SUPPLEMENTARY METHODS

Plasmids, strains, and growth conditions. To construct a series of pRS316-based plasmids harboring Om45-FLAG variants, we first constructed a vector with the 5'-UTR of *OM45*, a DNA fragment encoding the FLAG tag and termination codon just after unique *Xho*I and *Bam*HI sites, and the 3'-UTR of *OM45*. The appropriate fragments were amplified and joined by PCR, and the resulting DNA fragment was inserted into pRS316 to obtain the pRS316-OM45-FLAG vector. We then amplified DNAs for Om45, Om45-50, Om45-100 and Om45-150 with appropriate sets of primers, and the resulting fragments were inserted into the *Xho*I and *Xba*I sites of a pTNT vector (Promega) to construct plasmids for in vitro transcription/translation. At this stage, each ORF ends with a termination codon just after the *Bam*HI site and just before the *Xba*I site. After confirming the sequences, Om45-related fragments were separated by digesting those plasmids with *Xho*I and *Bam*HI, and the resulting fragments were inserted in the same sites of the pRS316-OM45-FLAG vector, yielding pRS316-OM45-FLAG, pRS316-OM45-50-FLAG, pRS316-OM45-100-FLAG, and pRS316-OM45-150-FLAG for Om45-FLAG, Om45-50-FLAG, Om45-100-FLAG, and Om45-150-FLAG, respectively.

pTNT-Om45 and Om45Δ10, Om45Δ20 or Om45Δ40, plasmids for in vitro translation of full-length and N-terminally truncated versions of Om45, were constructed as follows. DNA fragments coding Om45 or Om45 lacking either N-terminal 10, 20 or 40 residues was amplified by PCR using genomic DNA as a template and inserted into the *Xho*I/*Xba*I site of the pTNT-vector. pTNT-Om45-Om45-DHFR, a plasmid for in vitro translation of Om45-Om45-DHFR fusion protein was constructed as follows. A DNA fragment for Om45 without stop codon was amplified by PCR and inserted into the *Bam*HI/*Xho*I site of pGEM-4Z-DHFR [1]. A DNA fragment for Om45 without a stop codon was cloned into the *Xho*I/*Kpn*I site of the pTNT-vector, resulting in pTNT-OM45-w/o stop. A DNA fragment for Om45-DHFR was amplified from pGEM-4Z-Om45-DHFR and inserted into the *Kpn*I/*Xba*I site of pTNT-Om45-w/o stop.

pTNT-Tom70N-Om45 and Om45-100 plasmids for in vitro translation of fusion proteins consisting of the N-terminal 30 residues of Tom70 (Tom70N) and Om45 lacking the N-terminal 30 residues (Om45) or residues 31-100 of Om45 (Om45-100) were constructed as follows. A DNA fragment for Tom70N-Om45 or Tom70N-Om45-100 was amplified by PCR using a long primer including Tom70N as a forward primer, and was cloned into the *Xho*I/*Xba*I site of the pTNT-vector.

To construct plasmids for expression of C-terminally FLAG-tagged Tom70N-Om45 (Tom70N-Om45-FLAG) or Tom70N-Om45-100 (Tom70N-Om45-100-FLAG), a DNA fragment for Tom70N-Om45 or Tom70N-Om45-100 was amplified from pTNT-Tom70N-Om45 and was cloned into pIZ7 [2].

Yeast strains used in this study are W303-1A [3], *tim23-71,78*, *tim50-279,282,286* [4], 20T, 22T [5], GAL-TIM10 [6], GAL-TIM22 [1], GAL-TIM40 [7], PK81, PK82, PK83 [8], and *tom70*Δ*tom71*Δ [9] The genes for Tom40-HA and Tom40-FLAG were constructed by homologous recombination of W303-1A with the *3×HA-CgHIS3* or *3×FLAG-CgHIS3* gene cassettes amplified from pTYE249 or pTYE247, respectively [10].

Yeast cells were grown in YPGal (1% yeast extract, 2% polypeptone, and 2% galactose), YPD (1% yeast extract, 2% polypeptone, and 2% glucose), SCD (0.67% yeast nitrogen base without amino acids, 0.5% vitamin assay casamino acids, and 2% glucose) media with appropriate supplements. To isolate mitochondria for in vitro import unless otherwise stated, cells were cultured in lactate medium (0.36% yeast extract, 0.05% glucose, 0.06% $CaCl_2$, 0.06% NaCl, 0.22% $MgCl_2$, 0.36% KH_2PO_4, 0.36% NH_4Cl, 2.2% lactate).

Protease digestion. A hundred or 200 μg of mitochondria were suspended in 185 μl of SEM buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2, 1 mM EDTA). Then 5 μl of 5 mg/ml proteinase K (PK) or trypsin as well as 5 μl of 8% Triton X-100 or SEM buffer was added to the sample and incubated for 20 min on ice. Reaction was terminated by addition of 2 mM PMSF for PK or 0.1mg/mL Trypsin Inhibitor (SIGMA, 10,000 BAEE units/mg protein) and 2 mM PMSF for trypsin. To rupture the OM by osmotic swelling, mitochondria were suspended in EM buffer (10 mM MOPS-KOH,

pH 7.2, 1 mM EDTA), and rotated for 1 h at 4°C. The resulting mitoplasts were collected by centrifugation at 22,000 × *g* for 10 min at 4°C, resuspended in EM buffer, and subjected to protease treatment. After protease digestion, 1 ml of 10% trichloroacetic acid was added to precipitate proteins. Proteins were washed with ice-cold acetone and analyzed by SDS-PAGE and immunoblotting.

TEV protease treatment. One hundred fifty μg of 20T and 22T mitochondria were incubated in TEV buffer (250 mM sucrose, 20 mM MOPS-KOH, pH 7.2, 0.1 mg/ml BSA, 50 mM NaCl) with or without 48 μg and 60 μg of TEV protease (Sigma), respectively, at 30°C for 20 min. After washing with SEM buffer, the mitochondria were resuspended in Import buffer (3% BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS-KOH, pH 7.2, 5 mM methionine, 2 mM KH_2PO_4, 2 mM ATP, 2 mM NADH, 120 μg/ml creatine kinase, and 12 mM creatine phosphate) and used for *in vitro* import.

***In vitro* protein import.** Import of radiolabeled proteins into isolated mitochondria was performed as described previously [4]. Briefly, translation products were incubated with isolated mitochondria in Import buffer. Import reaction was stopped by addition of 10 μg/ml valinomycin or 500 μl ice-cold SEM buffer containing 50 μg/ml PK. Mitochondria were re-isolated by centrifugation and washed with SEM buffer. Proteins were analyzed by SDS-PAGE and radioimaging with a Typhoon 9200 image analyzer (GE Healthcare). To dissipate $\Delta\Psi$, mitochondria were preincubated with 10 μg/ml valinomycin or 100 μM CCCP. Treatment of mitochondria with Na_2CO_3 or Triton X-100 was performed as described previously [7].

Crosslinking. ^{35}S-labeled Om45-Om45-DHFR was incubated with mitochondria for 10 min at 25°C. Mitochondria were collected by centrifugation, washed with SEM buffer and then suspended in SEM buffer. One twelfth of the sample was mock-treated without DSG as a control. The rest of the sample was incubated with 200 μM DSG for 30 min on ice. DSG was quenched with 50 mM Tris-HCl, pH 7.5. One eleventh of the DSG-treated sample was left for a control. Mitochondria in the remaining sample were pelleted by centrifugation and were boiled in 50 μl of 1% SDS

buffer (1% SDS, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl). After a clarifying spin, the supernatant was diluted with 950 µl of 0.5% Triton buffer (0.5% Triton-X100, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and subjected to immunoprecipitation with antibodies against Tom40, anti-FLAG agarose resin (Sigma) or anti-HA antibody beads (Wako) overnight at 4°C. To precipitate anti-Tom40 antibodies, 10 µl of protein A-Sepharose beads were added to the sample. The beads were washed with 1 ml of 0.5% Triton buffer three times. The pulled-down proteins were eluted by boiling in SDS sample buffer and analyzed by SDS–PAGE and radioimaging.

Miscellaneous. Immunofluorescence microscopy observation was performed as described previously [11]. Generation of OM and IM vesicles and their separation were carried out as described previously [12].

1. Yamano K, Ishikawa D, Esaki M, Endo T (2005) Phosphate carrier has an ability to be sorted to either the TIM22 pathway or TIM23 pathway for its import into yeast mitochondria. *J Biol Chem* **280**: 10011-10017

2. Izawa T, Tsuboi T, Kuroha K, Inada T, Nishikawa S, Endo T (2012) Roles of Dom34:Hbs1 in nonstop protein clearance from translocators for normal organelle protein influx. *Cell reports* **2:** 447-453

3. Thomas BJ, Rothstein RJ (1989). Elevated recombination rates in transcriptionally active DNA, *Cell* **56**: 619–630

4. Tamura Y, Harada Y, Shiota T, Yamano K, Watanabe K, Yokota M, Yamamoto H, Sesaki H, Endo T (2009). Tim23–Tim50 pair coordinates functions of translocators and motor proteins in mitochondrial protein import. *J Cell Biol* **184**: 129–141

5. Yamano K, Yatsukawa Y, Esaki M, Aiken-Hobbs AE, Jensen RE, Endo T (2008) Tom20 and Tom22 share the common signal recognition pathway in mitochondrial protein import. *J Biol Chem* **283**: 3799–3807

6. Shiota T, Maruyama M, Miura M, Tamura Y, Yamano K, Esaki M, Endo T (2012) The Tom40 assembly process probed using the attachment of different intra-mitochondrial sorting signals. *Mol Biol Cell* **23**: 3936-3947

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

8. Gambill BD, Voos W, Kang PJ, Miao B, Langer T, Craig EA, Pfanner N (1993) A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. *J Cell Biol* **123**: 109–117

9. Kondo-Okamoto N, Shaw JM, Okamoto K (2008) Tetratricopeptide repeat proteins Tom70 and Tom71 mediate yeast mitochondrial morphogenesis. EMBO reports 9: 63-69

10. Yoshihisa T, Yunoki-Esaki K, Ohshima C, Tanaka N, Endo T (2003) Possibility of cytoplasmic pre-tRNA splicing: the yeast tRNA splicing endonuclease mainly localizes on the mitochondria. Molecular biology of the cell 14: 3266-3279

11. Nishikawa S, Hirata A, Nakano A (1994) Inhibition of endoplasmic reticulum (ER)-to-Golgi transport induces relocalization of binding protein (BiP) within the ER to form the BiP bodies. *Mol Biol Cell* **5:** 1129–1143

12. Tamura Y, Onguka O, Itoh TK, Endo T, Iijima M, Claypool SM, Sesaki H (2012) Phosphatidylethanolamine biosynthesis in mitochondria: Phosphatidylserine (PS) trafficking is independent of a PS decarboxylase and intermembrane space proteins Ups1p and Ups2p. *J Biol Chem* **287**: 43961–43971