Current Biology 20, 1227–1232, July 13, 2010 ©2010 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2010.05.058

Report

Cooperation of Stop-Transfer and Conservative Sorting Mechanisms in Mitochondrial Protein Transport

Maria Bohnert,[1,2] Peter Rehling,[1,3] Bernard Guiard,[4] Johannes M. Herrmann,[5] Nikolaus Pfanner,[1,6,*] and Martin van der Laan[1]

[1]Institut für Biochemie und Molekularbiologie, Zentrum für Biochemie und Molekulare Zellforschung (ZBMZ)
[2]Fakultät für Biologie, Universität Freiburg, 79104 Freiburg, Germany
[3]Abteilung für Biochemie II, Universität Göttingen, 37073 Göttingen, Germany
[4]Centre de Génétique Moléculaire, Centre National de la Récherche Scientifique, 91190 Gif-sur-Yvette, France
[5]Cell Biology, Universität Kaiserslautern, 67663 Kaiserslautern, Germany
[6]Centre for Biological Signalling Studies (*bioss*), Universität Freiburg, 79104 Freiburg, Germany

Summary

The mitochondrial inner membrane is a highly protein-rich membrane with central importance for oxidative phosphorylation and metabolite transport [1]. A large number of inner-membrane proteins are synthesized as preproteins with cleavable presequences [2–9]. Opposing mechanisms of preprotein insertion into the membrane have been debated: stop-transfer with arrest in the inner membrane versus conservative sorting via the matrix [3, 8, 10]. We dissected the membrane insertion of a multispanning ABC transporter. The N-terminal membrane domain was laterally released from the presequence translocase of the inner membrane (TIM23 complex) by a stop-transfer mechanism, whereas the subsequent domain was imported via the matrix heat-shock protein 70 (mtHsp70) motor and exported by the oxidase assembly (OXA) translocase. These observations lead to an unexpected solution to the controversial debate about mitochondrial preprotein sorting. Stop-transfer and conservative sorting are not mutually exclusive pathways but represent sorting mechanisms that cooperate in the membrane integration of a protein with complex topology. We conclude that the multispanning protein is inserted in a modular manner by the coordinated action of two inner-membrane preprotein translocases.

Results and Discussion

Most mitochondrial proteins are synthesized with N-terminal presequences on cytosolic ribosomes. These preproteins are imported by the translocase of the outer mitochondrial membrane (TOM complex) and the TIM23 complex of the inner membrane [2–9]. Initial transport of a preprotein through the TIM23 channel is driven by the membrane potential $\Delta\psi$. Further translocation into the matrix requires cooperation of the TIM23 complex with the import motor that contains mtHsp70 as the ATP-driven core component. Two conflicting models of how cleavable preproteins are inserted into the inner membrane have been discussed [3, 8]. In the "stop-transfer pathway," a hydrophobic segment located behind the presequence arrests translocation in the TIM23 complex, and the preprotein is laterally released into the lipid phase of the inner membrane [3, 10–13]. In contrast, in the "conservative-sorting pathway," the preprotein is imported into the matrix in an mtHsp70-dependent manner and is subsequently exported into the inner membrane by the OXA translocase [3, 14–17]. The OXA machinery has been conserved from the prokaryotic ancestor of mitochondria and mediates cotranslational inner-membrane insertion of proteins that are encoded by the mitochondrial genome [18–20], yet only limited information on a role of OXA in membrane insertion of authentic nuclear-encoded preproteins is available (OXA-dependent biogenesis of authentic imported proteins has been reported for members of the OXA protein family itself [16, 17, 21, 22]). Experimental observations favoring one or the other pathway have been reported, although the various studies typically used different preproteins to demonstrate stop-transfer versus conservative-sorting mechanisms, leading to the current view that there are two different classes of cleavable inner-membrane proteins and that these use two fundamentally different sorting pathways [3, 8].

The mitochondrial inner membrane contains many multispanning proteins. Defining their sorting mechanism requires an experimental dissection of membrane insertion of distinct transmembrane segments. In the import studies performed so far, researchers have typically analyzed whether termini or attached tags achieved the expected topology. However, the addition of nonmitochondrial tags can interfere with the correct topogenesis of mitochondrial proteins [23]. To study the topogenesis of individual transmembrane segments without tagging, we screened cleavable multispanning inner-membrane proteins in order to find a model substrate whose topology was known, that was efficiently imported into isolated mitochondria, and that was suitable for topological mapping. The mitochondrial multidrug-resistance-like protein Mdl1 fulfilled all requirements. Mdl1 belongs to the family of mitochondrial ABC transporters, which are inner-membrane proteins with a cleavable presequence, six transmembrane segments, and a nucleotide binding domain (NBD) in the matrix [24, 25]. The topology of Mdl1 (Figure 1A) can be deduced from the X-ray structure of a homologous *Staphylococcus aureus* protein [25]. The precursor of Mdl1 was efficiently imported into isolated yeast mitochondria, and the presequence was removed (Figure S1A). Import of Mdl1 was impaired in mitochondria lacking the outer membrane receptor Tom70, Tom20, or Tom22 (Figures S1B and S1C), demonstrating that Mdl1 was transported by the TOM complex. We then employed conditional yeast mutants with impaired activities of the TIM23 complex (*tim17-5*) or the TIM22 complex (*tim54-16*). Import of Mdl1 was inhibited in *tim17-5* mitochondria (Figure S1D), which are defective in translocation of TIM23- and motor (mtHsp70)-dependent preproteins into the matrix [12]. Import of Mdl1 into *tim54-16* mitochondria [26], which show a defect in the import of metabolite carriers via the TIM22 machinery, was not reduced (Figure S1E). We conclude that the precursor of Mdl1 was

*Correspondence: nikolaus.pfanner@biochemie.uni-freiburg.de

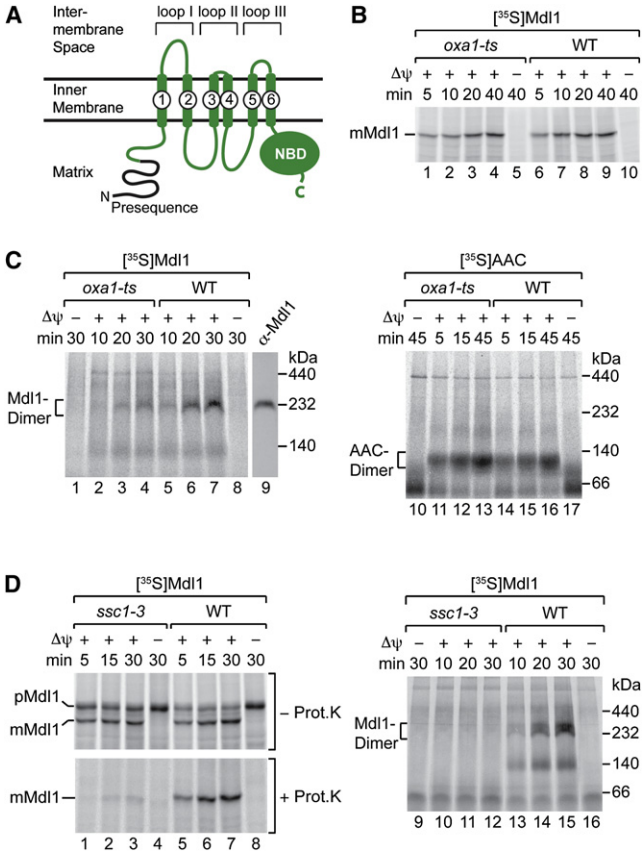

Figure 1. Differential Requirement for Import Motor and OXA at Distinct Stages of Mdl1 Biogenesis

(A) Schematic representation of Mdl1 topology. The Mdl1 precursor contains a 59 residue amino-terminal presequence that directs the preprotein to mitochondria [30]. The mature protein consists of a transmembrane domain with six membrane-spanning segments and a nucleotide-binding domain (NBD) located in the matrix. Two hydrophilic loops (I and III) are exposed to the intermembrane space, whereas loop II connecting transmembrane segments 3 and 4 is very short and comprises only two amino acid residues. Mitochondrial ABC transporters are half-transporters that assemble into homodimeric complexes (with 12 transmembrane segments) [25].

(B) Mdl1 precursor was imported into wild-type (WT) and *oxa1-ts* mutant mitochondria, and treatment with proteinase K as described in Experimental Procedures followed. Import reactions were analyzed by SDS-PAGE and digital autoradiography.

(C) Assembly of the Mdl1 dimer requires Oxa1. Mdl1 and AAC were imported into WT and *oxa1-ts* mitochondria. Mitochondria were treated with proteinase K, solubilized with digitonin, and subjected to blue native electrophoresis. "α-Mdl1" indicates detection of Mdl1 by immunoblotting.

(D) Dependence of Mdl1 biogenesis on the import motor. Mdl1 was imported into WT and *ssc1-3* mutant mitochondria, and treatment with proteinase K followed. Where indicated, the proteinase K treatment was omitted. Analysis was performed by SDS-PAGE (lanes 1–8) or blue native electrophoresis (lanes 9–16). Abbreviations are as follows: p, precursor; m, mature.

See also Figure S1.

imported via the presequence pathway by the TOM and TIM23 machineries.

In mitochondria isolated from an *oxa1* yeast mutant, import of Mdl1 to a protease-protected location was not affected (Figure 1B). To study a possible role of Oxa1 in a subsequent export of Mdl1 from the matrix into the inner membrane, we monitored the formation of the mature Mdl1 dimer by blue native electrophoresis and observed an inhibition of Mdl1 dimerization in *oxa1-ts* mitochondria (Figure 1C; see also Figure S1F). Import and dimerization of the ADP and ATP carrier (AAC), which is transported by the TIM22 complex, were not impaired in *oxa1-ts* mitochondria (Figure 1C). For comparison, in mutants of the TIM22 complex (*tim54-16* mitochondria), the dimerization of AAC, but not Mdl1, was inhibited (Figures S1G and S1H). Taken together, these results indicate that Mdl1 is imported into mitochondria via the TOM and TIM23 complexes and requires the OXA translocase for assembly into the inner membrane, suggesting that Mdl1 uses the conservative sorting pathway via the matrix.

Preproteins transported into the matrix require the mtHsp70 import motor, whereas preproteins that are laterally sorted by a stop-transfer mechanism can be imported despite an inactivation of mtHsp70 [3, 8–10, 13, 15]. Import of Mdl1 revealed a differential dependence on mtHsp70. Translocation to a protease-protected location and assembly to the dimer were strongly inhibited in the mtHsp70 yeast mutant, *ssc1-3* (Figure 1D), yet processing of Mdl1 to the mature form, i.e., removal of the presequence, was not blocked in *ssc1-3* mitochondria (Figure 1D, lanes 1–3, upper panel).

We established an assay to monitor the insertion of different transmembrane segments of Mdl1 into the inner membrane. Mdl1 contains two loops that protrude into the intermembrane space: loop I, located between transmembrane segment 1 (TM1) and TM2, and loop III, between TM5 and TM6 (Figure 1A). We imported ^{35}S-labeled Mdl1, opened the intermembrane space by swelling the mitochondria, and treated the resulting mitoplasts with proteinase K. Thereby four major fragments were generated; these are labeled with TM in Figure 2A. Antibodies against the C-terminal NBD of Mdl1 labeled the two larger fragments (Figure 2A, lane 3), demonstrating that they represented C-terminal fragments. Their size corresponded to the expected cleavage in loops I and III; this was confirmed by synthesis of the two predicted fragments TM2-6$_{NBD}$ and TM6$_{NBD}$ in vitro and comparison of their gel mobility to the fragments generated in mitoplasts (Figure S2A). A further two fragments, TM2-5 and TM1, were identified by a combination of size mapping and import of truncated versions of Mdl1 into mitochondria (Figures S2A and S2B and Supplemental Experimental Procedures).

We employed the fragmentation assay to determine the membrane insertion of radiolabeled Mdl1 in *oxa1-ts* mitochondria. Remarkably, the fragmentation pattern of the mutant mitochondria was indistinguishable from that of wild-type mitochondria (Figure 2B), in contrast to the clear impairment of *oxa1-ts* mitochondria in the formation of dimeric Mdl1 (Figure 1C). Two possibilities were conceivable: (i) membrane insertion of Mdl1 is independent of Oxa1; or (ii) the fragmentation assay does not monitor membrane insertion of the middle portion of Mdl1 (TM3-TM4) because loop II, which consists of only two amino acid residues, is too small to be accessible to the added protease, and thus a possible effect of Oxa1 on the insertion of the middle portion is missed.

To address the second possibility, we inserted the 15-residue sequence of the cleavable loop III between TM3 and TM4. The resulting Mdl1-loop construct was imported to a protease-protected location and dimerized (Figure 2C and Figure S2C). The fragmentation assay of Mdl1-loop yielded several fragments that were similar to fragments derived from wild-type Mdl1 (Figure 2D); however, TM2-6$_{NBD}$ was only observed in small amounts after short import times (Figure 2D, lane 1). Instead, two new fragments were formed (Figure 2D,

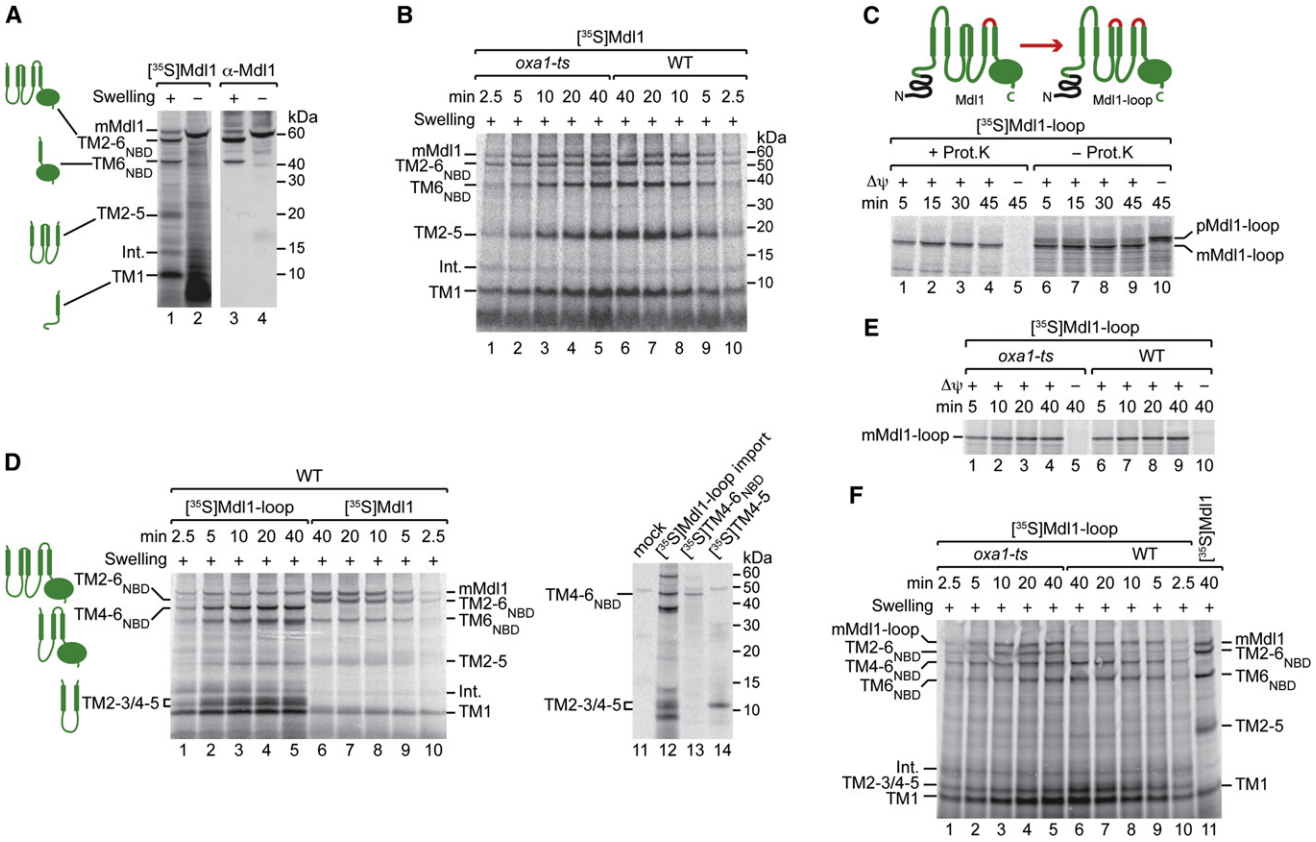

Figure 2. Oxa1 Mediates Membrane Insertion of Transmembrane Segments 3 and 4 of Mdl1

(A) Proteolytic fragmentation assay to analyze membrane insertion and topology of imported Mdl1 as described in the Experimental Procedures. Int., fragment derived from an import intermediate of Mdl1. "α-Mdl1" indicates detection of Mdl1 by immunoblotting.

(B) Import of Mdl1 into energized wild-type (WT) and *oxa1-ts* mitochondria was followed by topology mapping as in (A).

(C) Schematic representation of Mdl1-loop and its import into mitochondria.

(D) Comparison of proteolytic fragments generated from Mdl1 and Mdl1-loop that were imported in mitochondria (lanes 1–10). Fragments derived from imported Mdl1-loop (lane 12) were compared to Mdl1 fragments synthesized in vitro (lanes 13 and 14). TM4-6_{NBD}, amino acids 267–710; TM4-5, amino acids 267–377 of the Mdl1-loop precursor.

(E) Import of Mdl1-loop into *oxa1-ts* and wild-type mitochondria.

(F) Topology mapping of imported Mdl1-loop in *oxa1-ts* and wild-type mitochondria. For comparison, formation of TM2-6_{NBD} from wild-type Mdl1 is shown in lane 11.

See also Figure S2.

lanes 2–5). Size mapping confirmed that the larger fragment represented the expected fragment TM4-6_{NBD} (Figure 2D, lanes 12 and 13). The smaller new fragment, migrating as a double band, corresponded in size to in-vitro-synthesized TM4-5 (Figure 2D, lane 14) as well as to the similarly sized TM2-3. Irrespective of the exact assignment to TM2-3 and/or TM4-5, the formation of TM4-6_{NBD} and TM2-3/TM4-5 indicates that the middle loop was accessible to proteinase K in mitoplasts.

Mdl1-loop was transported to a protease-protected location in *oxa1-ts* mitochondria (Figure 2E). The fragmentation assay, however, revealed a striking difference in wild-type mitochondria. The fragment TM2-6_{NBD} of Mdl1-loop was observed in *oxa1-ts* mitochondria in considerable amounts (Figure 2F, lanes 2–5) in contrast to the situation in wild-type mitochondria (Figure 2F, lanes 6–9), indicating that cleavage of the middle loop was delayed in *oxa1-ts* mitochondria. Because the topogenesis of loop III itself is not affected in *oxa1-ts* mitochondria (Figure 2B and Supplemental Experimental Procedures), we conclude that membrane insertion of the middle portion of Mdl1, but not of the N- and C-terminal transmembrane portions, depends on Oxa1.

These results raised the possibility that the topogenesis of Mdl1 involves not only conservative sorting via the OXA translocase but also a stop-transfer mechanism. Because the dependence on mtHsp70 is a critical factor distinguishing lateral release into the inner membrane from translocation into the matrix, we asked which domains of Mdl1 made import dependent on mtHsp70. Full-length Mdl1 contains the large C-terminal NBD that is exposed to the matrix, explaining why transport of Mdl1 to a protease-protected location is virtually blocked in *ssc1-3* mitochondria (Figure 1D). However, import of TM1-6 that lacks NBD was still considerably inhibited in *ssc1-3* mitochondria (Figure 3A, lanes 1–3), indicating that parts of the transmembrane domain depended on mtHsp70. In contrast, the import of TM1-2 into *ssc1-3* mitochondria occurred with an efficiency close to that of wild-type mitochondria (Figure 3A, lanes 9–11). The fragmentation assay showed that TM1-2 was inserted into the inner membrane of *ssc1-3* mitochondria like in wild-type mitochondria (Figure 3B). Import and membrane insertion of TM1-2 were also not impaired in *oxa1-ts* mitochondria (Figure 3C). Thus, the truncated form of Mdl1, which contained only presequence, TM1

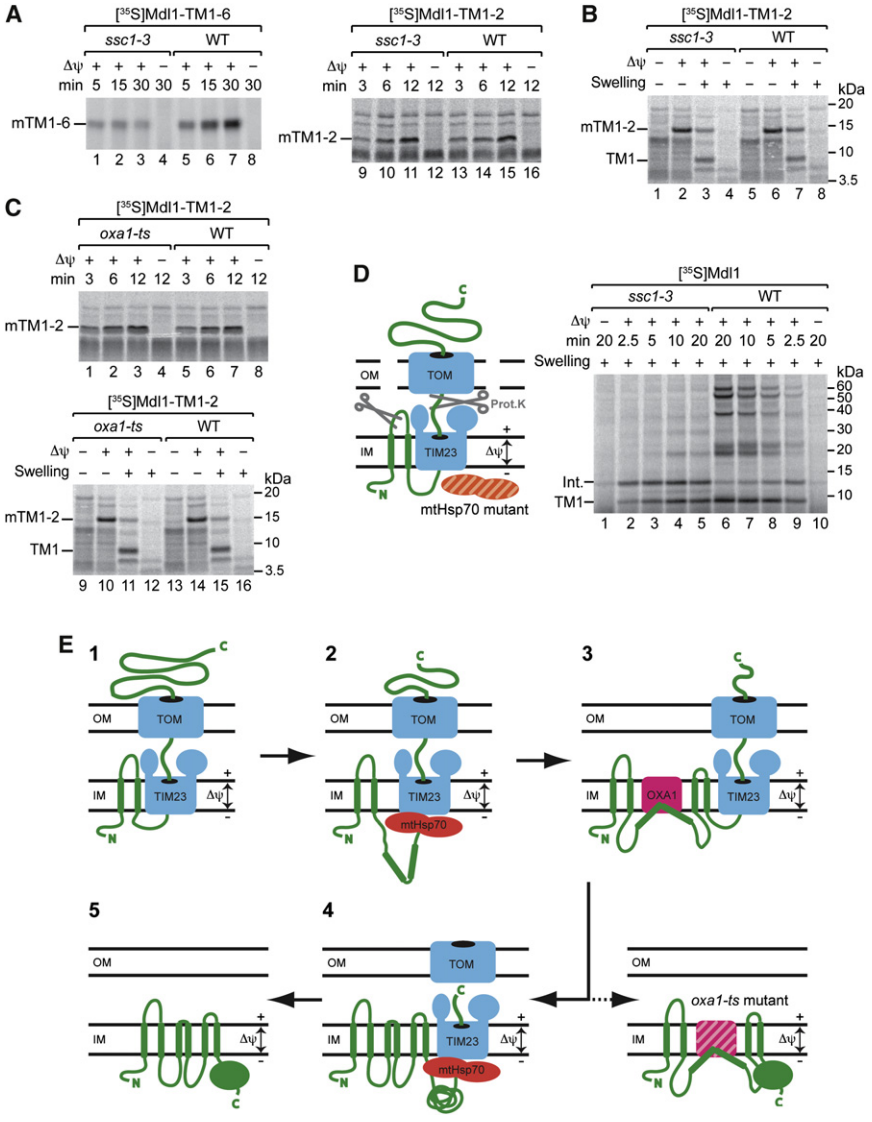

Figure 3. Integration of Transmembrane Segments 1 and 2 of Mdl1 into the Inner Membrane Is Independent of mtHsp70 and OXA

(A) Mdl1-TM1-6 and Mdl1-TM1-2 were imported into wild-type (WT) and *ssc1-3* mutant mitochondria, and treatment with proteinase K followed.

(B) Import of Mdl1-TM1-2 into wild-type and *ssc1-3* mitochondria was followed by topology mapping as described in Figure 2 and Experimental Procedures.

(C) Mdl1-TM1-2 was imported into wild-type and oxa1-*ts* mutant mitochondria (lanes 1-8) and subjected to proteolytic fragmentation (lanes 9-16).

(D) Import and topology mapping of full-length Mdl1 in wild-type and *ssc1-3* mitochondria. The import intermediate that accumulates in *ssc1-3* mutant mitochondria is depicted schematically. Abbreviations are as follows: OM, outer membrane; IM, inner membrane; and Int., fragment derived from an import intermediate of Mdl1 (under wild-type conditions, Int. shows the characteristic behavior of an intermediate, i.e., formation after short import times and decreasing intensity with increasing import time; in *ssc1-3* mitoplasts Int. accumulates, consistent with arrest of the preprotein in the import site).

(E) Hypothetical model for the biogenesis of Mdl1 (steps 1–5).

and TM2, was inserted into the inner membrane in an mtHsp70- and Oxa1-independent manner, fulfilling the characteristics of a protein following the stop-transfer pathway.

Does the sorting of full-length Mdl1 also involve a stop-transfer mechanism? The results we had obtained so far suggested that TM1 and TM2 were laterally released and correctly inserted into the inner membrane in *ssc1-3* mitochondria, whereas import of the subsequent portions (TM3 and TM4) should depend on mtHsp70 and thus portions of the preprotein should be stuck in the import site of the mutant mitochondria (Figure 3D, model). To test this prediction, we imported full-length Mdl1 into *ssc1-3* mitochondria, opened the outer membrane, and performed a fragmentation assay. The fragment TM1 was formed in a time- and Δψ-dependent manner (Figure 3D), indicating that inner-membrane insertion of the N-terminal portion of Mdl1 indeed does not require mtHsp70. Formation of the larger fragments was strongly inhibited (Figure 3D), demonstrating that Mdl1 was not fully imported.

Taken together, our findings indicate the following topogenesis pathway for the multi-spanning ABC transporter (Figure 3E). The first two transmembrane segments of Mdl1 are imported by a stop-transfer mechanism, i.e., lateral release from the TIM23 machinery without a dependence on mtHsp70. The subsequent TM3 and TM4 are translocated into the matrix in an mtHsp70-dependent manner and exported into the inner membrane by the OXA machinery (conservative sorting), thus identifying the ABC transporter as genuine nuclear-encoded substrate of the OXA translocase. TM5 and TM6 are OXA-independent and thus likely use the stop-transfer mechanism (though an OXA-independent export mechanism cannot be excluded). Why are TM3 and TM4 not arrested in the inner membrane like TM1 and TM2? TM3 is less hydrophobic, shorter than TM1 and TM2, and flanked by only a few charged amino acids. TM4 contains two proline residues that impair lateral sorting via the TIM23 machinery [11]. Together, these characteristics favor a transfer of TM3 and TM4 via the conservative-sorting route [3].

This study solves the controversial debate on the mechanism of mitochondrial protein sorting. Stop-transfer and conservative sorting are not conflicting views of mitochondrial protein biogenesis, but both mechanisms complement each other in the topogenesis of multispanning proteins. Two translocase complexes (TIM23, OXA) cooperate in the step-wise integration of a polytopic membrane protein. YidC, the Oxa1 homolog in bacteria, can mediate the insertion of proteins by itself or in cooperation with the Sec translocon [19, 27–29]. During evolution of mitochondria from a bacterial endosymbiont, the vast majority of mitochondrial genes were transferred to the nucleus, and the Sec translocon was lost in most species, whereas the OXA and YidC machinery was retained [2, 3, 8]. Since most mitochondrial proteins are imported from the eukaryotic cytosol, translocases such as the TIM23 complex have to transport preproteins in the opposite

direction from that of OXA and YidC, which export proteins from the mitochondrial matrix and bacterial cytosol, respectively. Thus, translocases that transport proteins in opposite directions cooperate in the insertion of a protein with complex topology. Our study reveals a coordinated mechanism of membrane integration that involves the modular insertion of distinct domains of a multispanning protein from different sides of the membrane.

Experimental Procedures

Import of Mdl1 into Isolated Yeast Mitochondria

Precursors of Mdl1 and derivatives were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. One import reaction typically contained 30–60 μg isolated mitochondria (protein amount) in 100 μl import buffer (3% [w/v] bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 10 mM MOPS-KOH [pH 7.2], 4 mM ATP, and 4 mM NADH). To minimize indirect effects of temperature-sensitive mutants, we grew cells at low temperature and induced phenotypes by in vitro heat shock of isolated mitochondria; mitochondria from *oxa1-ts*, *ssc1-3*, *tim54-16* or *tim17-5* mutants and corresponding wild-type mitochondria were incubated for 12–15 min at 37°C unless stated otherwise. Import was started by addition of 10% (v/v) reticulocyte lysate to the prewarmed import reaction mixture (25°C). The reaction was terminated by the addition of 8 μM antimycin A, 1 μM valinomycin, and 20 μM oligomycin and transfer on ice. Proteinase K (50 μg/ml) was added, and reactions were incubated for 15 min on ice. Proteinase K was inactivated by 2 mM phenylmethylsulfonyl fluoride (PMSF). Mitochondria were washed and resuspended in SEM buffer (250 mM sucrose, 10 mM MOPS [pH 7.2], and 1 mM EDTA), and proteins were precipitated by the addition of 15% trichloroacetic acid. Samples were heated to 60°C for 5 min and incubated on ice for 15 min. Precipitated proteins were recovered by centrifugation at 20,000 *g*, and pellets were washed with 1 M Tris base. Pellets were resuspended in Laemmli buffer by vigorous shaking at 37°C and subjected to SDS-PAGE. Alternatively, mitochondria were solubilized in digitonin-containing buffer and separated by blue native gradient gels [26].

Topology Mapping by Proteolytic Fragmentation of Membrane-Inserted Mdl1

The precursors of Mdl1 and derivatives were imported into isolated mitochondria. For hypo-osmotic swelling, mitochondria were resuspended in SEM buffer and diluted 1:10 or 1:20 with ice-cold EM buffer (10 mM MOPS [pH 7.2], and 1 mM EDTA). Control mitochondria were diluted with ice-cold SEM buffer (no swelling). After 10 min on ice, proteinase K (25 μg/ml) was added to swollen and control mitochondria. Proteinase K was inactivated by the addition of 2 mM PMSF. Proteins were precipitated and separated on a 12% NuPAGE gel (Invitrogen), and digital autoradiography followed. For generation of antibodies against Mdl1, rabbits were immunized with a synthetic peptide derived from the C-terminal NBD of Mdl1. The identity of the fragments generated by proteinase K was determined by a combination of in silico analysis with transmembrane topology prediction and a 3D model of Mdl1 [25], recognition by anti-NBD antibodies, size mapping using in vitro synthesized fragments, and mitochondrial import and analysis of Mdl1 constructs. Predicted fragments were synthesized by in vitro transcription and translation: TM2-6$_{NBD}$ (residues 148–695 of Mdl1 precursor), TM6$_{NBD}$ (residues 373–695 of Mdl1), TM2-5 (amino acids 148–362 of Mdl1), TM1 (amino acids 60–128 of Mdl1), TM2-6$_{NBD}$-loop (residues 148–710 of Mdl1-loop), TM4-6$_{NBD}$-loop (residues 267–710 of Mdl1-loop), and TM4-5-loop (residues 267–377 of Mdl1-loop). For mock controls, no external mRNA was added to the translation reaction.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online doi:10.1016/j.cub.2010.05.058.

Acknowledgments

We thank A. Chacinska, K. Wagner, and O. Schmidt for discussion. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 746, the Excellence Initiative of the German Federal and State Governments (EXC 294 *bioss*; GSC-4 Spemann Graduate School), the Gottfried Wilhelm Leibniz Program, Landesforschungspreis Baden-Württemberg, Fonds der Chemischen Industrie, and a Boehringer Ingelheim Fonds predoctoral fellowship (to M.B.).

Received: April 3, 2010
Revised: May 24, 2010
Accepted: May 25, 2010
Published online: June 17, 2010

References

1. Palmieri, F., Agrimi, G., Blanco, E., Castegna, A., Di Noia, M.A., Iacobazzi, V., Lasorsa, F.M., Marobbio, C.M., Palmieri, L., Scarcia, P., et al. (2006). Identification of mitochondrial carriers in *Saccharomyces cerevisiae* by transport assay of reconstituted recombinant proteins. Biochim. Biophys. Acta *1757*, 1249–1262.
2. Dolezal, P., Likic, V., Tachezy, J., and Lithgow, T. (2006). Evolution of the molecular machines for protein import into mitochondria. Science *313*, 314–318.
3. Neupert, W., and Herrmann, J.M. (2007). Translocation of proteins into mitochondria. Annu. Rev. Biochem. *76*, 723–749.
4. Oka, T., and Mihara, K. (2005). A railroad switch in mitochondrial protein import. Mol. Cell *18*, 145–146.
5. Ryan, M.T., and Hoogenraad, N.J. (2007). Mitochondrial-nuclear communications. Annu. Rev. Biochem. *76*, 701–722.
6. Alder, N.N., Jensen, R.E., and Johnson, A.E. (2008). Fluorescence mapping of mitochondrial TIM23 complex reveals a water-facing, substrate-interacting helix surface. Cell *134*, 439–450.
7. Koehler, C.M. (2004). New developments in mitochondrial assembly. Annu. Rev. Cell Dev. Biol. *20*, 309–335.
8. Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009). Importing mitochondrial proteins: machineries and mechanisms. Cell *138*, 628–644.
9. Endo, T., and Yamano, K. (2009). Multiple pathways for mitochondrial protein traffic. Biol. Chem. *390*, 723–730.
10. Glick, B.S., Brandt, A., Cunningham, K., Müller, S., Hallberg, R.L., and Schatz, G. (1992). Cytochromes c_1 and b_2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. Cell *69*, 809–822.
11. Meier, S., Neupert, W., and Herrmann, J.M. (2005). Proline residues of transmembrane domains determine the sorting of inner membrane proteins in mitochondria. J. Cell Biol. *170*, 881–888.
12. Chacinska, A., Lind, M., Frazier, A.E., Dudek, J., Meisinger, C., Geissler, A., Sickmann, A., Meyer, H.E., Truscott, K.N., Guiard, B., et al. (2005). Mitochondrial presequence translocase: Switching between TOM tethering and motor recruitment involves Tim21 and Tim17. Cell *120*, 817–829.
13. van der Laan, M., Meinecke, M., Dudek, J., Hutu, D.P., Lind, M., Perschil, I., Guiard, B., Wagner, R., Pfanner, N., and Rehling, P. (2007). Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. Nat. Cell Biol. *9*, 1152–1159.
14. Hartl, F.U., and Neupert, W. (1990). Protein sorting to mitochondria: Evolutionary conservations of folding and assembly. Science *247*, 930–938.
15. Herrmann, J.M., Neupert, W., and Stuart, R.A. (1997). Insertion into the mitochondrial inner membrane of a polytopic protein, the nuclear-encoded Oxa1p. EMBO J. *16*, 2217–2226.
16. Hell, K., Herrmann, J.M., Pratje, E., Neupert, W., and Stuart, R.A. (1998). Oxa1p, an essential component of the N-tail protein export machinery in mitochondria. Proc. Natl. Acad. Sci. USA *95*, 2250–2255.
17. Sato, T., and Mihara, K. (2009). Topogenesis of mammalian Oxa1, a component of the mitochondrial inner membrane protein export machinery. J. Biol. Chem. *284*, 14819–14827.
18. Hell, K., Neupert, W., and Stuart, R.A. (2001). Oxa1p acts as a general membrane insertion machinery for proteins encoded by mitochondrial DNA. EMBO J. *20*, 1281–1288.
19. Kuhn, A., Stuart, R., Henry, R., and Dalbey, R.E. (2003). The Alb3/Oxa1/YidC protein family: Membrane-localized chaperones facilitating membrane protein insertion? Trends Cell Biol. *13*, 510–516.
20. Fiumera, H.L., Broadley, S.A., and Fox, T.D. (2007). Translocation of mitochondrially synthesized Cox2 domains from the matrix to the intermembrane space. Mol. Cell. Biol. *27*, 4664–4673.

21. Funes, S., Nargang, F.E., Neupert, W., and Herrmann, J.M. (2004). The Oxa2 protein of *Neurospora crassa* plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family. Mol. Biol. Cell *15*, 1853–1861.
22. Reif, S., Randelj, O., Domanska, G., Dian, E.A., Krimmer, T., Motz, C., and Rassow, J. (2005). Conserved mechanism of Oxa1 insertion into the mitochondrial inner membrane. J. Mol. Biol. *354*, 520–528.
23. Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H.E., Schönfisch, B., Perschil, I., Chacinska, A., Guiard, B., et al. (2003). The proteome of *Saccharomyces cerevisiae* mitochondria. Proc. Natl. Acad. Sci. USA *100*, 13207–13212.
24. Young, L., Leonhard, K., Tatsuta, T., Trowsdale, J., and Langer, T. (2001). Role of the ABC transporter Mdl1 in peptide export from mitochondria. Science *291*, 2135–2138.
25. Zutz, A., Gompf, S., Schägger, H., and Tampé, R. (2009). Mitochondrial ABC proteins in health and disease. Biochim. Biophys. Acta *1787*, 681–690.
26. Wagner, K., Gebert, N., Guiard, B., Brandner, K., Truscott, K.N., Wiedemann, N., Pfanner, N., and Rehling, P. (2008). The assembly pathway of the mitochondrial carrier translocase involves four preprotein translocases. Mol. Cell. Biol. *28*, 4251–4260.
27. van Bloois, E., Haan, G.J., de Gier, J.W., Oudega, B., and Luirink, J. (2006). Distinct requirements for translocation of the N-tail and C-tail of the *Escherichia coli* inner membrane protein CyoA. J. Biol. Chem. *281*, 10002–10009.
28. Celebi, N., Yi, L., Facey, S.J., Kuhn, A., and Dalbey, R.E. (2006). Membrane biogenesis of subunit II of cytochrome *bo* oxidase: Contrasting requirements for insertion of N-terminal and C-terminal domains. J. Mol. Biol. *357*, 1428–1436.
29. du Plessis, D.J., Nouwen, N., and Driessen, A.J. (2006). Subunit a of cytochrome o oxidase requires both YidC and SecYEG for membrane insertion. J. Biol. Chem. *281*, 12248–12252.
30. Gompf, S., Zutz, A., Hofacker, M., Haase, W., van der Does, C., and Tampé, R. (2007). Switching of the homooligomeric ATP-binding cassette transport complex MDL1 from post-translational mitochondrial import to endoplasmic reticulum insertion. FEBS J. *274*, 5298–5310.

Current Biology Volume *20*

Supplemental Information

Cooperation of Stop-Transfer and Conservative Sorting Mechanisms in Mitochondrial Protein Transport

Maria Bohnert, Peter Rehling, Bernard Guiard, Johannes M. Herrmann, Nikolaus Pfanner, and Martin van der Laan

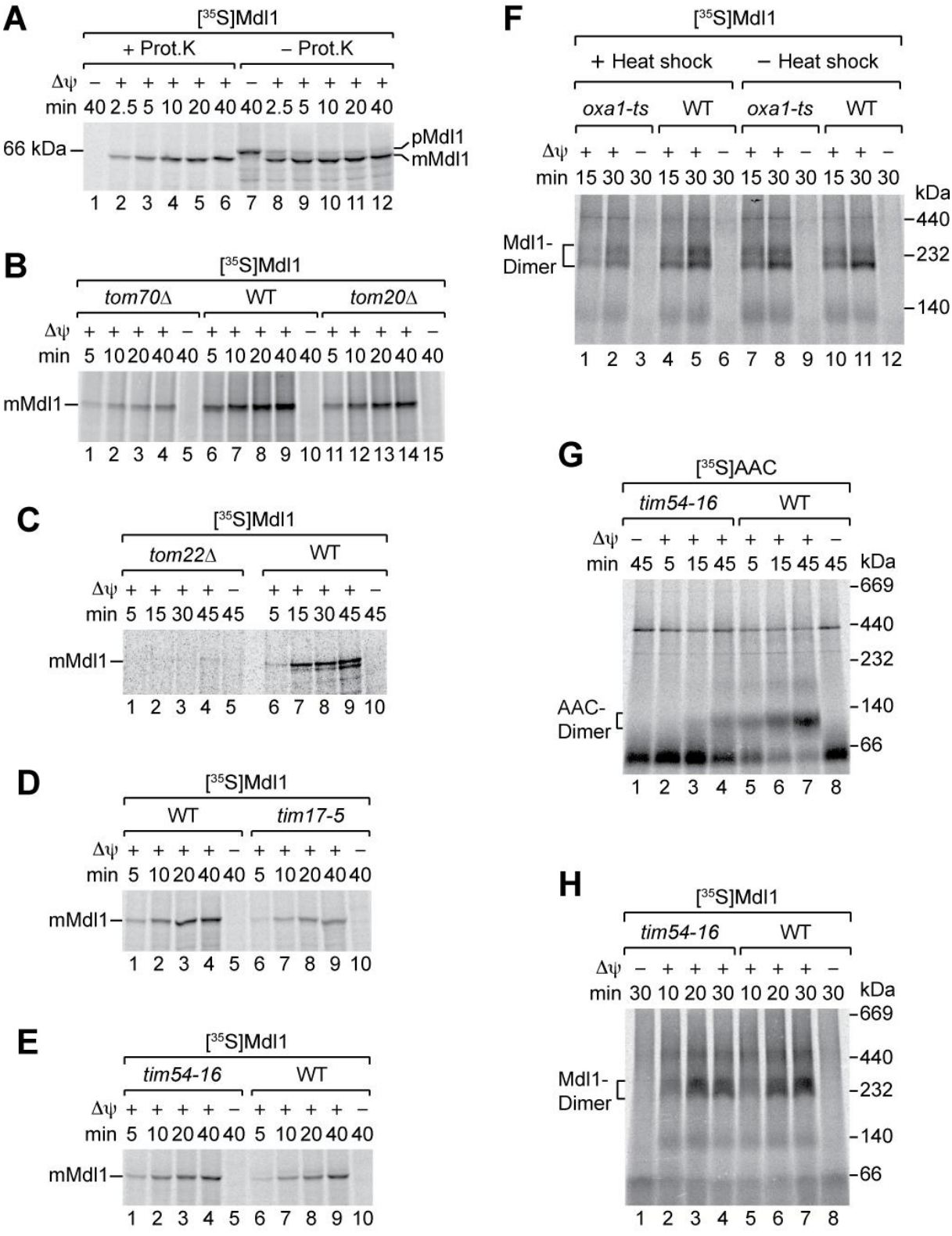

Figure S1. Import of Mdl1 into Mitochondria Involves the TOM and TIM23 Machineries. Related to Figure 1

(A) The radiolabeled precursor of Mdl1 was imported into wild-type mitochondria in the presence or absence of a membrane potential ($\Delta\psi$). Import reactions were stopped by the addition of antimycin A, oligomycin and valinomcyin and split into halves. One half was treated with 50 μg/ml proteinase K on ice (lanes 1-6), the other half was left untreated (lanes 7-12). Samples were subsequently analyzed by SDS-PAGE and digital autoradiography. The position of the 66 kDa marker band is indicated on the left. pMdl1, Mdl1 precursor; mMdl1, mature Mdl1.

(B) Import of [^{35}S]Mdl1 precursor into mitochondria isolated from yeast strains lacking either Tom70 (*tom70*Δ, lanes 1-5) or Tom20 (*tom20*Δ, lanes 11-15) and the corresponding wild-type mitochondria (WT, lanes 6-10) as described in (A). All samples were proteinase K-treated after import.

(C) [^{35}S]Mdl1 precursor was imported into mitochondria devoid of the central import receptor Tom22 (*tom22*Δ, lanes 1-5) and the corresponding wild-type mitochondria (WT, lane 6-10).

(D, E) Radiolabeled Mdl1 was imported into mitochondria isolated from the temperature-sensitive *tim17-5* (D) or *tim54-16* (E) yeast strains (the mitochondria were incubated for 15 min at 37°C prior to the import reaction at 25°C). After termination of the import reactions, Mdl1 precursor proteins not imported into mitochondria were digested with proteinase K (50 μg/ml). The samples were subjected to SDS-PAGE and digital autoradiography.

(F) Wild-type yeast and *oxa1-ts* mutant yeast were grown at 21°C. Mitochondria were isolated and either incubated for 12 min at 37°C (*in vitro* heat shock, samples 1-6) or not (no heat shock, samples 7-12). Radiolabeled Mdl1 precursor was imported into the mitochondria at 25°C. After proteinase K-treatment, mitochondria were re-isolated, solubilized in digitonin-containing buffer, separated by blue native electrophoresis and analyzed by digital autoradiography.

(G, H) Radiolabeled ADP/ATP carrier (AAC) (G) or Mdl1 (H) were imported into wild-type or *tim54-16* mutant mitochondria (after *in vitro* heat shock). After proteinase K-treatment, mitochondria were re-isolated and solubilized in digitonin-containing buffer. Mitochondrial extracts were applied to a blue native gradient gel (4-16% polyacrylamide). Radioactive protein complexes were detected by digital autoradiography.

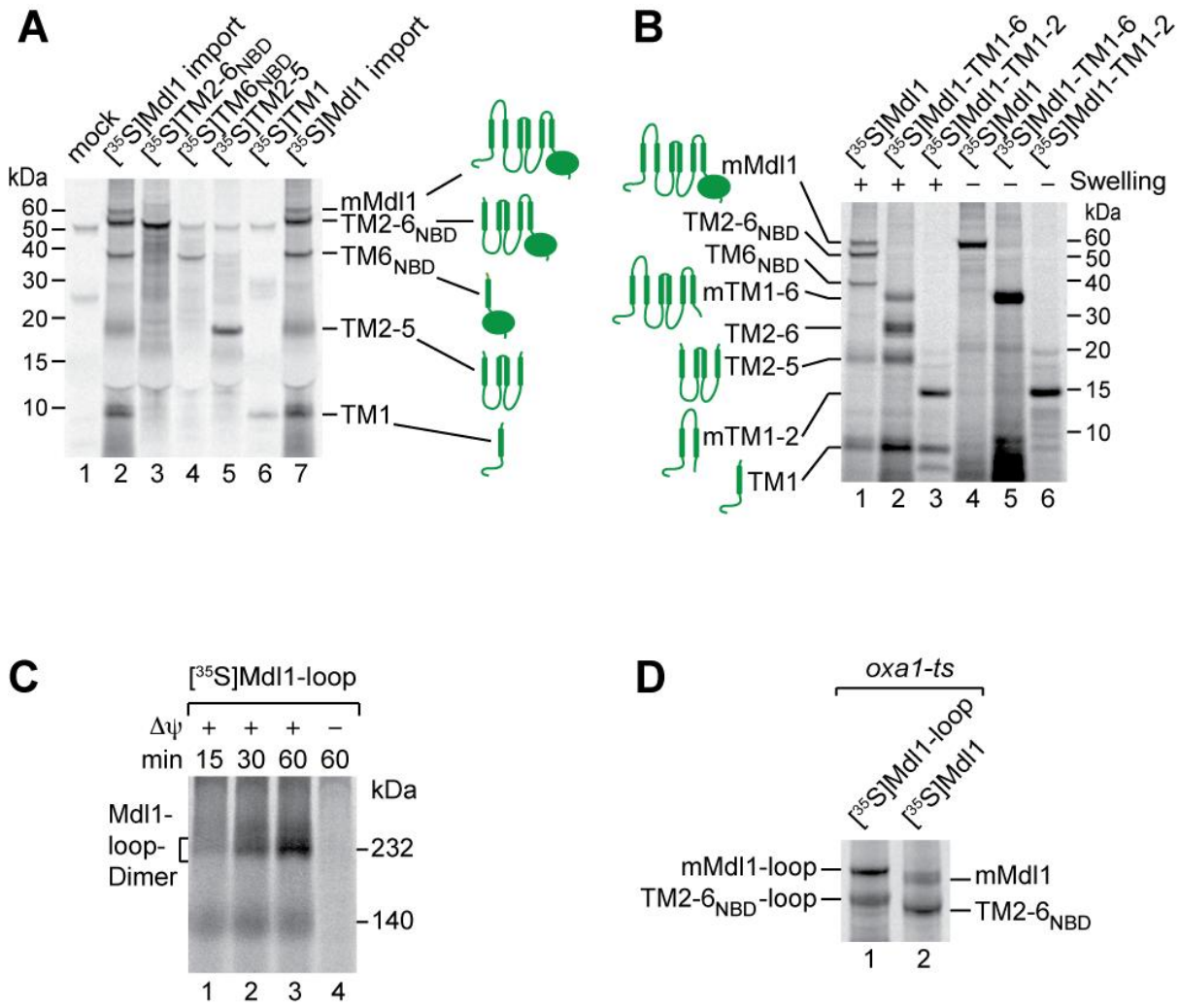

Figure S2. Identification of Proteolytic Fragments of Membrane-Inserted Mdl1 Generated in the Topology Mapping Assay. Related to Figure 2

(A) Isolated wild-type mitochondria were incubated with radiolabeled Mdl1 precursor (lanes 2 and 7). Mitochondria were resuspended in hypo-osmotic buffer to rupture the outer membrane and proteinase K (25 μg/ml) was added. Intermembrane space loops of membrane-inserted Mdl1 accessible to proteinase K were cleaved generating a characteristic fragment pattern. The predicted proteolytic fragments of Mdl1 were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine (lanes 3-6) and directly compared to the fragments generated in the topology mapping assay on a NuPAGE gel followed by digital autoradiography. A schematic representation of the identified proteolytic fragments of Mdl1 is shown on the right. mMdl1, mature Mdl1; TM, transmembrane segment; NBD, nucleotide binding

domain; mock, rabbit reticulocyte translation reaction not programmed with external RNA.

(B) Proteolytic fragments generated from membrane-inserted full-length and truncated Mdl1. ^{35}S-labeled full-length Mdl1 ([^{35}S]Mdl1), a C-terminally truncated form comprising the six transmembrane-spanning segments ([^{35}S]Mdl1-TM1-6), and a precursor protein consisting of the N-terminal two transmembrane segments ([^{35}S]Mdl1-TM1-2) were imported into wild-type mitochondria. Samples were subjected to hypo-osmotic swelling where indicated and further processed and analyzed as in (A). A ~8 kDa fragment was generated from full-length and both truncated versions of Mdl1, indicating that it represents the N-terminus and transmembrane segment 1 (TM1). A ~19 kDa fragment was generated from full-length Mdl1 and Mdl1-TM1-6, but not from Mdl1-TM1-2, indicating that it corresponds to transmembrane segments 2 to 5 (TM2-5) (see also (A)). A ~27 kDa fragment only generated from Mdl1-TM1-6 likely contains transmembrane segments 2 to 6 (TM2-6). The two larger fragments only formed from full-length Mdl1 contain the C-terminal NBD (see Figure 2A).

(C) [^{35}S]Mdl1-loop was imported into wild-type mitochondria. After proteinase K-treatment, mitochondria were re-isolated and subjected to blue native electrophoresis and digital autoradiography.

(D) [^{35}S]Mdl1-loop (lane 1) and [^{35}S]Mdl1 (lane 2) were imported into isolated *oxa1-ts* mitochondria after induction of the temperature-sensitive phenotype *in vitro*. Upon hypo-osmotic swelling and proteinase K-treatment (25 μg/ml), samples were subjected to SDS-PAGE and digital autoradiography.

Supplemental Experimental Procedures

Yeast Strains and Growth Conditions

Saccharomyces cerevisiae mutants *oxa1-ts*, *ssc1-3*, *tom70*Δ, *tom20*Δ, *tom22*Δ, *tim17-5*, and *tim54-16* [12, 26, S1-S4] and the corresponding wild-type strains have been described before. Mutant and corresponding wild-type strains were grown at 21°C - 23°C (*oxa1-ts*, *tim17-5*, *tim54-16*, *tom70*Δ, *tom20*Δ, *tom22*Δ) or 19°C (*ssc1-3*) in YPG medium (1% [w/v] yeast extract, 2% [w/v] peptone, and 3% [w/v] glycerol) or - in the case of *tom22*Δ - YPS medium (1% [w/v] yeast extract, 2% [w/v] peptone, and 2% [w/v] sucrose) and mitochondria were isolated.

Generation of Mdl1-Derived Precursor Proteins

For *in vitro* transcription/translation the open reading frame (ORF) encoding the Mdl1 precursor protein (695 amino acid residues) was amplified from genomic DNA of the *S. cerevisiae* strain YPH499 and cloned into pGEM4Z (Promega) downstream of the SP6 promoter using the restriction enzymes *Eco*RI and *Hind*III resulting in plasmid pGEM4Z-MDL1. For generation of the Mdl1-loop precursor (710 amino acid residues), an *Xba*I restriction site was first introduced into the *MDL1* ORF (5'-*TCTAGA*-3', nucleotides 757-762) by PCR mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). This modification led to the amino acid substitutions W253S and K254S at the tip of the intermembrane space loop II. The resulting plasmid was termed pGEM4Z-MDL1-*Xba*I. Two complementary oligonucleotides encoding the amino acid sequence of the intermembrane space loop III (15 amino acid residues) were synthesized with *Xba*I overhangs. Oligonucleotides were mixed, heated to 95°C and slowly cooled down to room temperature for annealing. The resulting double-stranded DNA fragment was ligated into *Xba*I-digested pGEM4Z-MDL1-*Xba*I to obtain pGEM4Z-MDL1-loop, which was used for *in vitro* transcription/translation. For generation of Mdl1-TM1-2, primers were designed to amplify plasmid pGEM4Z-MDL1 except for the part of the *MDL1* ORF that encodes amino acids 195-695. Both primers were flanked by *Hind*III sites and the forward primers introduced a stop codon after codon 194. The PCR product obtained with these primers was digested with *Hind*III and circularized by ligation of *Hind*III overhangs. This procedure led to the generation of a truncated *MDL1* ORF in

pGEM4Z encoding amino acid residues 1 to 194 (pGEM4Z-MDL1-TM1-2). The precursor protein synthesized from this plasmid consists of the N-terminal matrix domain including the presequence followed by TM1 and TM2 with the connecting intermembrane space loop I and a stretch of 17 further amino acids that is highly positively charged and potentially important for the topology of TM2. A pGEM4Z plasmid encoding *MDL1*-TM1-6 (pGEM4Z-MDL1-TM1-6) was generated via the same strategy. The resulting precursor protein Mdl1-TM1-6 (amino acids 1 to 420) consists of the entire transmembrane domain with TM1 – TM6 and 27 further amino acids of the C-terminal NBD.

Monitoring of Inner Membrane Insertion of Mdl1 by Proteolytic Fragmentation

We established a proteolytic fragmentation assay to determine the transmembrane topology of radiolabeled imported Mdl1 upon rupture of the outer membrane by hypo-osmotic swelling (generation of mitoplasts [S5]). Sufficiently large intermembrane space loops of Mdl1 that connect transmembrane segments are accessible to externally added proteases after swelling. Fragments generated by this treatment can be visualized by autoradiography as long as they contain at least one methionine residue. This amino acid is supplied to the *in vitro* translation reaction in the ^{35}S-labeled form. Mdl1 contains multiple methionines distributed throughout the entire sequence. Fragments containing the C-terminal NBD were identified by comparison with proteolytic fragments of Mdl1 that were detected by an antibody against a C-terminal peptide in a Western Blot analysis (Figure 2A, lanes 3 and 4). These fragments were generated by proteolytic cleavage in the intermembrane space loop I between TM1 and TM2 (leading to TM2-6$_{NBD}$) and (additionally) loop III between TM5 and TM6 (leading to TM6$_{NBD}$). The size of the proteolytic fragments corresponded well to the size of the respective *in vitro* synthesized fragments of Mdl1 (Figure S2A, lanes 2-4).

To analyze further fragments obtained in the proteolytic assay, we imported a C-terminally truncated Mdl1 lacking the NBD, termed TM1-6. After swelling and protease treatment, the gel mobility of the two smaller fragments was unchanged (Figure S2B, lane 2 compared to lane 1), indicating that they did not contain the C-terminal NBD (TM2-6$_{NBD}$ and TM6$_{NBD}$ were larger than TM1-6 and of course not generated from TM1-6). The size of the two smaller fragments corresponded to the

size predicted for TM1 (plus the matrix-exposed N-terminus of the mature protein) and TM2-5. *In vitro* synthesis of the two predicted fragments revealed a similar gel mobility as the fragments generated in mitoplasts (Figure S2A, lanes 5-7). To further analyze the predicted TM1 fragment, we generated a smaller Mdl1 precursor that contained TM1, TM2 and the complete amino-terminus with the presequence, but lacked TM3-6 and the NBD. The resulting TM1-2 precursor protein was imported and led to the generation of a fragment with the same gel mobility as TM1 generated from full-length Mdl1 (Figure S2B, lane 3 compared to lane 1), indicating that TM1 is indeed generated from the amino-terminal region of Mdl1. We conclude that the four major fragments of Mdl1 were identified with high confidence by the combination of size mapping (based on cleavage of loops I and III) and assignment to C-terminal and non C-terminal portions of Mdl1. Further fragments derived from Mdl1 constructs were tentatively assigned according to the their size (TM2-6 and TM2-3/TM4-5).

In wild-type Mdl1, the intermembrane space loop between TM3 and TM4 (loop II) is too small to be cleaved by proteases. According to homology modeling based on the X-ray structure of the bacterial homolog Sav1866, loop II is predicted to form the shortest possible turn between two TMs and to consist of only two amino acid residues [25]. In order to monitor the membrane integration of TM3 and TM4 we inserted the amino acid sequence that forms loop III between TM3 and TM4 (the resulting precursor protein was termed Mdl1-loop). At its native location between TM5 and TM6 the positioning of loop III in the intermembrane space does not depend on Oxa1, since the protease accessibility of loop III is not affected in *oxa1-ts* mutant mitochondria (see Figure 2B, generation of fragments TM2-5 and $TM6_{NBD}$). Herrmann and Bonnefoy [S6] showed that repositioning of an intermembrane space domain within a protein sequence does not influence its dependence on Oxa1, indicating that the insertion of loop III between TM3 and TM4 does not artificially induce a dependence on the OXA translocase. In the proteolytic fragmentation assay, $TM2\text{-}6_{NBD}$ generated from Mdl1-loop should contain the extended loop in the middle portion and thus should be larger than $TM2\text{-}6_{NBD}$ generated from wild-type Mdl1. To test this prediction, we used a gel with high resolution in the 50-60 kDa range and directly compared the two forms of $TM2\text{-}6_{NBD}$ (Figure S2D). Indeed, Mdl1-loop and the corresponding $TM2\text{-}6_{NBD}$-loop were larger than Mdl1 and $TM2\text{-}6_{NBD}$, respectively.

Supplemental References

S1. Preuss, M., Leonhard, K., Hell, K., Stuart, R.A., Neupert, W., and Herrmann, J.M. (2001). Mba1, a novel component of the mitochondrial protein export machinery of the yeast *Saccharomyces cerevisiae*. J. Cell Biol. *153*, 1085-1096.

S2. Gambill, B.D., Voos, W., Kang, P.J., Miao, B., Langer, T., Craig, E.A., and Pfanner, N. (1993). A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. J. Cell Biol. *123*, 109-117.

S3. Hönlinger, A., Kübrich, M., Moczko, M., Gärtner, F., Mallet, L., Bussereau, F., Eckerskorn, C., Lottspeich, F., Dietmeier, K., Jacquet, M., et al. (1995). The mitochondrial receptor complex: Mom22 is essential for cell viability and directly interacts with preproteins. Mol. Cell. Biol. *15*, 3382-3389.

S4. van Wilpe, S., Ryan, M.T., Hill, K., Maarse, A.C., Meisinger, C., Brix, J., Dekker, P.J., Moczko, M., Wagner, R., Meijer, M., et al. (1999). Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase. Nature *401*, 485-489.

S5. Stojanovski, D., Pfanner, N., and Wiedemann, N. (2007). Import of proteins into mitochondria. Meth. Cell Biol. *80*, 783-806.

S6. Herrmann, J. M., and Bonnefoy, N. (2004). Protein export across the inner membrane of mitochondria: the nature of translocated domains determines the dependence on the Oxa1 translocase. J. Biol. Chem. *279*, 2507-2512.