Cell Metabolism

Supplemental Information

Mic10 Oligomerizes to Bend Mitochondrial Inner Membranes at Cristae Junctions

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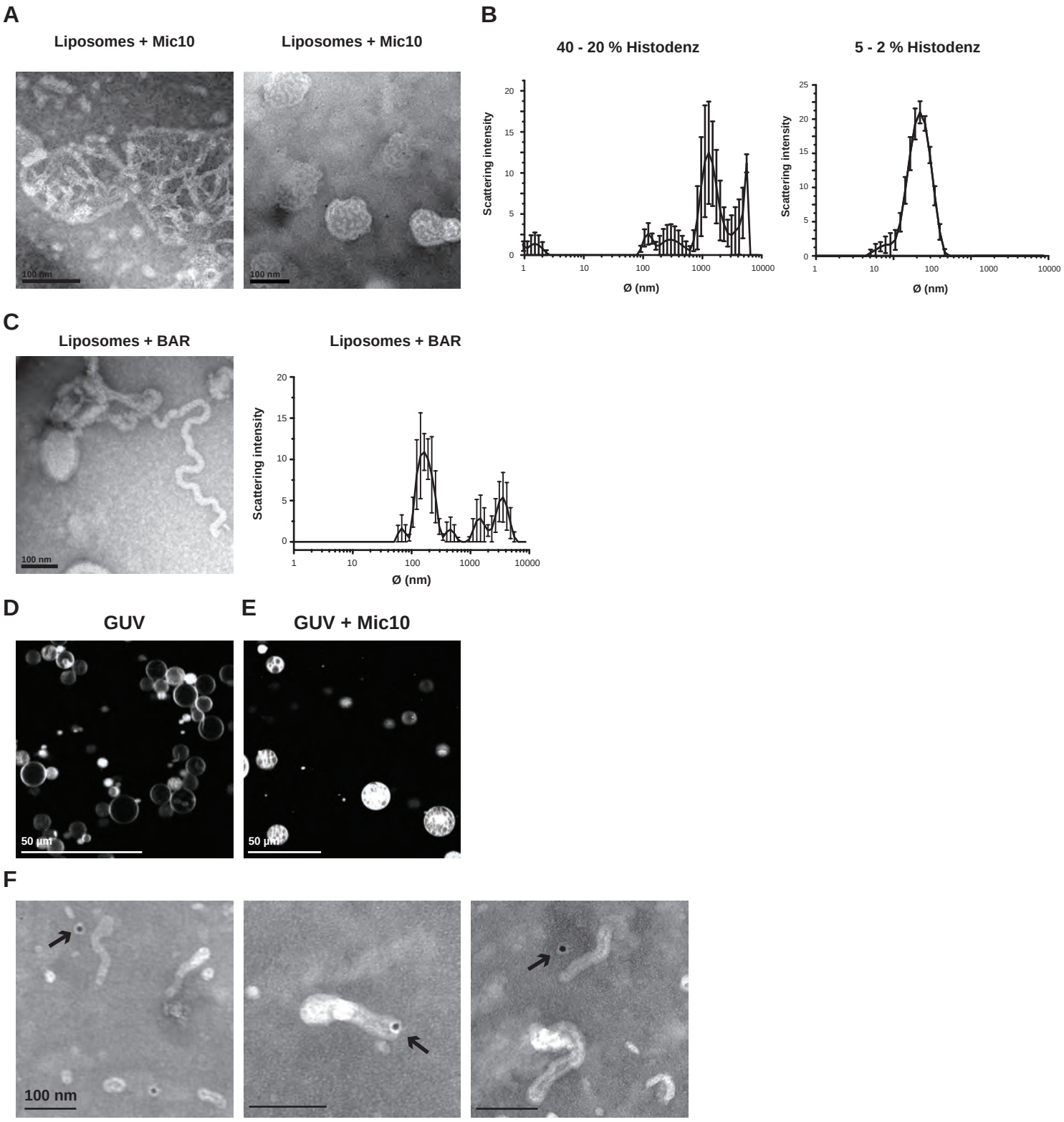
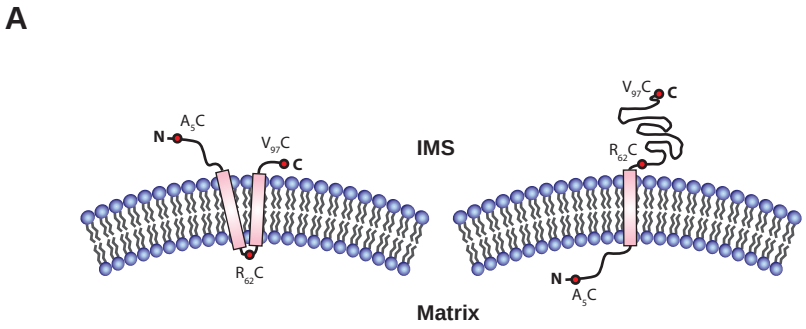
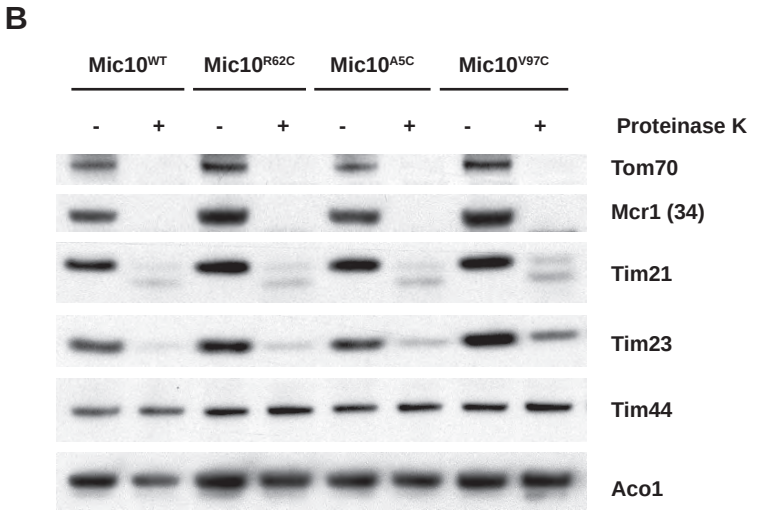


Figure S1. Vesicle deformation.
(A) Tubular membrane structures as observed after reconstitution of Mic10 into LUVs (scale bar 100 nm).
(B) Size distribution of Mic10 containing LUVs after flotation assay. Indicated layers were analyzed by dynamic light scattering. Error bars represent SEM.
(C) Membrane deformation of LUVs by endophilin BAR domain (scale bar 100 nm)(left). Size distribution of LUVs after incubation with endophilin BAR domain analyzed by dynamic light scattering (right). Error bars represent SEM.
(D) GUVs generated from LUVs by electroswelling (scale bar 50 µm).
(E) GUVs generated from Mic10 containing LUVs by electroswelling (scale bar 50 µm).
(F) Mic10 induced membrane tubules after immuno-gold labeling of Mic10 (arrows indicate gold particles which can be up to ~20 nm away from the epitope due to the size of the linking antibodies.)





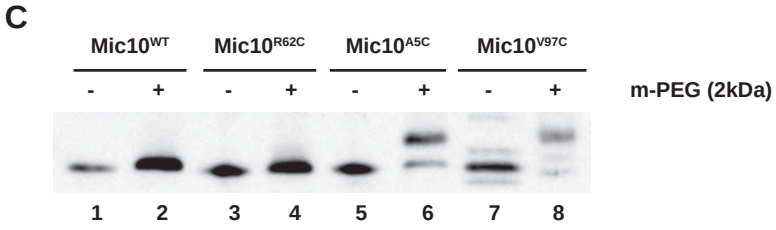


Figure S2. Topology of Mic10 in the mitochondrial inner membrane.

(A) Cartoon of the different possibilities how Mic10 spans the inner membrane with either one or two transmembrane helices under the assumption that the C-terminus is located within the intermembrane space.

(B) Mitochondria isolated from mic10Δ + Mic10WT, mic10Δ + Mic10^{R62C}, mic10Δ + Mic10^{A5C} and mic10Δ + Mic10^{V97C} cells were subjected to hypoosmotic treatment (swelling) and Proteinase K (Prot. K) digestion where indicated. Samples were analysed by SDS-PAGE and immunodecoration. Tom70 and Mcr1 are outer membrane proteins whereas Tim44 and Aco1 are located in the mitochondrial matrix. Tim23 and Tim21 are both integral inner membrane proteins but antibodies are directed against their IMS part.

(C) Western blot analysis of WT and indicated cysteine mutants of Mic10 in un-swollen mitochondria. Lanes 6 and 8 indicate that the OM is partially permeable for m-PEG. Lane 4 shows that the IM is not permeable for m-PEG.

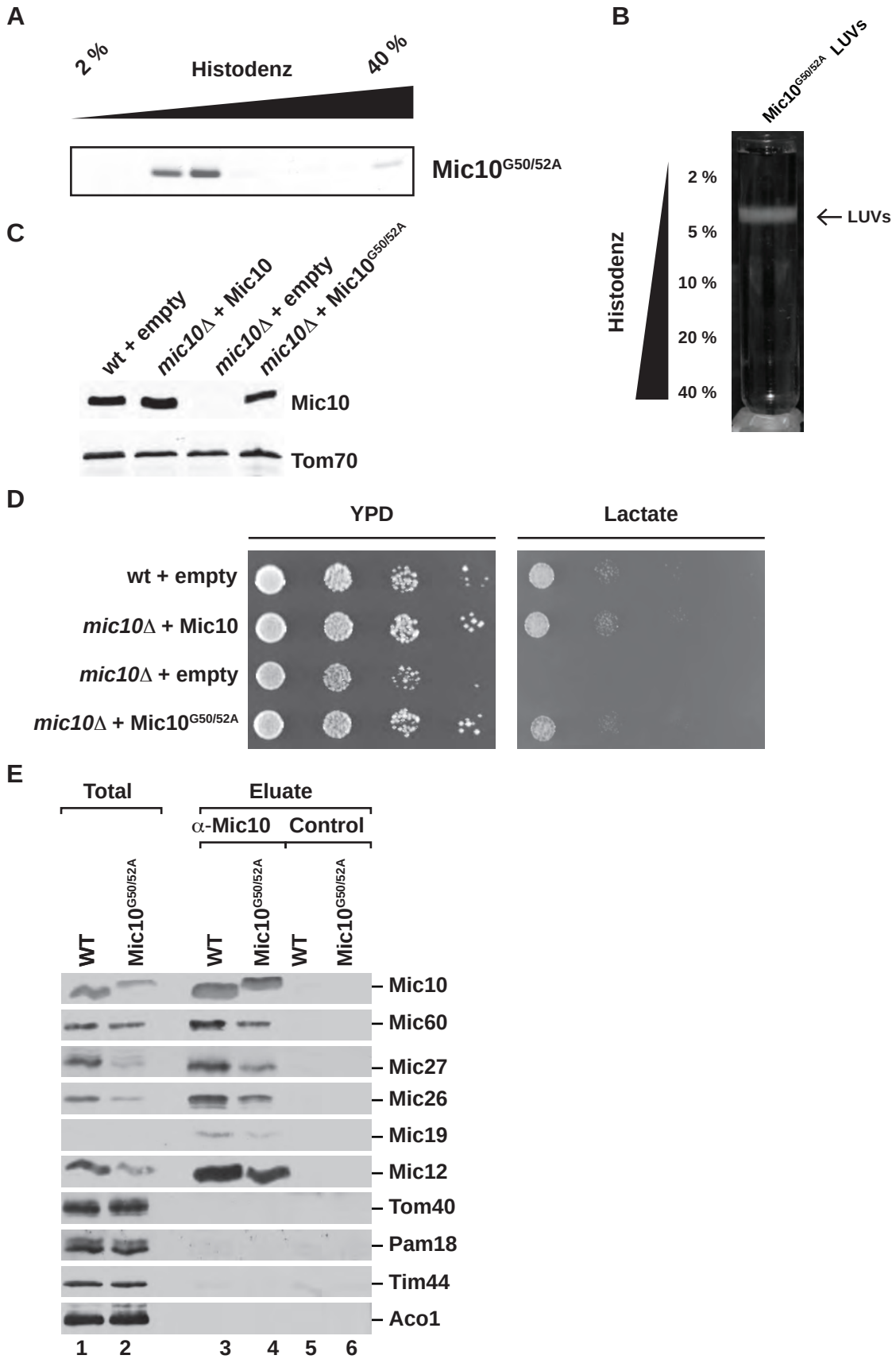


Figure S3. Mic10G50/52A can be reconstituted into model membranes and is normally inserted into the MICOS complex.

(A) Coomassie stained SDS gel of the fractionated density gradient flotation shown in (B).

(B) Flotation assay of Mic10G50/52A containing LUVs.

(C) Steady state levels of different Mic10 variants expressed in yeast cells analyzed by Western blot (upper panel). The outer mitochondrial protein Tom70 was used as a loading control (bottom panel).

(D) Growth test of indicated Mic10 strains on glucose (left) and lactate (right).

(E) Co-purification of MICOS subunits with Mic10WT (lane 3) and Mic10G50/52A (lane 4). All MICOS subunits are co-purified by both Mic10 variants whereas no control proteins are detectable after co-immunoprecipitation.

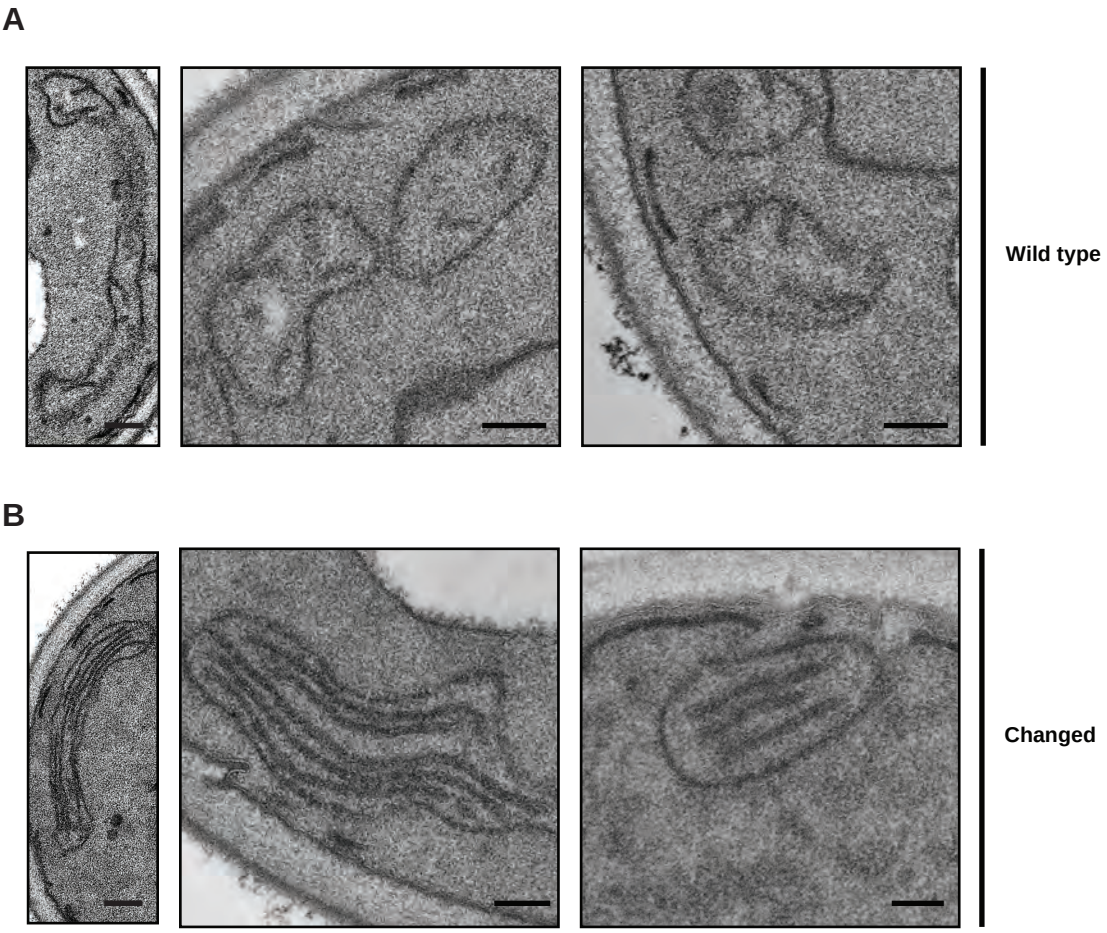


Figure S4. Mitochondrial morphology.
Representative images of mitochondria that serve as a baseline to distinguish between wild type (A) and changed (B) inner membrane morphology.

Supplemental Information

Supplemental Experimental Procedures

Cloning and expression of Mic10. Primers containing relevant restriction enzyme sites were used for polymerase chain reaction (PCR) to generate yeast Mic10. The PCR product was successfully cloned into pPROEX HTC vector containing N-terminal His_6-tag. After expression of Mic10 in *E. coli* strain BL21(DE3) (1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 3h, 37°C) cells were lysed, inclusion bodies were isolated (Meinecke et al., 2006) and subsequently dissolved in resuspension buffer containing 8 M urea, 100 mM NaCl, 10 mM Tris-HCl, 2 mM DTT, pH 8.0. The mixture was applied to the NiNTA-agarose column and eluted with resuspension buffer supplemented with 500 mM imidazole. Isolated Mic10 was further subjected on HiLoad 16/600 Superdex 200 size exclusion column (GE Healthcare, Piscataway, NJ, USA). Separated fractions were analyzed by SDS-PAGE and immunoblotting.
All single mutants G48A, G50A, G52A, G70A, G72A, G74A, G76A, G78A and a double mutant G50A/G52A were obtained by site-directed mutagenesis. The overexpressed mutant proteins were subjected to the same purification steps as the wild type protein.

Liposome flotation assay and carbonate extraction. For density flotation assays, nonionic Histodenz (SIGMA-ALDRICH) 40/20/10/5/2 percent gradients were prepared in 100 mM NaCl, 10 mM Tris, pH 8.0 buffer. Proteoliposomes were loaded below the 40 % layer, centrifuged at 150,000 x g for 45 min and ultimately collected at the interfaces of the layers. It should be noted that 45 min is not enough to reach equilibrium of flotated samples. Therefore, the different layers likely correspond to different shapes (different surface to volume ratios) of the deformed vesicles. After precipitation with 10 % trichloroacetic acid (TCA) the samples were resuspended in SDS sample buffer and subjected for SDS-PAGE analysis.
For sodium carbonate extraction assay proteoliposomes collected from the interfaces were treated by 20 mM Na_2CO_3 and incubated for 30 min on ice. Samples were centrifuged at 150,000 x g for 45 min and the supernatants were TCA precipitated. Ultimately, membrane and TCA pellets were resuspended in SDS sample buffer and analyzed by SDS-PAGE.

Dynamic light scattering. Samples were measured using a Zetasizer Nano S (Malvern Instruments). Data were obtained from at least three independent measurements. Each measurement consists of 20 repetitions over 10 minutes. Errors were calculated as the standard deviation from the averaged values.

Blue native electrophoresis. Approximately 2.5 µg of $Mic10^{WT}$ and $Mic10^{G50/52A}$ in buffer R were mixed with 20:1 v/v 5 % Coomassie G-250 and NativePAGE 4x sample buffer (Invitrogen) and separated on a NativePAGE 3-12 % Bis-Tris gel (Invitrogen) at a constant 150 V. One third of the electrophoresis was performed with pre-chilled dark blue cathode buffer and the rest with light blue cathode buffer. The bands were visualized by Coomassie G-250 staining.

Yeast strains and growth. The *mic10* open reading frame was cloned into pRS413 under the control of its native promoter and terminator. Mutants were generated by site-directed mutagenesis. *mic10*Δ (Mat a, his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0; mio10::kanMX4) (Open Biosystems, Thermo Biosystems, Huntsville, AL) cells were either transformed with the empty plasmid or plasmids containing wild type or respective mutant alleles of *mic10*. The expression was verified by whole cell extracts, SDS-PAGE and Western blotting. Cell growth

was assessed by spotting serial dilutions on selective glucose or Lactate plates (0.67 % yeast nitrogen base without amino acids, 0.07 % complete supplement mixture lacking Histidine (MP Biomedicals), 25 g/l agar and 2% glucose or 3 % Lactate, pH 5.0) and incubation at 30°C or 18°C, respectively.

Immunogold staining of Mic10 proteoliposomes. Mic10 containing liposomes were shortly spotted on carbon-coated grids (Agar scientific, Essex) and transferred to drops of Tris-Buffered Saline (TBS) washing solution. Afterwards, the grids were incubated with the primary anti-His antibody (Sigma-Aldrich) 1:6000 dilution for 1hour at 25°C followed by washing and subsequent incubation with the secondary Anti-Mouse IgG- Gold (colloidal gold 10 nm) antibody (Sigma-Aldrich) 1:100 dilution for 45 min at 25°C as recommended by manufacturers. Subsequently, the grids were washed with TBS and eventually stained with 5 % (w/v) uranyl acetate solution. Electron microscopic imaging was performed as described above.

Construction of cysteine mutants and preparation of mitochondria. All Mic10 cysteine mutants R62C, A5C and V97C were obtained by site-directed mutagenesis from the plasmid containing wild type alleles of *MIC10*. The resulting plasmids and plasmid containing wild type Mic10 were transformed into a *mic10*Δ strain (Mat a, his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0; mio10::kanMX4) (Open Biosystems, Thermo Biosystems, Huntsville, AL, USA) using the lithium acetate method.
Mitochondria were isolated by using Zymolyase treatment, Dounce homogenization, and subsequent differential centrifugation according to Meisinger et al. (2006) (Meisinger et al., 2006) from cells grown on selective 3% glycerol media (0.67 % yeast nitrogen base without amino acids, 0.07 % complete supplement mixture lacking Histidine (MP Biomedicals).

Mic10 co-immunoprecipitation. Mitochondria were solubilised in 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10% glycerol, 0.5 mM EDTA and 1 % digitonin. The supernatant obtained after centrifugation was loaded on ProteinA sepharose coupled with a-Mic10 serum. After binding the beads were washed with solubilization buffer containing 0.3 % digitonin and bound proteins were eluted with 100 mM glycine pH 2.8. Samples were analyzed by SDS-PAGE and Western blotting.

References

Meisinger, C., Pfanner, N., and Truscott, K.N. (2006). Isolation of yeast mitochondria. Methods Mol. Biol. *313*, 33–39.