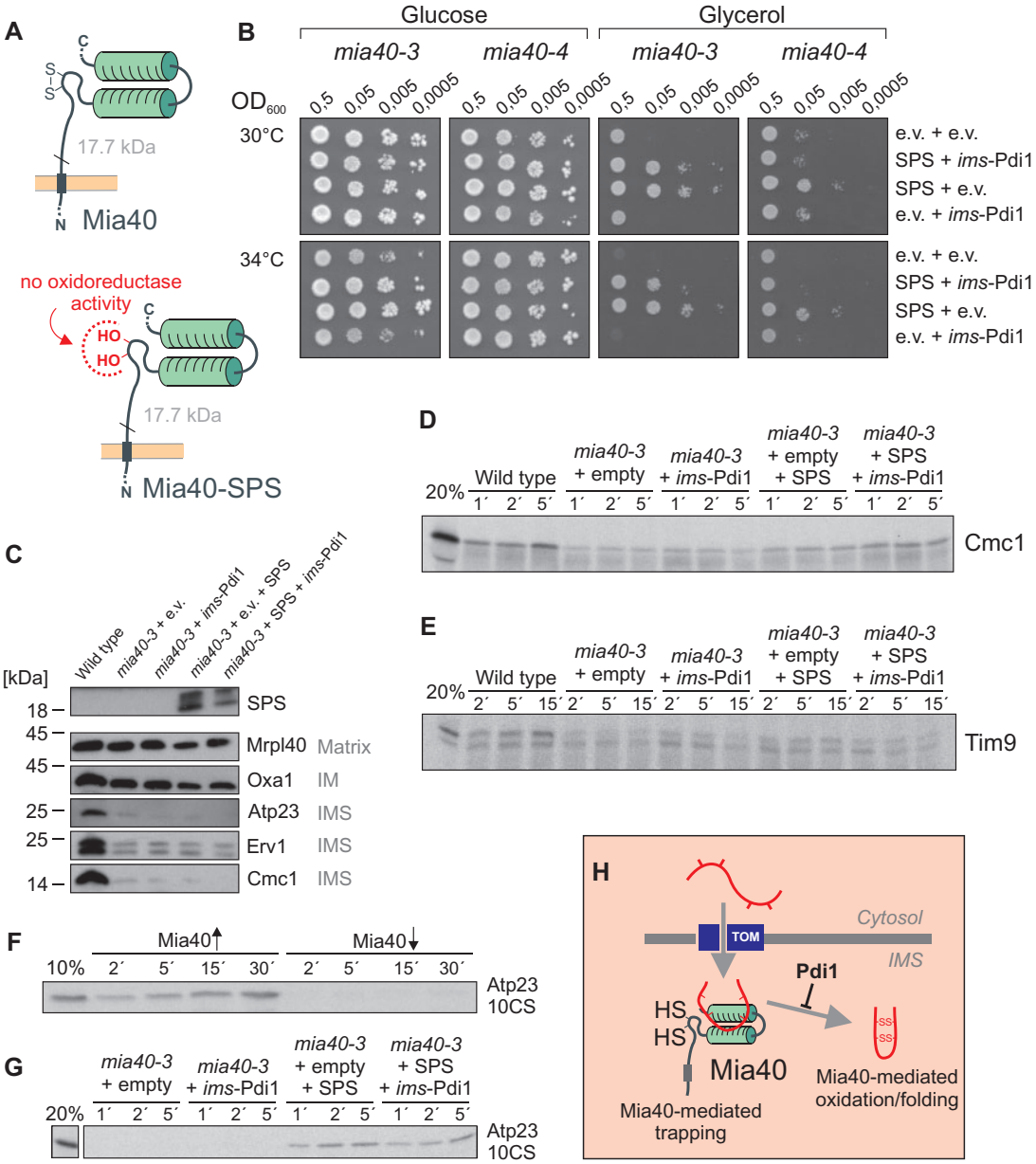

Fig. 7. The expression of Pdi1 does not support Mia40-mediated import of IMS proteins. (*A*) Schematic representation of the Mia40-SPS mutant (Peleh et al. 2016). (*B*) Temperature-sensitive *mia40-3* and *mia40-4* cells were transformed with empty vector (e.v.), plasmids expressing Mia40-SPS and/or IMS-directed Pdi1. The growth at 30 and 34°C on glucose- and glycerol-containing plates was analyzed. (*C*) Expression of Pdi1 in the IMS does not suppress the biogenesis defect of *mia40-3* mutants. Cell extracts of the indicated strains were analyzed by Western blotting. Whereas the amounts of matrix and inner membrane proteins such as Mrpl40 and Oxa1 were not considerably altered, IMS proteins such as Atp23, Erv1, and Cmc1 are strongly depleted in the *mia40-3* mutants. Expression of *ims*-Pdi1 and/or Mia40-SPS did not restore the levels of these proteins. (*D*–*G*) Radiolabeled Cmc1, Tim9 and Atp23 10CS were incubated with isolated mitochondria for the times indicated. Mitochondria were re-isolated and treated with proteinase K in order to remove nonimported proteins. Proteins were resolved by SDS-PAGE and visualized by autoradiography. (*H*) Schematic representation of the functions of Mia40 during protein biogenesis. *ims*-Pdi1 does not stimulate protein import. The observed negative effect of *ims*-Pdi1 on cell viability in *mia40-3* and *mia40-4* cells suggests that Pdi1 counteracts Mia40-mediated oxidation and folding of proteins that occurs subsequent to the import reaction.

oxidase whose oxidase/isomerase activities were extensively studied in the past (Huber-Wunderlich and Glockshuber 1998; Jonda et al. 1999; Denoncin et al. 2013). We therefore wanted to test whether these stronger oxidizing thioredoxin family members are able to carry out a Mia40-dependent function.

The redox potential of Pdi1 was measured to be around −180 mV (Lundstrom and Holmgren 1993), which is much less negative than the −285 mV of the Mia40s CPC motif (Tienson et al. 2009). The redox potentials of thioredoxin family members is largely determined by the two variable residues in the CXXC motif (Huber-Wunderlich and

Glockshuber 1998; Mossner et al. 1998, 1999; Jonda et al. 1999; Maskos et al. 2003). In the past, a collection of mutants of the *E. coli* DsbA and thioredoxin (Trx) proteins were generated and characterized that covered a wide range of different redox potentials (Fig. 8A). These oxidoreductases thus ranged from predominantly oxidizing to reducing proteins.

We fused HA-tagged versions of DsbA and Trx as well as the different mutants of these proteins to the IMS-targeting signal of Mia40 and expressed these proteins in the *MIA40* shuffle strain. None survived the loss of the *MIA40*-containing *URA3* plasmid (fig. 8B), indicating that not a single thioredoxin family member was able to replace the function of Mia40. This was confirmed by experiments in *mia40-3* mutants, which showed that none of these thioredoxin family members rescued the temperature-sensitive growth phenotype of the mutant (fig. 8C), although they were efficiently expressed and stable (fig. 8D). Expression of many DsbA and thioredoxin variants even reduced the fitness of *mia40-3* cells with a pronounced negative effect (fig. 8C, cf. DsbA [CPGC], DsbA [CGHC], DsbA [CGPC], Trx [CGHC], Trx [CPYC]). This is reminiscent to the negative effect observed upon *ims*-Pdi1-expression (fig. 7B). Hence, we conclude that regardless of their specific redox potential, thioredoxin-like proteins are not able to exhibit the function of Mia40 in the IMS nor do they support the growth of temperature-sensitive *mia40* mutants.

The Mia40 Redox State Is Largely Unaffected by Thioredoxin-Like Proteins

Next, we tested the redox states of these IMS-located DsbA and Trx (fig. 9A). Incubation with the alkylating reagent $mmPEG_{24}$ showed that DsbA and Trx are largely reduced in the IMS. The *ims*-Trx (CGHC) mutant was slightly more oxidized than the wild type, but mostly reduced. When the redox state of Mia40 was analyzed in these mutants (fig. 9A, lower panel), we found that in all strains about half of Mia40 was fully oxidized and half was shifted by two $mmPEG_{24}$ moieties, characteristic for a situation in which the CPC motif is reduced (Peleh et al. 2016, 2017). Thus, none of these thioredoxins showed a considerable effect on Mia40 in this background (which contains a functional Erv1 protein). In summary, regardless of the redox potential of the different thioredoxin family members, these proteins were unable to take over the redox activity of Mia40, nor did any of them considerably influence the Mia40 redox state in the presence of a functional Erv1 oxidase.

Discussion

Mia40 is a unique and peculiar oxidoreductase. It has a much simpler fold than the widely distributed thioredoxin-like proteins (to which it is structurally unrelated), binds its substrates as long-lasting reaction intermediates via hydrophobic and covalent dithiol interactions, has very low isomerase activity and does not efficiently interact with monothiols such as GSH. These features are perfect adaptations to its specific tasks in mitochondria. In this study we demonstrate that neither the oxidoreductase domain of Pdi1, nor DsbA or thioredoxin can functionally replace Mia40. Thus, Mia40 plays a specific and distinct role that sets it apart from members of the widespread family of thioredoxin-like oxidoreductases.

We observed that the IMS-targeted versions of Pdi1, DsbA and thioredoxin were largely reduced in mitochondria even in the presence of functional Erv1. This suggests that Erv1 does not efficiently oxidize these thioredoxin-like proteins or that they are rapidly reduced again by GSH. Erv1 comprises a central redox-active disulfide in proximity to its FAD cofactor which exchanges electrons with its second redox-active disulfide that is part of a flexible shuttle arm (Hofhaus et al. 2003; Stojanovski et al. 2008; Ang and Lu 2009; Tienson et al. 2009; Bien et al. 2010; Lionaki et al. 2010; Guo et al. 2012; Neal et al. 2015). The shuttle arm forms an amphipathic helix very similar to the Mia40 interaction motif present in IMS proteins (Sideris and Tokatlidis 2007; Milenkovic et al. 2009), allowing electron transfer by a substrate mimicry reaction (Banci et al. 2011). We show in this study, however, that Erv1 can be replaced by Ero1, the sulfhydryl oxidase of the ER, or by Pdi1, albeit resulting in severely compromised mutants.

It was previously shown that overexpression of the Erv1 homolog Erv2 can rescue the phenotype of an otherwise lethal Δ*ero1* mutant (Sevier et al. 2001). We observed that mutants that lack Erv1 but express IMS-localized Pdi1 or Ero1, show very low levels of Mia40 substrates and are unable to respire. Thus, even in the presence of Pdi1 or Ero1, Erv1 remains essential for efficient biogenesis of Mia40 substrates. This suggests that *ims*-Ero1 and *ims*-Pdi1 can either not efficiently oxidize Mia40 or/and they are unable to exhibit another function of Erv1 such as its proposed role in the biogenesis of cytosolic iron sulfur proteins (Lange et al. 2001). Unfortunately, the poor growth of these mutants did not allow us to study these processes in this strain biochemically.

The results shown in this study suggest that thioredoxin-like proteins are not only unable to functionally replace Mia40, but even often have negative effects when present in the IMS. Efficient protein oxidation in the IMS that contains high levels of reduced GSH is only possible, because this compartment shows limited levels of glutaredoxin (Kojer et al. 2012, 2015; Kritsiligkou et al. 2017). Increasing amounts of thioredoxin-like proteins, regardless of their specific redox potential, will presumably equilibrate the GSH with the protein thiol pool, which explains why these proteins do not support productive protein folding in the IMS. Thus, during evolution, it was necessary to replace the thioredoxin-based folding system of the endosymbiont with a novel system that met the specific requirements of the organelle. Although the Mia40 protein is not a universal component of eukaryotic biology, it is found conserved in deeply diverging supergroups such as the opisthokonts and archaeplastids (fig. 2), suggesting it was already encoded by LECA. One way or the other, some eukaryotic lineages have evidently evolved independent solutions. In trypanosomes for instance, Mia40s function might be carried out by a component of the MICOS complex (Kaurov et al. 2018). Others remain to be explored, but one of the major steps in the emergence of the mitochondrion, and a salient difference in comparison to any free-living bacteria, was the need to import proteins into the IMS.

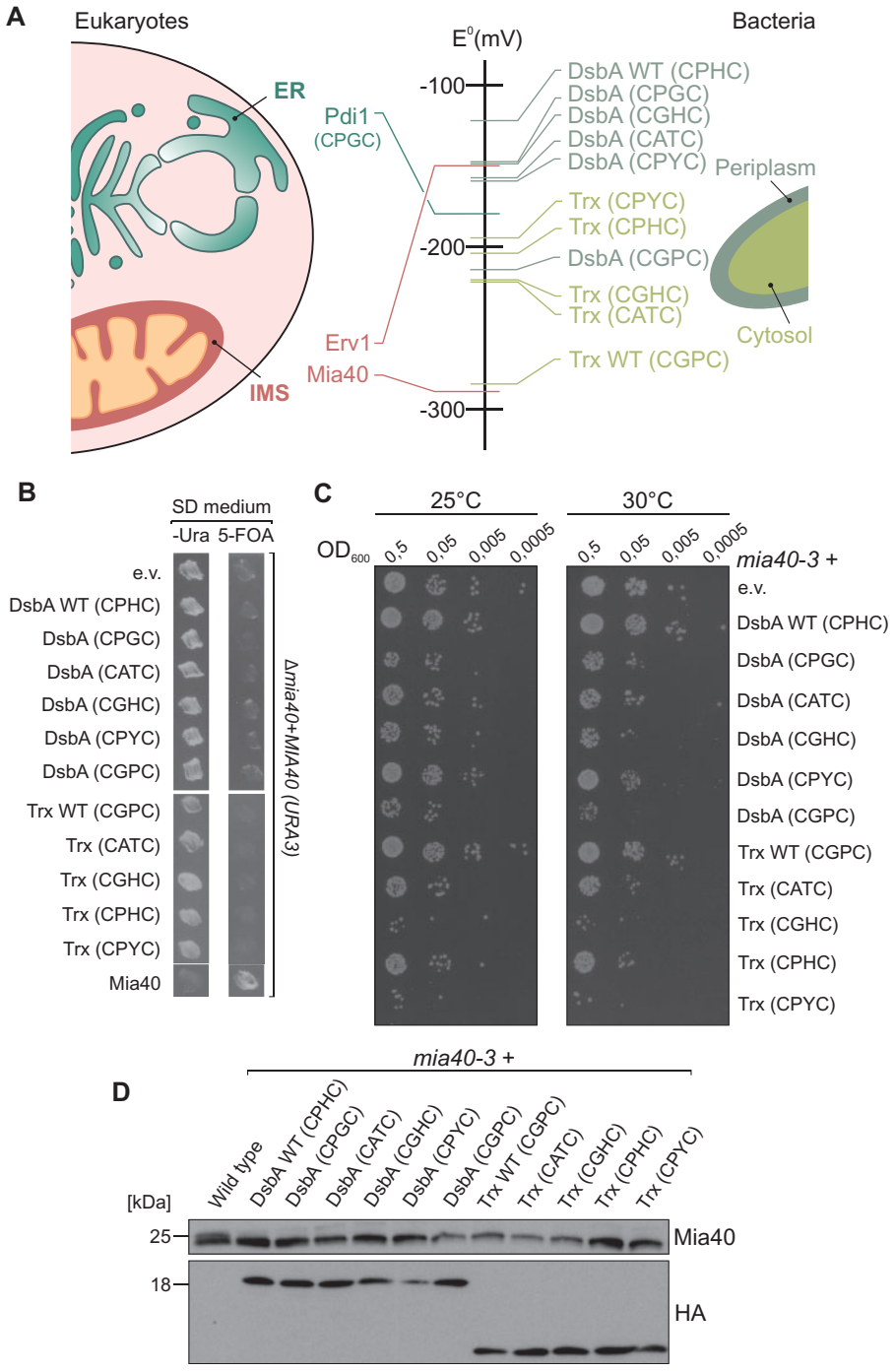

Fig. 8. Thioredoxin-like proteins fail to rescue Mia40 mutants. (*A*) Overview about the published redox potentials of different DsbA and thioredoxin (Trx) mutants, as well as of the redox-sensitive catalytically relevant thiol pairs in Pdi1, Erv1 and Mia40. (*B*) DsbA and Trx variants (and Mia40 for control) were expressed from *LEU2*-containing plasmids in the IMS of mitochondria in a Mia40 shuffle mutant. In none of these strains, with exception of the positive control, the *MIA40*-containing *URA3* plasmid could be lost. This positive control is a Mia40-expressing *LEU2* plasmid that rendered the *MIA40*-containing *URA3* plasmid dispensable. Thus, Mia40 remains essential in the presence of thioredoxin-like proteins, regardless of their specific redox properties. e.v., empty vector. (*C*) The DsbA and Trx variants were expressed in the IMS of *mia40-3* cells. Growth of these strains was tested at 25 and 30°C on selective glucose-containing media. Neither of these thioredoxin-like proteins rescued the growth of the mutant but several variants caused severe growth defects. (*D*) The expression levels of DsbA and Trx proteins were tested by Western blotting using HA-specific antibodies.

We propose that the evolution of the mitochondria-specific disulfide relay system is intimately tied to the transition of the endosymbiont into the mitochondrion and the evolution of protein import. Given the functional homology of the eukaryotic PDIs and the bacterial DsbA proteins (Humphreys et al. 1995), the ER represents the primary localization of the bacterial-like disulfide relay system. The similarity of bacterial and eukaryotic signal peptides renders such a

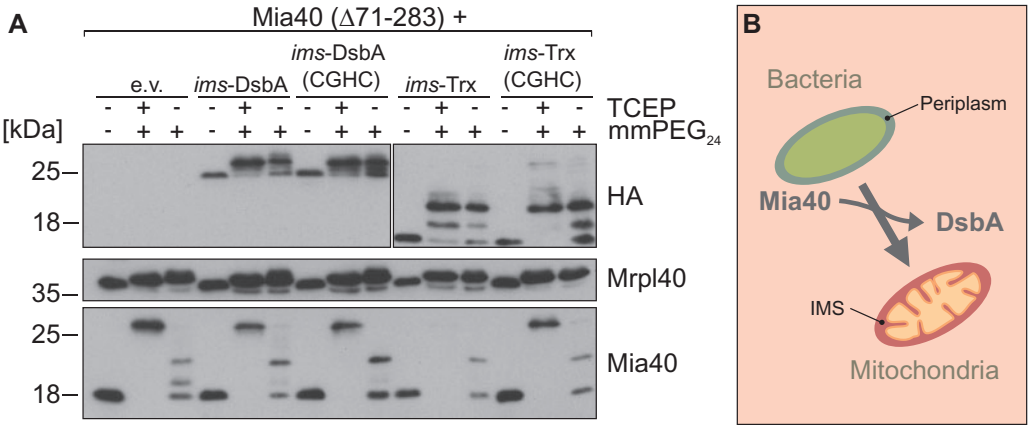

Fig. 9. The redox state of Mia40 is hardly influenced by DsbA or Trx expression. (*A*) Cell extracts of the indicated strains were generated by acid precipitation. The redox states of the *ims*-DsbA/*ims*-Trx proteins and of Mia40 Δ71-283 were analyzed by alkylation with $mmPEG_{24}$. The DsbA and Trx proteins were largely, but not completely reduced. In all strains, a considerable fraction of Mia40 was in the oxidized form that was not modified with $mmPEG_{24}$ unless pretreated with TCEP. Expression of *ims*-DsbA or *ims*-Trx did not increase Mia40 oxidation, but, if it had an influence at all, resulted in more reduced Mia40. (*B*) The acquisition of the Mia40 system was a critical step in the evolution of eukaryotic cells. Thioredoxin-like proteins, such as the DsbA of the bacterial periplasm, cannot exhibit a Mia40-like activity. Our observations here even show that the expression of thioredoxins in the IMS has the potential to compromise the biogenesis of IMS proteins. Thus, the transfer of genes for IMS proteins from the genome of the endosymbiont into the nucleus made it necessary to replace the thioredoxin-like system with the unique and unrelated mitochondrial disulfide relay that is very well suited to promote the import and folding of precursor proteins from the cytosol into the IMS.

localization plausible (Garg and Gould 2016) and is congruent with the hypothesis that the ER evolved from outer membrane vesicles the endosymbiont was secreting (mitochondria do so until today) into the cytosol of the archaeal host cell (Gould et al. 2016), meaning that the ER and bacterial periplasm are evolutionary (and to a degree functionally) homologous compartments. Coupled to the promiscuous nature of mitochondrial protein import during the early stages of organellar protein import evolution, a mitochondrial specific disulfide relay system was likely a necessity.

Inertness against GSH, and slow oxidation kinetics coupled to the high affinity towards substrates, Mia40 presumably serves as the perfect import receptor for IMS proteins and outcompeted or replaced the proteobacterial copy once present in the IMS. Either way, the development of a specific mitochondrial disulfide relay system obviously represented a crucial step in the evolution of the eukaryotic cell. A next step in the characterization of this unique, eukaryotic disulfide relay system is the analysis of less well-studied unicellular eukaryotes in order to screen for variations of the structure and function of Mia40 and Erv1 (Alcock et al. 2012; Peleh et al. 2017; Specht et al. 2018) and to better understand how the mitochondria disulfide relay evolved during eukaryotic evolution.

Materials and Methods

Screening for Orthologs of Yeast Proteins Across Eukaryotic Diversity

To screen for homologs of the four *Saccharomyces cerevisiae* proteins Mia40, Erv1, Ero1, and Pdi1, complete genomes of 150 eukaryotes from NCBI and JGI (supplementary table 1, Supplementary Material online) were downloaded. A reciprocal best BLAST approach was then employed to find homologs of the yeast proteins in each of the 150 eukaryotic genomes with the cut offs of 30% sequence identity and an e-value of 1e-10. The pipeline was implemented using in-house scripts written in Python v3.6.3.

Yeast Strains and Plasmids

Yeast strains used in this study were based on the wild type strain YPH499, including the regulatable *GAL*-Mia40 strain (Mesecke et al. 2005). Shuffle strains for *ERV1* and *MIA40* as well as *mia40-3* and *mia40-4* heat-sensitive mutants were described before (Lisowsky 1992; Chacinska et al. 2004; Bien et al. 2010; Peleh et al. 2016). Yeast strains were either grown in synthetic media containing 2% glucose or galactose, or in YP (1% yeast extract, 2% peptone) medium containing 2% galactose or glucose (Peleh et al. 2015).

To express Ero1, Pdi1 or the different DsbA and Trx variants in the IMS, the sequence of these proteins (corresponding to their residues 56–424 for Ero1; 372–492 for Pdi1; 20–208 for the DsbA variants and 2-109 for the Trx variants) in addition to a HA tag was cloned using *BamHI* and *SmaI* (Pdi1, Ero1) or *BamHI* and *XmaI* restriction sites in frame into the single copy vector pRS315 or pRS314 (Sikorski and Hieter 1989) harboring an *MIA40* promoter and a sequence corresponding to the amino acid residues 1–70 of yeast Mia40 (Peleh et al. 2016). For the redox shift experiments of Mia40, a Mia40 mutant was used with a shortened membrane anchor to better resolve the blotting of the modified Mia40 species as described before (Peleh et al. 2016).

Plasmid Shuffling

Deletion mutants (Δ*mia40* or Δ*erv1*) containing the corresponding gene on a pRS316 plasmid with URA3 marker were used. The strains were transformed with an additional pRS314/15 plasmid containing the desired gene using a lithium acetate-based method (Gietz et al. 1992). After

transformation, cells were grown on selective medium, containing uracil, but lacking leucine or tryptophan. After several rounds of growth on uracil-containing media, cells were tested for the loss of the pRS316 plasmid by replica plating on medium lacking uracil but containing 5-FOA. Experimental procedures on the isolation of mitochondria, immunoprecipitation and Western blotting were reported previously (Peleh et al. 2014).

Alkylation Shift Experiments for Redox State Detection

To analyze the redox state of cysteine residues, 2 OD_{600} of cells were harvested from cultures grown to mid log phase (OD 0.6–0.8) by centrifugation (17,000 × g, 3 min). Cells were resuspended in 12% trichloro acetic acid on ice and opened by agitation with glass beads. Proteins were precipitated by centrifugation, lysed in 1% SDS and incubated in the presence of 15 mM $mmPEG_{24}$. If indicated, 50 mM NEM or 10 mM TCEP were added.

Protein Import into Mitochondria

Radiolabeled Cmc1, Tim9, and Atp23 10CS were synthesized in vitro using the TNT T7 Quick Coupled Transcription/Translation kit (Promega) (Weckbecker et al. 2012). The import reactions and their analyses were performed as described previously (Hansen et al. 2018) in import buffer containing 500 mM sorbitol, 50 mM Hepes pH 7, 480 mM KCl, 10 mM magnesium acetate, and 2 mM KH_2PO_4. Mitochondria were energized by addition of 2 mM ATP and 2 mM NADH before radiolabeled precursor proteins were added. To remove non-imported protein, mitochondria were treated with 100 µg/ml proteinase K for 30 min on ice after the import reactions.

Measurement of Oxygen Consumption Rates in Isolated Mitochondria

Mitochondrial oxygen consumption was measured using a clark electrode (Hansatech Instruments, Norfolk, United Kingdom). The 100 µg mitochondria were incubated in 0.6 M sorbitol, 1 mM $MgCl_2$, 5 mM EDTA, 20 mM Hepes pH 7.4. Oxygen consumption was induced by addition of 5 mM NADH and measured for 10 min.

Localization of *ims*-Pdi1

The mitochondrial sublocalization assay was performed by hypoosmotic swelling and proteinase K digest. 10 µg of mitochondria were incubated either in SH buffer (0.6 M sorbitol, 20 mM Hepes pH 7.4) or 20 mM HEPES pH 7.4 for 30 min on ice in the absence or presence of 100 µg/ml proteinase K. Proteinase digestion was stopped by addition of SH buffer containing 2 mM PMSF. Mitochondria were pelleted by centrifugation and resuspended in 50 µl Laemmli buffer.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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