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Mitochondrial protein import: precursor oxidation in a ternary complex with disulfide carrier and sulfhydryl oxidase

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The biogenesis of mitochondrial intermembrane space proteins depends on specific machinery that transfers disulfide bonds to precursor proteins. The machinery shares features with protein relays for disulfide bond formation in the bacterial periplasm and endoplasmic reticulum. A disulfide-generating enzyme/sulfhydryl oxidase oxidizes a disulfide carrier protein, which in turn transfers a disulfide to the substrate protein. Current views suggest that the disulfide carrier alternates between binding to the oxidase and the substrate. We have analyzed the cooperation of the disulfide relay components during import of precursors into mitochondria and identified a ternary complex of all three components. The ternary complex represents a transient and intermediate step in the oxidation of intermembrane space precursors, where the oxidase Erv1 promotes disulfide transfer to the precursor while both oxidase and precursor are associated with the disulfide carrier Mia40.

Introduction

Mitochondria use diverse mechanisms to import precursor protein (Jensen and Johnson, 2001; Endo et al., 2003; Koehler, 2004; Oka and Mihara, 2005; Dolezal et al., 2006; Neupert and Herrmann, 2007). The mitochondrial intermembrane space (IMS) contains an import and assembly (MIA) machinery, which transfers disulfide bonds to precursors and promotes their import and maturation (Chacinska et al., 2004; Koehler, 2004; Mesecke et al., 2005; Tokatlidis, 2005). The MIA pathway was reported to operate as a protein relay for disulfide bond formation like the disulfide relay systems of the bacterial periplasm (disulfide bond formation protein B [DsbB]–DsbA) and the ER (Ero1–protein disulfide isomerase [PDI]; Collet and Bardwell, 2002; Kadokura et al., 2004; Tu and Weissman, 2004; Sevier and Kaiser, 2006; Herrmann and Köhl, 2007). In each case, a disulfide-generating enzyme/sulfhydryl oxidase (DsbB, Ero1, and Erv1) oxidizes a disulfide carrier protein (DsbA, PDI, and Mia40). The oxidized disulfide carrier subsequently transfers the disulfide to the substrate, typically a newly synthesized protein arriving in the periplasm, ER, or IMS.

Mixed disulfides are generated between oxidase and disulfide carriers, as well as between carrier protein and substrate as direct means for the transfer of disulfide bonds. The current models of disulfide relays suggest an alternating association of the carrier protein with the substrate (reduction of the carrier) and the sulfhydryl oxidase (reoxidation of the carrier; Collet and Bardwell, 2002; Kadokura et al., 2004; Tu and Weissman, 2004; Mesecke et al., 2005; Sevier and Kaiser, 2006; Herrmann and Köhl, 2007). This implies that substrates, which receive more than one disulfide bond, require multiple cycles of the disulfide relay. An alternative, efficient mechanism would be that oxidative protein folding involves a direct cooperation of

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Abbreviations used in this paper: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; Dsb, disulfide bond formation protein; IMS, intermembrane space; MIA, mitochondrial IMS import and assembly; Tim, translocase of the inner mitochondrial membrane.

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all three partners in a ternary complex to promote the flow of electrons. However, such a ternary complex has not been shown experimentally.

We addressed the cooperation of sulfhydryl oxidase and disulfide carrier during oxidation of mitochondrial IMS precursors. Mia40 (Tim40), which contains three essential pairs of cysteines, forms a mixed disulfide with precursors in translocation across the outer membrane, drives their import into the IMS, and transfers disulfides to the proteins (Chacinska et al., 2004; Naoé et al., 2004; Terziyska et al., 2005, 2007; Grumbt et al., 2007; Milenkovic et al., 2007; Müller et al., 2008). Mesecke et al. (2005) found that upon release of the oxidized substrate, reduced Mia40 was reoxidized by Erv1 (also containing three pairs of cysteines), thus permitting new rounds of precursor import. Here, we report that, contrary to the view that Erv1 acts only on substrate-free Mia40, we have identified a ternary complex of Erv1, Mia40, and precursor. This ternary complex assists in the transfer of disulfide bonds to IMS precursors by spatially connecting sulfhydryl oxidase, carrier protein, and substrate.

Results and discussion

Disulfide-linked precursor–Mia40 intermediate in *erv1* mutant mitochondria

The import and assembly stages of the ^{35}S-labeled precursors of the small translocase of the inner mitochondrial membrane (Tim) proteins of the IMS were monitored by blue native electrophoresis. After disulfide-mediated interaction with Mia40, the oxidized proteins, which contain two disulfides each, assemble to the mature hexameric complexes (Tim9–Tim10 or Tim8–Tim13); Tim9 and Tim10 additionally assemble into the TIM22 complex of the inner membrane (Fig. 1 A; Chacinska et al., 2004; Curran et al., 2004; Milenkovic et al., 2007; Sideris and Tokatlidis, 2007; Müller et al., 2008). Blue native analysis of a yeast mutant of Erv1 revealed that precursors were efficiently bound to Mia40 in the mutant mitochondria, whereas the subsequent assembly into mature complexes was inhibited (Rissler et al., 2005; Müller et al., 2008), shown here for Tim10 with three different *erv1* mutants (Fig. 1 A). These results questioned the prevailing model that Erv1 was only needed for reoxidation of Mia40 to activate it for substrate binding, and suggested that Erv1 may play a role in later steps of substrate maturation.

To analyze the precursor–Mia40 conjugate under denaturing conditions, we used nonreducing SDS-PAGE (Milenkovic et al., 2007). Formation of the conjugate was impaired in *mia40-3* mutant mitochondria, and its gel migration was faster (Fig. 1 B, lanes 4–6) because of a C-terminal truncation of Mia40-3 (Chacinska et al., 2004). The conjugate was observed in similar amounts in *erv1* mutant mitochondria and wild-type mitochondria (Fig. 1 B, lanes 7–10), as well as after purification of tagged Mia40 from lysed mitochondria (Fig. 1 B, lanes 11–16). The total amount of imported precursor, determined by treatment of the mitochondria with protease, was decreased in *erv1* mutant mitochondria (Fig. 1 B, lanes 9 and 10), in agreement with the requirement of Erv1 for the Mia40-driven import reaction (Allen et al., 2005; Mesecke et al., 2005; Rissler et al., 2005). We hypothesized that in *erv1* mutants, the MIA reaction cycle was impaired such that imported precursors remained bound to Mia40, preventing assembly of the proteins and further rounds of precursor import.

A ternary complex of Erv1, Mia40, and precursor protein

The hypothesis raised the possibility that Erv1 may associate with the substrate–Mia40 complex. To capture a putative ternary complex, we used the trap mutant form Mia40-4, which efficiently binds substrate but is impaired in its release (Chacinska et al., 2004; Milenkovic et al., 2007; Müller et al., 2008). In *mia40-4* mitochondria, formation of the mature complexes (Tim9–Tim10, Tim8–13, and TIM22) is impaired, and precursor proteins accumulate at Mia40-4. We noticed an additional complex in the high molecular weight range of blue native gels upon import of ^{35}S-precursors (Fig. 1 C, Mia40–intermediate II). Mitochondria carrying His-tagged versions of Mia40 and Mia40-4 were lysed in digitonin and subjected to affinity purification. The amount of Erv1 bound to Mia40-4 was increased approximately twofold in the mutant (Fig. 1 D). The yield of copurification of the imported precursor of Tim9 with Mia40-4 was also enhanced (Fig. 1 D), and nonreducing SDS-PAGE revealed a precursor conjugate in the higher molecular weight range of *mia40-4* mitochondria (Fig. 1 E, lane 2). We conclude that both substrate and Erv1 were accumulated at the mutant protein Mia40-4.

In wild-type mitochondria, a ternary complex of Erv1, Mia40, and precursor would represent a short-lived intermediate in the reaction cycle for precursor oxidation. We searched for conditions to slow down the reaction and stabilize an intermediate complex with wild-type Mia40. Upon rupturing the outer membrane by swelling (mitoplasting), IMS components like the small Tim proteins were released, and the amount of Erv1 was reduced by approximately half (Erv1 was found in two fractions, soluble and peripherally membrane associated; Fig. 2 A, lanes 10 and 12; and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200804095/DC1). Upon import of Tim9 into mitoplasts, the formation of mature TIM complexes is impaired, whereas precursors accumulate at the membrane-bound Mia40 (Milenkovic et al., 2007). In addition to increased amounts of the Mia40–intermediate I, we observed the high molecular weight intermediate II (Fig. 2 A, lanes 5–8). Formation of the intermediate depended on the presence of precursors, as immunodecoration of the mature complexes did not reveal the high molecular weight complex (Fig. 2 A, lanes 9 and 10). Mia40–intermediate I disappeared with time, whereas the amount of intermediate II increased (Fig. 2 A, lanes 5–8), which indicates that the ^{35}S-precursor moved from intermediate I to intermediate II.

To analyze the composition of the intermediate, we used antibody-shift blue native electrophoresis upon accumulation of ^{35}S-precursors in mitoplasts. Antibodies against Mia40 as well as antibodies against Erv1 shifted the intermediate II complex, whereas intermediate I was shifted only by anti-Mia40 (Fig. 2 B, lanes 1 and 5; Erv1-specific antibodies also depleted intermediate II formed in the *mia40-4* mutant; Fig. S1). To exclude that intermediate II was associated with the membrane translocases (translocase of the outer mitochondrial membrane [TOM] and TIM22), we used antibodies shifting those complexes (Rehling

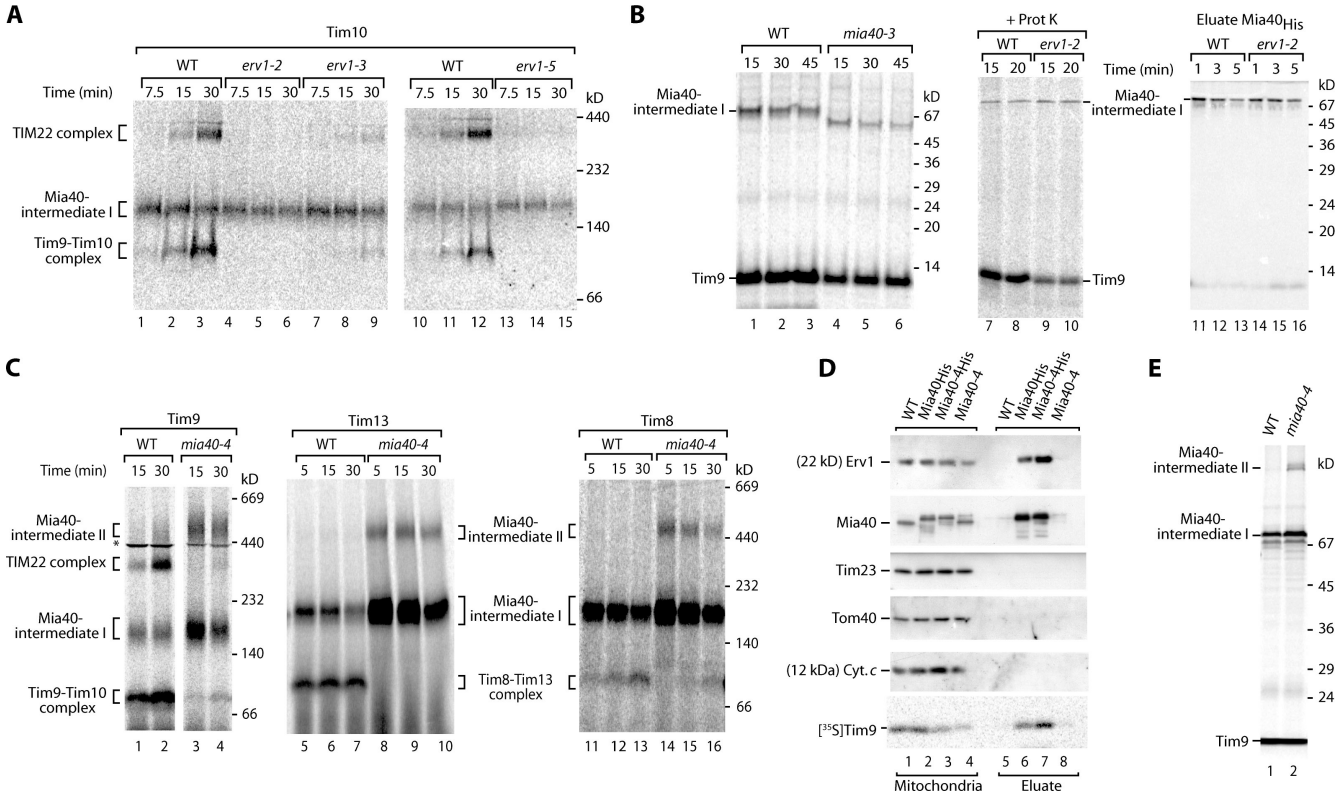

Figure 1. **Accumulation of precursor proteins at Mia40.** (A) Isolated mitochondria were incubated with ^{35}S-Tim10 and analyzed by native electrophoresis. WT, wild-type. (B) ^{35}S-Tim9 was imported into mitochondria (samples 1–10). Tim9-Mia40 was isolated by affinity purification from $Mia40_{His}$ mitochondria (right). The samples were analyzed by nonreducing SDS-PAGE. (C) ^{35}S-precursors were incubated with mitochondria and analyzed by native electrophoresis. Asterisk, nonspecific band. (D) Mia40 or $Mia40_{His}$ mitochondria were subjected to affinity purification, SDS-PAGE, and autoradiography (^{35}S-Tim9) or immunoblotting. Load, 2.5%; eluate, 100%. (E) ^{35}S-Tim9 was imported and analyzed by nonreducing SDS-PAGE.

et al., 2003; Wiedemann et al., 2003). Neither anti-Tom22 nor anti-Tim54 shifted intermediate II. Anti-Tim54 shifted the TIM22 complex as expected, whereas neither anti-Mia40 nor anti-Erv1 shifted the TIM22 complex or the Tim9–Tim10 complex (Fig. 2 B). The dicarboxylate carrier (DIC), which is imported independently of Mia40/Erv1 (Rehling et al., 2003; Chacinska et al., 2004), was not shifted by the antibodies (Fig. 2 B). We conclude that Erv1, Mia40, and precursor are present in the intermediate II complex.

The precursor–Mia40 conjugates were dissociated by addition of the reductant DTT (Fig. 2 C), which indicates that they were linked by disulfide bonds. To compare the conjugates formed in mitoplasts and *mia40-4* mutants, we performed affinity purifications. Upon separation by nonreducing SDS-PAGE, the migration of the precursor–Mia40 conjugates was indistinguishable between mitochondria, mitoplasts, and *mia40-4* mutants (Fig. 2 D).

To obtain independent evidence for a ternary complex, we used coimmunoprecipitation. ^{35}S-Tim9 imported into mitoplasts was immunoprecipitated with antibodies against Mia40, as well as Erv1, but not control antibodies (Fig. 2 E, lanes 1–5). To distinguish if ^{35}S-Tim9 was directly coprecipitated with Erv1 or via binding to Mia40, we used the mutant form Mia40-3, which is impaired in precursor binding (Chacinska et al., 2004; Müller et al., 2008). The coprecipitation of ^{35}S-Tim9 with anti-Erv1 was impaired in the *mia40-3* mutant, like that with anti-Mia40 (Fig. 2 E, lanes 6 and 8). Because in mitoplasts the precursor would have direct access to Erv1 without a need for Mia40-dependent translocation across the outer membrane (Milenkovic et al., 2007), we conclude that Mia40 is required for the interaction of Erv1 with the precursor. In *mia40-4* mitochondria, Tim9 was efficiently coprecipitated with anti-Erv1 as well as anti-Mia40 (Fig. 2 E), confirming the findings of the blue native analysis.

Collectively, we conclude that Erv1, Mia40, and precursor are present in a ternary complex, representing a transient intermediate stage during precursor oxidation, which is slowed down in mitoplasts and *mia40-4* mitochondria.

Erv1 is required for resolving the ternary complex

In *erv1* mutant mitochondria, the Mia40–intermediate II formed with an efficiency close to that of wild-type mitochondria (Fig. 3 A). Although the original *erv1-1* mutant showed only a minor effect on assembly of small Tim proteins, as described previously, the other *erv1* mutants were strongly impaired in the formation of the mature Tim9–Tim10 and TIM22 complexes (Figs. 1 A and 3 A; Allen et al., 2005; Rissler et al., 2005; Milenkovic et al., 2007; Müller et al., 2008). We reasoned that Erv1 may be rate-limiting for the turnover of intermediate II. When Erv1 was overexpressed, the amount of intermediate II was indeed decreased (Fig. 3 B). Moreover, overexpression of Erv1 in *mia40-4* cells restored growth at elevated temperatures (Fig. 3 C) and decreased the

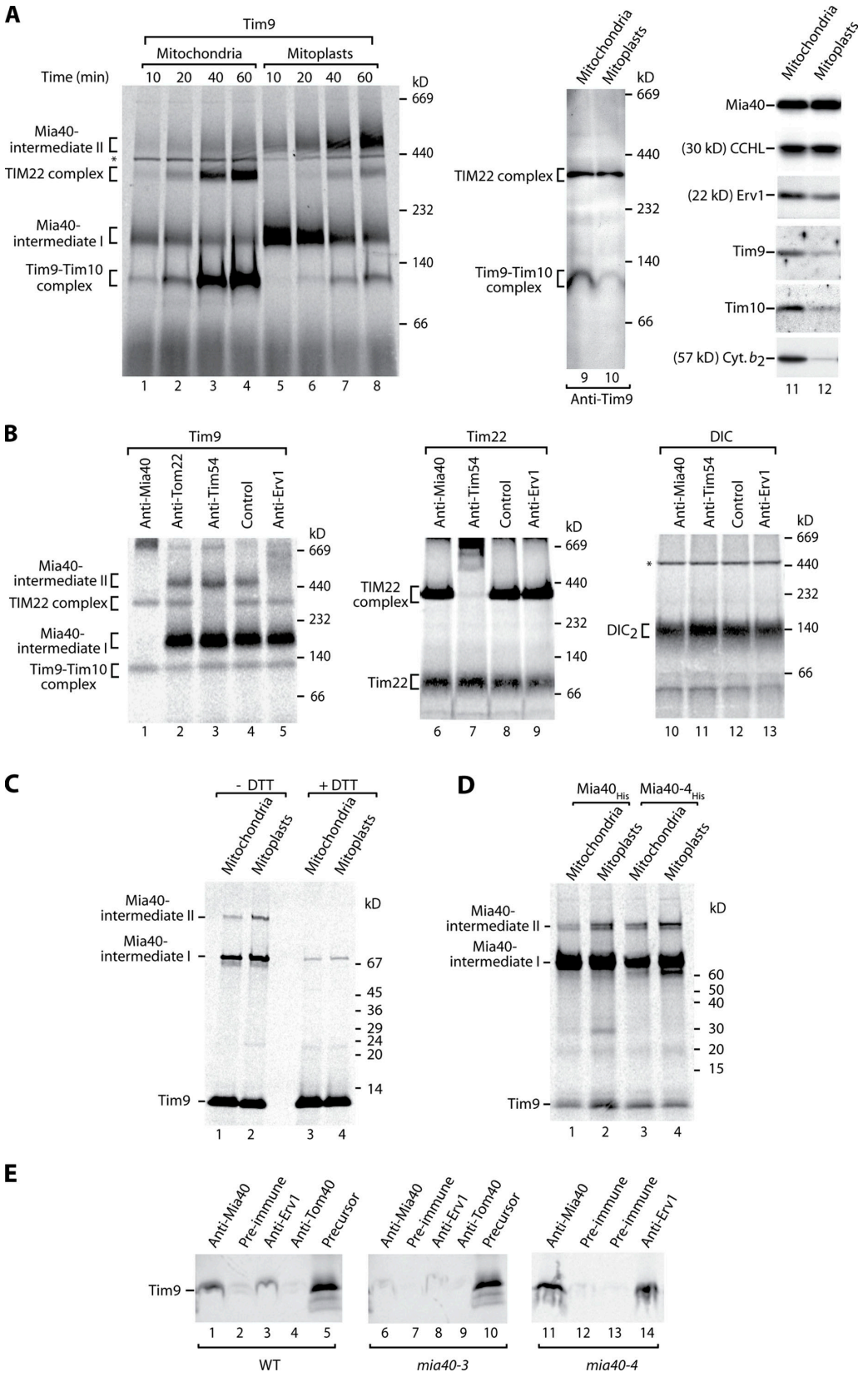

Figure 2. **Ternary complex of Mia40, Erv1, and substrate.** (A) ^{35}S-Tim9 was incubated with mitochondria or mitoplasts and analyzed by native electrophoresis (lanes 1–8). Immunoblotting of mitochondria and mitoplasts (lanes 9–12) is shown. Asterisk, nonspecific band. (B) After import of ^{35}S-precursors, mitoplasts were incubated with antisera and analyzed by native electrophoresis. Asterisk, nonspecific band. (C) ^{35}S-Tim9 was incubated with mitochondria or mitoplasts for 30 min. SDS-PAGE ± 50 mM DTT. (D) After import of ^{35}S-Tim9, Mia40, or Mia40$_{His}$, mitochondria or mitoplasts were subjected to affinity purification, and eluates were analyzed by nonreducing SDS-PAGE. (E) After import of ^{35}S-Tim9, mitoplasts (WT and *mia40-3*) or mitochondria (*mia40-4*) were subjected to immunoprecipitation.

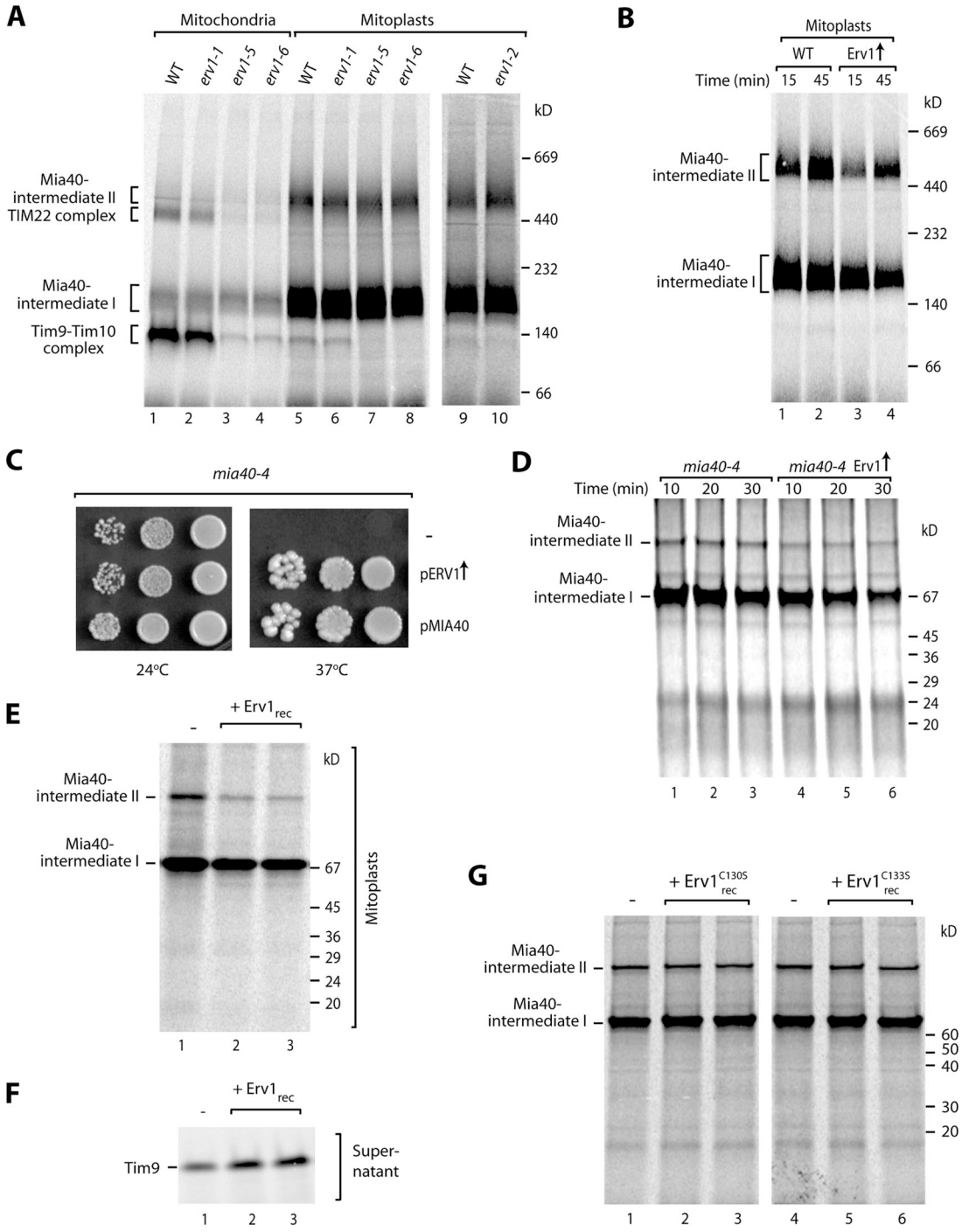

Figure 3. **Influence of Erv1 on the ternary complex.** (A) ^{35}S-Tim9 was incubated with mitochondria and mitoplasts for 30 min and analyzed by native electrophoresis. (B) Mitoplasts were incubated with ^{35}S-Tim9. (C) Growth of *mia40-4* cells overproducing Erv1. (D) ^{35}S-Tim9 was incubated with mitochondria and analyzed by nonreducing SDS-PAGE. (E–G) Mitoplasts were incubated with recombinant Erv1 forms. Pellets (E and G) and supernatants (F) were analyzed by nonreducing SDS-PAGE.

amount of intermediate II (Fig. 3 D). To directly test if Erv1 was needed for dissociation of intermediate II, we generated the intermediate in mitoplasts and added recombinant Erv1. Thereby, the amount of intermediate II was significantly decreased (Fig. 3 E), and released precursor was found in the supernatant fraction (Fig. 3 F). Mutant forms of Erv1 (Hofhaus et al., 2003) did not change the amount of intermediate II (Fig. 3 G). We conclude that Erv1 promotes a dissociation of the ternary complex.

Disulfide transfer in the ternary complex

Each small Tim protein contains two $CysX_3Cys$ motifs, forming two intramolecular disulfide bonds in the mature protein (Curran et al., 2002; Koehler, 2004; Webb et al., 2006). The fully oxidized proteins are released from Mia40 and assemble to hexameric complexes (Lu et al., 2004; Müller et al., 2008). To determine why Erv1 was needed for precursor release, we asked if it promoted disulfide formation on a Mia40-bound precursor. We monitored the

oxidation of single cysteines of Tim9 by modification with the alkylating agent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; 500 D). Mutant precursors with a replacement of one to four cysteines were used as standards (Fig. 4 A, lanes 1–5). By indirect thiol trapping, the number of oxidized cysteines after import into mitochondria was determined: first, free thiol groups were blocked by iodoacetamide, then the mitochondria were lysed and all disulfides were reduced, followed by modification of the originally oxidized cysteines by AMS. At a low temperature (15°C), a sequential oxidation of the cysteines was observed (Fig. 4 A, lanes 6–10). At physiological temperature (30°C), three species were dissected: reduced Tim9, Tim9 with one oxidized cysteine, and fully oxidized Tim9 (Fig. 4 B). In *erv1* mutant mitochondria, oxidation of the first cysteine was only mildly affected, but formation of the fully oxidized form was significantly inhibited (Fig. 4 B).

The first cysteine residue of Tim9 is required for binding to Mia40 (Milenkovic et al., 2007; Sideris and Tokatlidis, 2007). We imported mutant precursors lacking three, two, or one of the other cysteines and observed the generation of one, two, and three oxidized cysteines, respectively (Fig. 4 C). We hypothesized that species with an odd number of oxidized cysteines may represent Mia40-linked precursors. After affinity purification of His-tagged Mia40, bound precursors were eluted, and the number of originally oxidized cysteines was determined. The precursors lacking three or two cysteines contained one oxidized cysteine in the Mia40-bound form (Fig. 4 D), which reflects the mixed disulfide with Mia40. Of particular importance was the precursor $Tim9_{C55S}$. Here, two forms, one with a single oxidized cysteine and one with all three cysteines oxidized, were bound to Mia40 (Fig. 4 D). Thus, in addition to the mixed disulfide with Mia40, a further disulfide bond was transferred to the precursor bound to Mia40. In *erv1* mutant mitochondria, the oxidation of $Tim9_{C55S}$ was impaired with a particular defect in generation of the third oxidized cysteine (Fig. 4, E and F). We determined the number of oxidized cysteines in the Mia40-bound precursor of wild-type and *erv1* mutant mitochondria. Full oxidation of the precursor strongly depended on the activity of Erv1, whereas oxidation of the first cysteine (mixed disulfide with Mia40) was only moderately affected in the *erv1* mutant (Fig. 4 G). Thus, active Erv1 is critical for the efficient transfer of a further disulfide bond to the precursor, which is bound to Mia40 via a mixed disulfide, demonstrating the function of Erv1 in the ternary complex. We conclude that the association of Erv1 with the precursor–Mia40 complex permits an efficient transfer of disulfides in a ternary complex.

Conclusions

We report that the interactions of Mia40 with Erv1 and precursor are not mutually exclusive, but all three components associate in a ternary complex. This intermediate complex is a transient step in precursor oxidation and thus has escaped experimental detection in previous studies. We found two means to trap precursors in a ternary complex. First, a trap mutant form of Mia40 accumulated precursors and Erv1. Second, mitochondria were swollen, leading to a release of IMS components, including approximately half of Erv1, whereas the amount of membrane-bound Mia40 was unchanged. Erv1 was rate-limiting for dissociation of the precursor–Mia40 complex because overexpression as well as addition of purified Erv1 promoted substrate release from Mia40. The ternary complex thus accumulated in mitoplasts containing limiting amounts of Erv1.

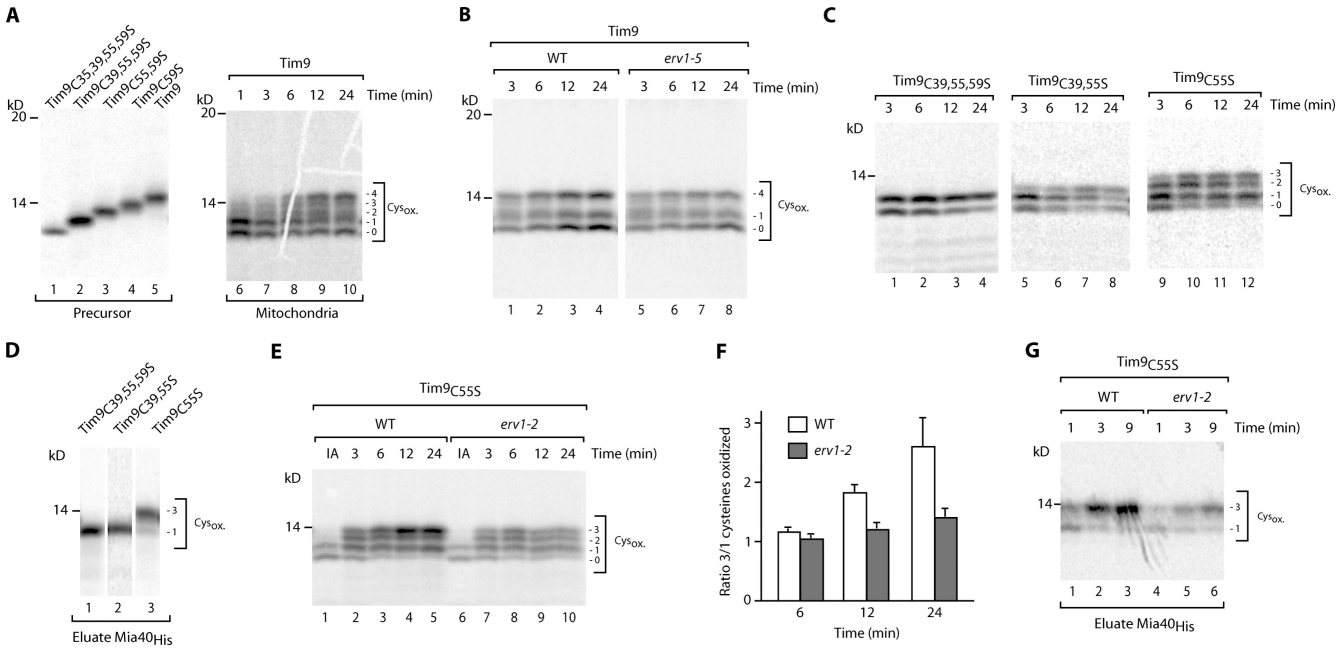

Figure 4. **Erv1 promotes disulfide transfer to Mia40-bound precursors.** (A) ^{35}S-Tim9 precursors were treated with AMS (left) or imported into mitochondria at 15°C, and subjected to indirect thiol trapping (right). Samples were analyzed by Tricine–SDS-PAGE. (B) ^{35}S-Tim9 was imported into mitochondria at 30°C and treated as described in A. (C) ^{35}S-Tim9 precursors were imported into WT mitochondria. (D) ^{35}S-Tim9 precursors were imported into $Mia40_{His}$ mitochondria and subjected to affinity purification and indirect thiol trapping. (E) ^{35}S-$Tim9_{C55S}$ was imported into mitochondria for the indicated times or for 24 min in the presence of iodoacetamide (IA). Samples were treated as described in A. (F) ^{35}S-$Tim9_{C55S}$ with one or three oxidized cysteines were quantified; error bars indicate SEM ($n = 5$). (G) ^{35}S-$Tim9_{C55S}$ was imported into $Mia40_{His}$ mitochondria and analyzed as in D.

Two possibilities are conceivable for the organization of the ternary complex. Erv1 may bind to the precursor or to Mia40. Our findings support the scenario that Erv1 does not directly bind the precursor but acts on Mia40 because the coprecipitation of precursor with Erv1 requires the presence of functional Mia40, and is enhanced by the Mia40-trap form. We suggest that Erv1 promotes maturation (oxidation) of Mia40-bound substrate in the ternary complex through the transfer of disulfide bonds to precursors via Mia40, and this serves as a prerequisite for substrate release.

Most disulfide relay components of mitochondria, bacteria, and ER share only limited homology on the primary structure level; however, basic functional mechanisms of disulfide shuttling may be related (Gerber et al., 2001; Sevier et al., 2001; Gross et al., 2002; Kadokura et al., 2004; Tu and Weissman, 2004; Inaba et al., 2006; Sevier and Kaiser, 2006). By splitting bacterial DsbB into two domains, disulfide transfer in a complex of three polypeptides, DsbA with the two DsbB domains was shown (Kadokura and Beckwith, 2002). Here, the central polypeptide contained one redox-active pair of cysteines; in the ternary complex, each cysteine was bound to a different partner polypeptide. Mia40 may function in a similar manner with one redox-active pair of cysteines (Grumbt et al., 2007). Alternatively, more than one cysteine residue of precursor and Mia40 may be involved in forming transient disulfides in the ternary complex. Several components of the ER and bacterial systems contain more than one redox-active pair of cysteines (Tsai and Rapoport, 2002; Kadokura et al., 2004; Kulp et al., 2006; Sevier and Kaiser, 2006). Moreover, more than one molecule of Mia40 may participate in the reaction, as human Mia40 forms a homo-oligomer linked by noncovalent interactions (Hofmann et al., 2005). In addition to disulfide-mediated interactions, noncovalent interactions and conformational changes likely contribute to the cooperation of the partners in the ternary complex.

The findings presented here change the view that the disulfide carrier (Mia40) strictly alternates between binding to Erv1 and precursor in a sequential relay system (Mesecke et al., 2005; Herrmann and Köhl, 2007). The identification of a ternary complex suggests that the flow of electrons between oxidase, carrier, and substrate does not require sequential association and dissociation of the components, but rather their concerted action within a single complex. Thus, two distinct redox reactions are spatially coupled, which results in a channeling mechanism for disulfide transfer. Moreover, for substrates that receive more than one disulfide, the binding of Erv1 to the Mia40–precursor complex may permit an efficient transfer of multiple disulfides in a ternary complex. We speculate that the concept of mitochondrial disulfide channeling, comprising oxidase, disulfide carrier, and substrate, provides mechanistic implications for the ER and bacterial systems.

Materials and methods

Strains

Strains generated and used in this study are derivatives of the wild-type YPH499 strain of yeast *Saccharomyces cerevisiae* (*MAT*a, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, and *lys2-801*). The temperature-sensitive *erv1-1* (YPH-BG-Erv1ts-F124S), *erv1-2* (YBG-0702b), *erv1-3* (YBG-0701a), and *erv1-5* (YPH-BGErv1ts-C159S) mutants have been described previously (Rissler et al., 2005; Milenkovic et al., 2007; Müller et al., 2008). The mutant strain *erv1-6* was generated by site-directed mutagenesis followed by plasmid shuffling in the background of the genomic deletion of *ERV1* (YPH-BG-Erv1ts-C176S; *MAT*a, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, and *erv1::ADE2* [pFL39-*erv1*-C176S]). The *erv1-6* mutant possesses the amino acid substitution Cys176Ser (Hofhaus et al., 2003).

To overproduce Erv1, the yeast YPH499 strain and *mia40-4* mutant strain were transformed with the plasmid p9014-8 harboring the *ERV1* gene under the control of its endogenous promoter and terminator in the multicopy Yep352 yeast vector. The suppression by p9014-8 was analyzed on YPD plates (1% [wt/vol] yeast extract, 2% [wt/vol] bactopeptone, and 2% [wt/vol] glucose). The C-terminal His_{10}-tagged versions of Mia40 were generated by genomic integration in a wild-type background, resulting in $Mia40_{His}$ (yAC53; *MAT*a, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, and *MIA40*$_{His10}$; Milenkovic et al., 2007), and in the *erv1-2* mutant background, resulting in *erv1-2* $Mia40_{His}$ (yAC55-81; *MAT*a, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, *MIA40*$_{His10}$, and *erv1::ADE2* [pFL39-*erv1-2*]). The Mia40-4int strain (BG-fomp2-7-int-4-12; *MAT*a, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, and *mia40-4*) was constructed by integration of the *mia40-4* allele (amino acids substitutions T313A, I316M, and F342L according to original open reading frame assignment in the *Saccharomyces* Genome Database [Chacinska et al., 2004]; T289A, I292M, and F318L according to the current open reading frame assignment) into the genome of YPH499. Subsequently, a His_{10} tag was introduced at the 3′ end of the *mia40-4* genomic coding sequence, giving rise to Mia40-4_{His} (yAC64-1; *MAT*a, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, and *mia40-4*$_{His10}$).

Mitochondrial procedures

Mitochondria were isolated from *S. cerevisiae* strains grown on YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] bactopeptone, and 3% [wt/vol] glycerol) at 19, 24, or 30°C by differential centrifugation, as described previously, and mitoplasts were generated by hypoosmotic swelling (Stojanovski et al., 2007). Mitochondrial precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and subjected to in vitro import assays (Stojanovski et al., 2007). Import reactions were performed at 25°C (dicarboxylate carrier and Tim9), 30°C (Tim8, Tim9, and Tim13), or 35°C (Tim10 and Tim22). Samples analyzed by nonreducing SDS-PAGE were solubilized in Laemmli buffer (minus reducing agent) containing 50 mM iodoacetamide. Carbonate extractions were performed as described previously (Stojanovski et al., 2007).

For coimmunoprecipitation experiments, ^{35}S-labeled Tim9 was imported into mitochondria or mitoplasts, and the reaction was stopped by the addition of 50 mM iodoacetamide. Mitochondria were isolated and solubilized in digitonin-containing buffer (1% digitonin, 20 mM Tris-Cl, pH 7.4, 50 mM NaCl, 10% [wt/vol] glycerol, and 50 mM iodoacetimide) for 20 min on ice. After clarification of the solubilized material, supernatants were isolated and incubated with IgGs coupled to protein–A Sepharose for 1 h at 4°C. Antibody-bound material was washed three times in digitonin-containing buffer, upon which Laemmli buffer was added to the samples. Samples were heated at 95°C for 5 min and analyzed by SDS-PAGE.

Recombinant $Erv1_{His}$ and its mutant versions (C130S and C133S) were purified via nickel–nitrilotriacetic acid–agarose as described previously (Hofhaus et al., 2003; Milenkovic et al., 2007). For release assays, recombinant Erv1 (1 and 2 µg per 50-µl reaction volume) was added, and samples were incubated at 30°C for 20 min. The reaction was stopped by the addition of 50 mM iodoacetamide. Analysis of protein complexes by blue native electrophoresis and antibody-shift/depletion analysis was performed as described previously (Wiedemann et al., 2003; Chacinska et al., 2004; Stojanovski et al., 2007). Affinity purification of $Mia40_{His}$ and Mia40-4_{His} was performed as described previously (Rissler et al., 2005; Milenkovic et al., 2007). Radiolabeled proteins were detected by digital autoradiography (Storm imaging system; GE Healthcare) and analyzed by ImageQuant software (GE Healthcare). Western blot analysis was performed using enhanced chemiluminescence.

Thiol-trapping experiments

Thiol modifications were performed as described previously (Jakob et al., 1999; Curran et al., 2002). Oxidation reactions of ^{35}S-precursors during their import into isolated mitochondria were blocked by modification of their free sulfhydryl groups with 50 mM iodoacetamide. After reisolation, mitochondria were solubilized in digitonin-containing buffer (1% digitonin, 20 mM Tris-Cl, pH 7.4, 50 mM NaCl, and 10% [wt/vol] glycerol) supplemented with 10 mM of the reducing agent Tris(2-carboxyethyl) phosphine hydrochloride

(TCEP) at 30°C for 15 min to reduce existing disulfide bonds. After protein precipitation, samples were dissolved in Laemmli buffer containing 15 mM AMS at 25°C for 30 min to alkylate liberated thiols, and were subsequently denatured at 60°C for 15 min. For affinity purification, ^{35}S-precursors were imported into $Mia40_{His}$ mitochondria, and free thiols were subsequently blocked with 50 mM iodoacetamide. After reisolation, mitochondria were solubilized in digitonin-containing buffer (1% digitonin, 20 mM Tris-Cl, pH 7.4, 50 mM NaCl, 10% [wt/vol] glycerol, and 50 mM iodoacetamide) and subjected to nickel–nitrilotriacetic acid–agarose affinity chromatography. Elution fractions were treated with 10 mM TCEP at 30°C. After precipitation, samples were dissolved in Laemmli buffer containing 15 mM AMS at 25°C for 30 min followed by incubation at 60°C for 15 min. In some figures, nonrelevant gel lanes were excised digitally, and the loading intensities of the precursors were used for standardization. Proteins were separated by Tricine–SDS-PAGE.

Online supplemental material

Fig. S1 demonstrates conditions to form the ternary complex. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200804095/DC1.

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References

Allen, S., V. Balabanidou, D.P. Sideris, T. Lisowsky, and K. Tokatlidis. 2005. Erv1 mediates the Mia40-dependent protein import pathway and provides a functional link to the respiratory chain by shuttling electrons to cytochrome *c*. *J. Mol. Biol.* 353:937–944.

Chacinska, A., S. Pfannschmidt, N. Wiedemann, V. Kozjak, L.K. Sanjuán Szklarz, A. Schulze-Specking, K.N. Truscott, B. Guiard, C. Meisinger, and N. Pfanner. 2004. Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. *EMBO J.* 23:3735–3746.

Collet, J.F., and J.C. Bardwell. 2002. Oxidative protein folding in bacteria. *Mol. Microbiol.* 44:1–8.

Curran, S.P., D. Leuenberger, W. Oppliger, and C.M. Koehler. 2002. The Tim9p-Tim10p complex binds to the transmembrane domains of the ADP/ATP carrier. *EMBO J.* 21:942–953.

Curran, S.P., D. Leuenberger, E.P. Leverich, D.K. Hwang, K.N. Beverly, and C.M. Koehler. 2004. The role of Hot13p and redox chemistry in the mitochondrial TIM22 import pathway. *J. Biol. Chem.* 279:43744–43751.

Dolezal, P., V. Likic, J. Tachezy, and T. Lithgow. 2006. Evolution of the molecular machines for protein import into mitochondria. *Science.* 313:314–318.

Endo, T., H. Yamamoto, and M. Esaki. 2003. Functional cooperation and separation of translocators in protein import into mitochondria, the double-membrane bounded organelles. *J. Cell Sci.* 116:3259–3267.

Gerber, J., U. Mühlenhoff, G. Hofhaus, R. Lill, and T. Lisowsky. 2001. Yeast Erv2p is the first microsomal FAD-linked sulfhydryl oxidase of the Erv1p/Alrp protein family. *J. Biol. Chem.* 276:23486–23491.

Gross, E., C.S. Sevier, A. Vala, C.A. Kaiser, and D. Fass. 2002. A new FAD-binding fold and intersubunit disulfide shuttle in the thiol oxidase Erv2p. *Nat. Struct. Biol.* 9:61–67.

Grumbt, B., V. Stroobant, N. Terziyska, L. Israel, and K. Hell. 2007. Functional characterization of Mia40p, the central component of the disulfide relay system of the mitochondrial intermembrane space. *J. Biol. Chem.* 282:37461–37470.

Herrmann, J.M., and R. Köhl. 2007. Catch me if you can! Oxidative protein trapping in the intermembrane space of mitochondria. *J. Cell Biol.* 176:559–563.

Hofhaus, G., J.E. Lee, I. Tews, B. Rosenberg, and T. Lisowsky. 2003. The N-terminal cysteine pair of yeast sulfhydryl oxidase Erv1p is essential for *in vivo* activity and interacts with the primary redox centre. *Eur. J. Biochem.* 270:1528–1535.

Hofmann, S., U. Rothbauer, N. Mühlenbein, K. Baiker, K. Hell, and M.F. Bauer. 2005. Functional and mutational characterization of human Mia40 acting during import into the mitochondrial intermembrane space. *J. Mol. Biol.* 353:517–528.

Inaba, K., S. Murakami, M. Suzuki, A. Nakagawa, E. Yamashita, K. Okada, and K. Ito. 2006. Crystal structure of the DsbB-DsbA complex reveals a mechanism of disulfide bond generation. *Cell.* 127:789–801.

Jakob, U., W. Muse, M. Eser, and J.C. Bardwell. 1999. Chaperone activity with a redox switch. *Cell.* 96:341–352.

Jensen, R.E., and A.E. Johnson. 2001. Opening the door to mitochondrial protein import. *Nat. Struct. Biol.* 8:1008–1010.

Kadokura, H., and J. Beckwith. 2002. Four cysteines of the membrane protein DsbB act in concert to oxidize its substrate DsbA. *EMBO J.* 21:2354–2363.

Kadokura, H., H. Tian, T. Zander, J.C. Bardwell, and J. Beckwith. 2004. Snapshots of DsbA in action: detection of proteins in the process of oxidative folding. *Science.* 303:534–537.

Koehler, C.M. 2004. The small Tim proteins and the twin Cx_3C motif. *Trends Biochem. Sci.* 29:1–4.

Kulp, M.S., E.M. Frickel, L. Ellgaard, and J.S. Weissman. 2006. Domain architecture of protein-disulfide isomerase facilitates its dual role as an oxidase and an isomerase in Ero1p-mediated disulfide formation. *J. Biol. Chem.* 281:876–884.

Lu, H., S. Allen, L. Wardleworth, P. Savory, and K. Tokatlidis. 2004. Functional TIM10 chaperone assembly is redox-regulated *in vivo*. *J. Biol. Chem.* 279:18952–18958.

Mesecke, N., N. Terziyska, C. Kozany, F. Baumann, W. Neupert, K. Hell, and J.M. Herrmann. 2005. A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. *Cell.* 121:1059–1069.

Milenkovic, D., K. Gabriel, B. Guiard, A. Schulze-Specking, N. Pfanner, and A. Chacinska. 2007. Biogenesis of the essential Tim9-Tim10 chaperone complex of mitochondria: site-specific recognition of cysteine residues by the intermembrane space receptor Mia40. *J. Biol. Chem.* 282:22472–22480.

Müller, J.M., D. Milenkovic, B. Guiard, N. Pfanner, and A. Chacinska. 2008. Precursor oxidation by Mia40 and Erv1 promotes vectorial transport of proteins into the mitochondrial intermembrane space. *Mol. Biol. Cell.* 19:226–236.

Naoé, M., Y. Ohwa, D. Ishikawa, C. Ohshima, S. Nishikawa, H. Yamamoto, and T. Endo. 2004. Identification of Tim40 that mediates protein sorting to the mitochondrial intermembrane space. *J. Biol. Chem.* 279:47815–47821.

Neupert, W., and J.M. Herrmann. 2007. Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* 76:723–749.

Oka, T., and K. Mihara. 2005. A railroad switch in mitochondrial protein import. *Mol. Cell.* 18:145–146.

Rehling, P., K. Model, K. Brandner, P. Kovermann, A. Sickmann, H.E. Meyer, W. Kühlbrandt, R. Wagner, K.N. Truscott, and N. Pfanner. 2003. Protein insertion into the mitochondrial inner membrane by a twin-pore translocase. *Science.* 299:1747–1751.

Rissler, M., N. Wiedemann, S. Pfannschmidt, K. Gabriel, B. Guiard, N. Pfanner, and A. Chacinska. 2005. The essential mitochondrial protein Erv1 cooperates with Mia40 in biogenesis of intermembrane space proteins. *J. Mol. Biol.* 353:485–492.

Sevier, C.S., and C.A. Kaiser. 2006. Conservation and diversity of the cellular disulfide bond formation pathways. *Antioxid. Redox Signal.* 8:797–811.

Sevier, C.S., J.W. Cuozzo, A. Vala, F. Aslund, and C.A. Kaiser. 2001. A flavoprotein oxidase defines a new endoplasmic reticulum pathway for biosynthetic disulphide bond formation. *Nat. Cell Biol.* 3:874–882.

Sideris, D.P., and K. Tokatlidis. 2007. Oxidative folding of small Tims is mediated by site-specific docking onto Mia40 in the mitochondrial intermembrane space. *Mol. Microbiol.* 65:1360–1373.

Stojanovski, D., N. Pfanner, and N. Wiedemann. 2007. Import of proteins into mitochondria. *Methods Cell Biol.* 80:783–806.

Terziyska, N., T. Lutz, C. Kozany, D. Mokranjac, N. Mesecke, W. Neupert, J.M. Herrmann, and K. Hell. 2005. Mia40, a novel factor for protein import into the intermembrane space of mitochondria is able to bind metal ions. *FEBS Lett.* 579:179–184.

Terziyska, N., B. Grumbt, M. Bien, W. Nuepert, J.M. Herrmann, and K. Hell. 2007. The sulfhydryl oxidase Erv1 is a substrate of the Mia40-dependent protein translocation pathway. *FEBS Lett.* 581:1098–1102.

Tokatlidis, K. 2005. A disulfide relay system in mitochondria. *Cell.* 121:965–967.

Tsai, B., and T.A. Rapoport. 2002. Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J. Cell Biol.* 159:207–216.

Tu, B.P., and J.S. Weissman. 2004. Oxidative protein folding in eukaryotes: mechanisms and consequences. *J. Cell Biol.* 164:341–346.

Webb, C.T., M.A. Gorman, M. Lazarou, M.T. Ryan, and J.M. Gulbis. 2006. Crystal structure of the mitochondrial chaperone TIM9-10 reveals a six-bladed α-propeller. *Mol. Cell.* 21:123–133.

Wiedemann, N., V. Kozjak, A. Chacinska, B. Schönfisch, S. Rospert, M.T. Ryan, N. Pfanner, and C. Meisinger. 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature.* 424:565–571.