Dual Role of Mitofilin in Mitochondrial Membrane Organization and Protein Biogenesis

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INVENTORY OF SUPPLEMENTAL INFORMATION

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Predicted Domains of MINOS Components, Related to Figures 1 and 3
Schematic representation of MINOS proteins. The number of predicted amino acid residues is indicated in the panel ‘Domain structure’ (including the presequence in case of mitofilin/Fcj1). The predicted molecular masses of the mature proteins are shown in the right panel (without presequence). Fcj1 carries an N-terminal mitochondrial targeting sequence (Rabl et al., 2009; Vögtle et al., 2009), followed by a predicted transmembrane segment, a central coiled-coil region and a conserved C-terminal mitofilin domain (Rabl et al., 2009). Mio10 has two predicted transmembrane segments connected by a short positively charged loop and has been highly conserved in evolution (28.4% identity / 45.1% similarity between S. cerevisiae Mio10 and the human UPF0327 protein c1orf151, termed hMIO10 [isoform a]). Aim5 contains a predicted transmembrane segment near the N-terminus. For Aim13 a central coiled-coil domain is predicted and a C-terminal CHCH motif-like domain with two conserved cysteine residues that align with the inner disulfide pair of classical CHCH motifs that contain four cysteine residues (Cavallaro, 2010). Aim37 has two predicted transmembrane segments and a C-terminal putative coiled-coil domain. Mio27 has two predicted transmembrane segments. Aim37 and Mio27 are homologous to each other (17.4% identity / 41.3% similarity) and to C. elegans MOMA-1 (10.6% identity / 30.1% similarity with Aim37 and 15.7% identity / 33.2% similarity with Mio27).

Figure S2. Steady-State Level and Redox State of Mia40 are not Affected in fcj1Δ Mitochondria, Related to Figure 2
(A) Steady-state levels of the indicated proteins in wild-type (WT) and fcj1Δ mutant mitochondria, analyzed by NuPAGE and immunodecoration.
(B) Redox state of Mia40. Wild-type and fcj1Δ mitochondria were subjected to denaturation in the presence of the reducing agent DTT (50 mM) or the thiol-modifying
agent iodoacetamide (IA, 50 mM) in Laemmli buffer at 60°C. Samples were analyzed by immunoblotting with Mia40-specific antibodies. Part. reduced, partially reduced.

**Figure S3. Protein Levels and Interactions in MINOS Mutant Mitochondria, Related to Figure 4**

(A) Serial dilutions of wild-type and mutant yeast strains were grown on synthetic complete medium containing 3% glycerol at 23°C or 30°C.

(B) Steady state protein levels of wild-type (WT), aim5Δ and aim37Δ mitochondria were analyzed by SDS-PAGE and Western blotting.

(C, D) Protein levels of wild-type mitochondria compared to aim13Δ or mio27Δ mitochondria were analyzed as described in (B).

(E) After solubilization with digitonin, wild-type, Fcj1ProtA and Fcj1ProtA mio10Δ mitochondria were subjected to IgG affinity chromatography. Bound proteins were eluted by TEV protease cleavage and analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. Load, 4%; eluate, 100%.

(F) Wild-type, Fcj1ProtA, Fcj1ProtA aim5Δ and Fcj1ProtA aim13Δ mitochondria were processed and analyzed as described in (E). Arrowhead, unspecific band.

(G) Wild-type, Aim5ProtA and Fcj1ProtA mitochondria were lysed with digitonin and separated by blue native electrophoresis. The TOM complex was decorated with antibodies directed against Tom40.

**Figure S4. Mitochondrial Morphology in MINOS Mutant Cells, Related to Figure 5**

(A) Wild-type (WT) yeast and indicated mutant cells grown in YPG medium were stained with 100 ng/ml 3,3’-dihexyloxacarbocyanine iodide (DiOC6) and observed with an Olympus BX61 microscope using an EGFP ET Filterset (Bandpass) from AHF Analysentechnik (excitation filter bandpass 470/40, emission filter bandpass 525/50, dichroic mirror 495 LP) and recorded with a high resolution CCD-Camera (F-View II). Bar, 5 µm.
(B) Isolated wild-type, *fcj1Δ* and *mio10Δ* mitochondria were lysed with Triton X-100 and treated with proteinase K or left untreated. Proteins were precipitated by TCA and analyzed by SDS-PAGE and Western blotting.

(C) Mitochondria isolated from wild-type, *mio10Δ* and *fcj1Δ* cells were incubated for 1 h at 37°C in the presence or absence of Bax (and tBid). Release of soluble intermembrane space proteins was monitored by separation of pellet (P) and supernatant (S) fractions, SDS-PAGE and immunoblotting. T, Total.

**Figure S5. Characterization of Ribosome-Nascent Chain Complexes (RNCs) formed by Tim9, Related to Figure 7**

(A) Tim9-RNCs were pelleted by centrifugation and treated with puromycin or RNase A as indicated. The samples were analyzed by non-reducing NuPAGE followed by autoradiography.

(B) Wild-type (WT) mitochondria were incubated with Tim9 or Tim9-RNC-127 for 5-10 min. Where indicated, samples were treated with DTT, proteinase K, RNase A or precipitated with hexadecyltrimethylammonium bromide (CTAB). Analysis was performed as described for (A).

(C) Mitochondria isolated from wild-type and *mia40-3* yeast cells were incubated with Tim9 or Tim9-RNCs for 10 min and analyzed as described for (A).

(D) Tim9-RNC-96 containing different combinations of cysteine-to-serine exchanges as indicated were imported into mitochondria for 5 min and analyzed as described for (A).

(E) Hypothetical model of MINOS and its role in inner membrane organization and protein biogenesis of mitochondria. OM, outer mitochondrial membrane; IMS, intermembrane space; IBM, inner boundary membrane.
Table S1. Proteins Identified in SILAC Affinity-Purification Mass Spectrometry Experiments Using Mitofilin/Fcj1 as Bait, Related to Figures 1 and 3

Mitochondria were isolated from *S. cerevisiae* cells expressing Fcj1ProtA grown in the presence of standard amino acids and from wild-type *S. cerevisiae* cells that were labeled by $[^{13}\text{C}_6/^{15}\text{N}_2]\text{lysine}$ and $[^{13}\text{C}_6/^{15}\text{N}_4]\text{arginine}$. After solubilization of the mitochondria with digitonin and IgG affinity chromatography, the elution fractions were combined and analyzed by mass spectrometry. Data listed were obtained by processing mass spectrometric data of triplicate experiments with the software MaxQuant (version 1.0.13.13; Cox and Mann, 2008). For identified proteins, the ratio of ‘light’ peptides ($^{12}\text{C}/^{14}\text{N}$; Fcj1ProtA mitochondria) over ‘heavy’ peptides ($^{13}\text{C}/^{15}\text{N}$; wild-type mitochondria) was determined. Potential Fcj1 interaction partners were required to have a sequence coverage of $\geq 5\%$ and a posterior error probability (PEP) of $\leq 0.01$. Proteins identified and quantified with $\geq$ three unique peptides and $\geq$ two SILAC pairs (Ratio L/H Count) in each replicate and exhibiting a normalized protein abundance ratio of $\geq 15$ determined over all replicates were considered candidate proteins of Category I. Further proteins with normalized abundance ratios of $\geq 10$ across all replicates identified and quantified based on at least two unique peptides and SILAC pairs in each experiment were classified as Category II candidates. See Experimental Procedures and Supplemental Experimental Procedures.
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von der Malsburg et al., Figure S1
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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids

Saccharomyces cerevisiae strains are derivatives of YPH499 or BY4741. Yeast strains expressing Fcj1 or Aim5 with a C-terminal Protein A-tag were generated by homologous recombination using a cassette that encodes the Protein A moiety, a Tobacco Etch Virus (TEV) protease cleavage sequence and a HIS3 marker gene. The deletion strains \textit{fcj1\textDelta}, \textit{mio10\textDelta}, \textit{aim5\textDelta}, \textit{aim13\textDelta}, \textit{aim37\textDelta} and \textit{mio27\textDelta}, in which the indicated ORFs had been replaced by a \textit{kanMX4} module, were obtained from Euroscarf (Frankfurt). The deletion cassettes including the flanking regions were amplified from genomic DNA of these strains and transformed into the Fcj1\textsubscript{ProtA} strain yielding Fcj1\textsubscript{ProtA} mio10\textDelta, Fcj1\textsubscript{ProtA} aim5\textDelta, Fcj1\textsubscript{ProtA} aim13\textDelta, Fcj1\textsubscript{ProtA} aim37\textDelta and Fcj1\textsubscript{ProtA} mio27\textDelta, respectively. For overexpression of Fcj1, a PCR fragment containing the \textit{FCJ1} ORF together with its promoter and terminator regions was cloned into plasmid pRS426, yielding pRS-\textit{FCJ1} (pAC-18-3; 1807). The resulting plasmid and the empty control vector were transformed into YPH499. The strain expressing Mia40 fused to a C-terminal decahistidine tag and the strain \textit{mia40-3} have been described (Chacinska et al., 2004; Milenkovic et al., 2007; Stojanovski et al., 2008).

Growth of Yeast Cells

For isolation of mitochondria, \textit{S. cerevisiae} cells were grown on YPG medium (1\% [w/v] yeast extract, 2\% [w/v] bacto-peptone, 3\% [v/v] glycerol) at 19, 24 or 30\textdegree C. Fcj1 overexpressing cells (transformed with plasmid pRS-\textit{FCJ1}) and control cells (transformed with the empty vector pRS426) were precultured in selective minimal medium (0.67\% [w/v] yeast nitrogen base, 0.07\% [w/v] CSM amino acid mix minus uracil, 3\% [v/v] glycerol) at 24\textdegree C. Cells were subsequently transferred into YPG medium and growth was continued for 24 h at 19\textdegree C. For spot tests (serial dilutions), agar plates containing 0.67\% (w/v) yeast nitrogen base, 0.07\% (w/v) SC amino acid mix, and 3\% (w/v) glycerol were used and incubated at different temperatures as indicated.
For electron microscopy, cells were inoculated in medium containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (v/v) L-lactate (pH 5.0) and grown for 24 h at 30°C and 200 rpm. Subsequently the cultures were diluted to an OD_{660} of 0.1 in minimal medium (Van Dijken et al., 1976) containing 2% (v/v) L-lactate (pH 5.0) and 20 mg/l of the appropriate amino acids (L-leucine, histidine, L-methionine and uracil). After incubation for 16 h the strains were harvested at an OD_{660} of 1.0, except for the fcj1Δ and mio10Δ strains that reached an optical density of only 0.5.

**SILAC**

Wild-type (YPH499 arg4Δ) and Fcj1ProtA (YPH499) yeast cells were subjected to stable isotope labeling with amino acids in cell culture (SILAC) by growth in synthetic medium (0.67% [w/v] bacto-yeast nitrogen base, amino acid mix containing histidine, tryptophan, adenine, methionine, uracil, isoleucine, tyrosine, phenylalanine, leucine, valine, threonine and proline, 3% [v/v] glycerol) supplemented with 0.1 mg/ml ampicillin. Wild-type cells were labeled using 22.84 mg/l [^{13}C_6/^{15}N_4] L-arginine (Arg10) and 23.52 mg/l [^{13}C_6/^{15}N_2] L-lysine (Lys8) (Euriso-top, Gif-sur-Yvette, France) and Fcj1ProtA cells were grown using the corresponding [^{12}C/^{14}N] amino acids (18 mg/l arginine and 22.5 mg/l lysine).

**Mass Spectrometry and Data Analysis**

Tryptic digests of affinity-purified and acetone-precipitated proteins were performed in 60% (v/v) methanol and 20 mM NH_4HCO_3. Peptide mixtures of triplicate experiments were analyzed by nano-HPLC/ESI-MS/MS using an UltiMate™ 3000 HPLC system (Dionex LC Packings, Idstein, Germany) and an LTQ-Orbitrap XL instrument (Thermo Fisher Scientific, Bremen, Germany) operated in a data-dependent mode. MS spectra over the mass range of m/z 300 – 1500 were acquired in the Orbitrap with a mass resolution of 30,000 at m/z 400. Simultaneously, fragmentation of multiply charged peptides was performed by low-energy CID in the linear ion trap using a "Top6" method with a dynamic exclusion window of 45 sec.
For data analysis, the software MaxQuant (version 1.0.13.13; Cox and Mann, 2008) was employed. Peptide and protein identification was based on searching MS/MS data against a decoy version of the *Saccharomyces* Genome database ([www.yeastgenome.org](http://www.yeastgenome.org)) containing the original and a shuffled sequence of each protein using Mascot (version 2.2, Matrix Science; Perkins et al., 1999). Proteins were identified with at least one unique peptide, a minimum peptide length of six amino acids and a false discovery rate of < 0.01 on peptide and protein levels. Mass tolerances for precursor and fragment ions were 7 ppm and 0.5 Da, respectively. Two missed tryptic cleavages were allowed. Arg10 and Lys8 were set as heavy labels, methionine oxidation as variable modification. For the determination of SILAC-based protein abundance ratios (light/heavy), only unique peptides were considered. The options "re-quantify" and "filter labeled amino acids" in MaxQuant were enabled; low-scoring peptides were disregarded for quantification. To correct for systematic deviations between replicates introduced by experimental variances in sample processing, protein ratios were normalized by MaxQuant as described (Cox and Mann, 2008).

**Electron Microscopy**

For cryo-ultramicrotomy cells were fixed in 1% (w/v) formaldehyde and 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 4 h on ice and subsequently incubated in 2.3 M sucrose (Tokuyasu 1973, 1978). A small droplet of the cell suspension was plunge frozen in liquid nitrogen. Sections were cut using a Reichert Ultracut at -80°C and collected on 100 mesh grids covered with a carbon coated formvar film. After extensive washing with water, the sections were stained with 0.2% (w/v) methylcellulose in 1% (w/v) uranylacetate in water, dried and viewed in a Philips CM12 at 80 kV.

For diaminobenzidine (DAB) staining and quantitative analysis cells were fixed with 3% (v/v) glutaraldehyde in sodium cacodylate buffer (pH 7.2) for 1 h on ice, and afterwards incubated for 1 h in 0.1 M Tris buffer (pH 7.5) with 2 mg/ml DAB (pH 7.5) and 0.06% (v/v) H₂O₂ at 37°C (Veenhuis et al., 1976). For postfixation cells were
treated with 1.5% (w/v) KMnO₄ for 20 min at room temperature, followed by incubation in 0.5% (w/v) uranylacetate overnight and subsequent embedding in Epon 812. For each strain 100 random cell sections were imaged. The number of cell sections with sheet-like, stacked mitochondrial cristae and the total number of crista junctions per cell section were determined.

**Purification of Yeast Mitochondria and Outer Membrane Vesicles**

Mitochondria were isolated by differential centrifugation (crude mitochondrial fraction) and subsequent sucrose density centrifugation (highly purified mitochondria) as described in Meisinger et al. (2006).

For isolation of outer membrane vesicles, gradient purified mitochondria (30 mg protein) from wild-type yeast were resuspended in hypoosmotic buffer containing 5 mM potassium phosphate, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at a protein concentration of 2 mg/ml (Zahedi et al., 2006). After incubation on ice for 20 min, swollen mitochondria were treated by 20 strokes in a glass-Teflon potter. The samples were loaded on top of a two-step sucrose gradient containing 1 ml 60%, 4 ml 32% and 1 ml 15% (w/v) sucrose in EM buffer (10 mM MOPS/KOH, pH 7.2, 1 mM EDTA and 1 mM PMSF) and centrifuged for 1 h at 134,000 x g (2°C) in a swing-out rotor. Outer membrane vesicles were collected from the 32-15% interphase. The sucrose concentration was then adjusted to 50% (w/v) and the sample was loaded on the bottom of a centrifuge tube. 5 ml 32% (w/v) sucrose in EM buffer and 1.5 ml EM buffer were carefully poured onto the sample. After further centrifugation for 6 h at 240,000 x g (2°C), highly pure outer membrane vesicles were recovered from the 32-0% interphase.

**Hypoosmotic Swelling Assay and Proteinase K treatment**

50 μg of mitochondria (protein amount) suspended in SEM buffer (250 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EDTA) were diluted 1:8 to 1:20 fold in EM buffer (10 mM MOPS, pH 7.2, 1 mM EDTA) and left on ice for 30 min. Mitochondria were reisolated by
centrifugation and resuspended in import buffer (3% [w/v] bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 2 mM KH₂PO₄, 5 mM methionine, 10 mM MOPS, pH 7.2). Proteinase K was added to a final concentration of 50 µg/ml and mitochondria were incubated on ice for 15 min. Proteinase K was inactivated by addition of 2 mM PMSF.

To determine if the proteins were digested upon lysis of mitochondria, 30 µg of mitochondria (protein amount) were solubilized in TS buffer (1% [v/v] Triton X-100, 250 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EDTA, 150 mM NaCl) by shaking for 5 min at 4°C. The samples were incubated with 50 µg/ml proteinase K (final concentration) for 15 min on ice. 2 mM PMSF was added. Proteins were recovered by trichloroacetic acid (TCA) precipitation and analyzed by SDS-PAGE.

**Outer Membrane Permeabilization Assay**

Permeabilization of the outer mitochondrial membrane through treatment with Bax was performed according to Sanjuán Szklarz et al. (2007). 80 µg isolated mitochondria (protein amount) were incubated in release buffer (250 mM sucrose, 150 mM KCl, 10 mM MOPS-KOH, pH 7.2) containing 100 nM Bax and 10 nM tBid for 60 min at 37°C shaking at 1,000 rpm. Supernatant and pellet were separated by centrifugation (12,000 x g, 10 min, 4°C) and the pellet was washed in SEM buffer (250 mM sucrose, 1 mM EDTA and 10 mM MOPS, pH 7.2). Proteins were precipitated with trichloroacetic acid and separated by SDS-PAGE.

**Carbonate Extraction**

To separate membrane-integral and soluble proteins, isolated mitochondria were incubated for 30 min on ice with 0.1 M Na₂CO₃ under mild (pH 10.8) or harsh (pH 11.5) conditions (Truscott et al., 2003; Wiedemann et al., 2006) and subsequently ultracentrifuged for 60 min at 100,000 x g at 4°C. After separation of pellet and supernatant, samples were precipitated with trichloroacetic acid and analyzed by SDS-PAGE.
Affinity Chromatography

For IgG affinity chromatography, 2 mg (protein amount) of isolated wild-type mitochondria (control) and mitochondria expressing Protein A-fusion constructs were solubilized in digitonin buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 10% [v/v] glycerol, 1% [w/v] digitonin, 2 mM PMSF, 2 mM Pefabloc and 4 μg/ml Leupeptin). After a clarifying spin (12,000 x g, 10 min, 4°C), mitochondrial extracts were incubated with 100 μl of pre-equilibrated human IgG-coupled sepharose beads for 90 min. Unbound proteins were removed by washing the beads ten times with washing buffer (20 mM Tris-HCl, pH 7.4, 60 mM NaCl, 0.5 mM EDTA, 10% [v/v] glycerol, 0.3% [w/v] digitonin, 2 mM PMSF). Bound proteins were eluted with TEV protease either by incubation over night at 4°C or at room temperature for 2.5 h.

Efficiency of co-isolation of MINOS proteins with Fcj1 ProtA from different yeast strains was determined from Western Blot analysis of affinity purifications (as shown in Figures S3E and F) using Multigauge software. Total and eluate signals were quantified to calculate the relative amounts of MINOS proteins recovered with Fcj1. The values obtained were corrected for the efficiency of Fcj1 (bait) recovery in wild-type and different mutants. The yield of MINOS proteins co-isolated with Fcj1 in wild-type was set to 100%. Three independent experiments were analyzed; the bar diagram in Figure 4D shows mean values and standards errors of the means (SEM).

Purification of histidine-tagged Mia40 was performed essentially as described (Milenkovic et al., 2007). Mitochondria were solubilized in digitonin-containing buffer (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 10% [v/v] glycerol, 50 mM NaCl, 1% [w/v] digitonin, 20 mM imidazole, 1 mM PMSF, 50 mM iodoacetamide). After a clarifying spin, mitochondria were incubated for 1-2 h with Ni²⁺-NTA agarose at 4°C. After washing (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM iodoacetamide, 20-40 mM imidazole), bound proteins were eluted with elution buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 50 mM iodoacetamide, 400 mM imidazole).
Size Exclusion Chromatography

1 mg of wild-type mitochondria (protein amount) were solubilized in digitonin-containing buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 10% [v/v] glycerol, 1% [w/v] digitonin), at a protein concentration of 2 mg/ml. After a clarifying spin (12,000 x g, 10 min, 4°C), soluble proteins were subjected to size exclusion chromatography using a TSK4000W column. Protein complexes were separated in a buffer composed of 20 mM Tris, pH 7.4, 150 mM NaCl, 5% (v/v) glycerol and 0.075% (w/v) digitonin. 500 µl fractions were collected and precipitated with TCA for analysis.

Assessment of the Redox State of Mia40

Mia40 contains six cysteine residues, two arranged in a CPC motif and a twin Cx₉C motif. The Cx₉C motifs form stable disulfide bonds, whereas the cysteine residues in the redox-active CPC motif (involved in binding of precursor proteins) are more readily accessible for reduction. Thus, two forms of Mia40 exist in mitochondria, fully oxidized and partially reduced, differing with respect to the oxidation state of the redox-active CPC motif (Mesecke et al., 2005; Grumbt et al., 2007; Banci et al., 2009; Kawano et al., 2009). The thiol-reactive agent iodoacetamide (IA) reacts with free sulfhydryl groups and thereby prevents further oxidation. Treatment with iodoacetamide allows an assessment of the ratio between oxidized and partially reduced Mia40. Mia40 can be fully reduced by treatment with dithiothreitol (DTT) under denaturing conditions.

Preparation of Mitochondrial Precursor Proteins

For import into mitochondria, precursor proteins were either synthesized in rabbit reticulocyte lysate in the presence of [³⁵S]methionine or produced in Escherichia coli and purified. To produce recombinant Tim10 protein with a decahistidine-tag, the Tim10 ORF was cloned into the pET10N-vector (Truscott et al., 2001). The resulting plasmid was transformed into the E. coli strain BL21 and expression of the recombinant protein was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, resuspended in lysis buffer (500 mM NaCl, 20 mM Tris-
HCl, pH 8.0) supplemented with 0.5 mM PMSF, 50 μg/ml lysozyme, 0.25% Triton X-100, 5 μg/ml DNase I, and 0.5 mM MgCl₂, and lysed by sonication. Bacterial debris was pelleted at 20,000 x g for 30 min at 4°C. The supernatant was incubated with 50 mM DTT for 20 min at 50°C and mixed with Ni²⁺-NTA Agarose beads for affinity purification. Beads were washed once with lysis and twice with wash buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8.0, 40 mM imidazole). Tim10 was eluted with elution buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 250 mM imidazole, 20 mM DTT, 20% [v/v] glycerol). Recombinant purified Tim10 and ³⁵S-labeled Tim8 and Tim9 precursors were precipitated using a saturated ammonium sulfate solution and subsequently denatured in urea-containing buffer (8 M urea, 30 mM MOPS-KOH, pH 7.2, 10-20 mM DTT).

Protein Import into Isolated Mitochondria

For import of small Tim proteins, radiolabeled and purified preproteins were incubated with mitochondria in import buffer (250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 5 mM methionine, 10 mM KH₂PO₄, 10 mM MOPS-KOH, pH 7.2). For import of ³⁵S-labeled b₂-DHFR, F₁β, Su9-DHFR, ADP/ATP carrier (AAC) and Tom40, the import buffer included 3% BSA, 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 5 mM methionine, 2 mM KH₂PO₄, 10 mM MOPS-KOH, pH 7.2, 2 mM NADH and 4 mM ATP. In the case of AAC and Tom40, the import buffer additionally contained 20 mM creatine phosphate and 0.1 mg/ml creatine kinase. Small Tim proteins import reactions were stopped by the addition of 50 mM iodoacetamide to block free thiol groups (Milenkovic et al., 2007; Müller et al., 2008) and by putting the samples on ice. Import of the presequence-containing preproteins b₂-DHFR, F₁β, Su9-DHFR, as well as AAC was stopped by the addition of AVO mix (8 μM antimycin A, 1 μM valinomycin, 20 μM oligomycin final concentrations) (Stojanovski et al., 2007). Tom40 import reactions were stopped by putting the samples on ice. Where indicated, samples were treated with 50 μg/ml proteinase K after the import reaction. Mitochondria were washed once in SEM buffer and solubilized in Laemmli buffer containing either 50 mM iodoacetamide (non-reducing conditions) or 50 mM dithiothreitol (DTT) or 1% β-mercaptoethanol (reducing
conditions). For analysis of import reactions by blue native electrophoresis, mitochondria were solubilized in digitonin buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 10% [v/v] glycerol, 50 mM NaCl, 1% [w/v] digitonin, 1 mM PMSF). When small Tim proteins had been imported, 50 mM iodoacetamide was added to the digitonin buffer.

Miscellaneous
Mitochondrial membrane potential was assessed using the membrane potential sensitive dye 3,3′-dipropylthiadicarbocyanine iodide [DiSC$_3$(5)] (Geissler et al., 2000). Further details of generation of RNCs and release of nascent chains by puromycin are described by Fünfschilling and Rospert (1999), Jiang et al. (2009) and Ziehr et al. (2010). Proteins and RNCs were separated by denaturing SDS- or precast Nu-(Novex Midi Bis-Tris Gel, Invitrogen) polyacrylamide gel electrophoresis (SDS-PAGE or NuPAGE). Protein complexes were separated on 6-16.5% polyacrylamide gradient gels and analyzed by blue native electrophoresis (Schägger and von Jagow, 1991; Chacinska et al., 2004). Western blot analysis was performed using enhanced chemiluminescence (ECL). $^{35}$S-labeled proteins were visualized by digital autoradiography (Storm Imaging System; GE Healthcare) and analyzed by ImageQuant software. In some figures, non-relevant gel parts were excised digitally. Transmembrane segments were predicted using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Coiled-coil regions were predicted using PCOILS (http://toolkit.tuebingen.mpg.de/pcoils) (Lupas et al., 1991). Mitochondrial targeting sequences were predicted using Mitoprot (http://ihg.gsf.de/ihg/mitoprot.html) (Claros and Vincens, 1996). Tertiary structure predictions were performed using HHpred (http://toolkit.tuebingen.mpg.de/hhpred) (Söding, 2005). Homology search was performed by BLAST (http://expasy.org/tools/blast/).
SUPPLEMENTAL REFERENCES


