Table S1 | Yeast strains used in this study

Strain	Genotype
JSY7452*	*MATα ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100*
JSY7453*	*MATα ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 mfb1::HIS3*
JSY8272	*MATα ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 tom70::TRP1*
JSY8275	*MATα ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 mfb1::HIS3 tom70::TRP1*
JSY8278	*MATα ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 tom71::HIS3*
JSY8281	*MATα ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 mfb1::HIS3 tom71::HIS3*
JSY8283	*MAT***a** *ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 tom70::TRP1 tom71::HIS3*
JSY8286	*MAT***a** *ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 mfb1::HIS3 tom70::TRP1 tom71::HIS3*
JSY8323	*MATα ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 tom20::TRP1*
tom5Δ	*MAT***a** *ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 tom5::CgHIS3*
MNMS-1C	*MAT***a** *ade2-1 leu2-3 his3-11,15 trp1-1 ura3-1 can1-100 mas17::HIS3 [pGAL:MAS17(URA3)]*

*These strains have been used previously (Kondo-Okamoto *et al*, 2006). Gene disruptions were confirmed by PCR. *tom5Δ* (Esaki *et al*, 2004) and MNMS-1C (Nakai *et al*, 1995) were provided kindly by T. Endo (Nagoya University, Nagoya, Japan).

Table S2 | Plasmids used in this study

Plasmid	Construct name	Insert
B1220*	pYX142-*mtGFP*$^{+}$	*N. crassa ATP9(1-69) + brighter GFP*
B1641*	pYX142-*mtRFPm*	*N. crassa ATP9(1-69) + monomeric DsRed*
B1788*	pRS416-*MFB1-3HA*	438 bp 5' + *MFB1(1-465)* + *3xHA* + 400 bp 3'
B1800*	pRS416-*MFB1(1-465)-GFP*	438 bp 5' + GAATTC + *MFB1(1-465)* + *GFP*$^{+}$ + 400 bp 3'
B1865*	pYX142-*MFB1(1-465)-3HA*	*MFB1(1-465)* + *3xHA*
B1870	pRS416-*MFB1*	438 bp 5' + *MFB1(1-465)* + 400 bp 3'
B2055*	pRS416-*TMS-MFB1-3HA-GFP*	438 bp 5' + GAATTC + *TOM20(1-50)* + *MFB1(2-465)* + *3xHA* + *GFP*$^{+}$ + 400 bp 3'
B2119	pRS415-*TOM70*	476 bp 5' + *TOM70(1-617)* + 267 bp 3'
B2120	pRS415-*TOM71*	342 bp 5' + *TOM71(1-639)* + 347 bp 3'
B2149	pRS415-*TOM70(R171A)*	476 bp 5' + *TOM70(1-617)(R171A)* + 267 bp 3'
B2169	pRS415-*TOM70(1-575)*	476 bp 5' + *TOM70(1-575)* + 267 bp 3'
B2176	pRS415-*TOM70(1-605)*	476 bp 5' + *TOM70(1-605)* + 267 bp 3'
B2267	pRS415-*TOM71(ΔTPR1-3)*	342 bp 5' + *TOM71(1-125)* + *TOM71(209-639)* + 347 bp 3'
B2268	pRS415-*TOM70(HCS)*	476 bp 5' + *TOM70(1-78)* + *LEVLFQGPAS* + *TOM70(79-617)* + 267 bp 3'
B2272	pRS415-*TOM71(R200A)*	342 bp 5' + *TOM71(1-639)(R200A)* + 347 bp 3'
B2285	pRS415-*TOM70(ΔTPR1-3)*	476 bp 5' + *TOM70(1-97)* + *TOM70(179-617)* + 267 bp 3'
B2286	pRS415-*TOM71(1-597)*	342 bp 5' + *TOM71(1-597)* + 347 bp 3'
B2288	pRS415-*TOM71(1-627)*	342 bp 5' + *TOM71(1-627)* + 347 bp 3'
B2289	pRS425-*TOM71*	342 bp 5' + *TOM71(1-639)* + 347 bp 3'
KOB1	pRS314-*MFB1-3HA*	438 bp 5' + *MFB1(1-465)* + *3xHA* + 400 bp 3'
KOB2	pRS314-*MFB1(1-465)-GFP*	438 bp 5' + GAATTC + *MFB1(1-465)* + *GFP*$^{+}$ + 400 bp 3'

*These plasmids have been used previously (Kondo-Okamoto *et al*, 2006). Constructs cloned in pYX142 are expressed under the control of the strong constitutive *TPI* promoters. All PCR-amplified fragments were verified by sequencing. Brighter GFP and monomeric DsRed variants were provided kindly by M. Niederweis (Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany) and R. Tsien (University of California, San Diego, San Diego, CA, USA), respectively.

SUPPLEMENTAL METHODS

Immunoprecipitation. Physical interactions of Mfb1 with Tom71, Tom70, Tom20, Tom22, and Tom40 were examined by immunoprecipitation as described previously (Kondo-Okamoto *et al*, 2006). Mitochondria isolated from *mfb1Δ* cells expressing Mfb1-3HA or wild-type Mfb1 (0.4 mg of protein, Fig 1A), or *mfb1Δ* and *mfb1Δ tom70Δ* cells overexpressing Mfb1-3HA (0.6 mg of protein, Fig 1B) were pelleted, resuspended in 0.6 ml lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100) containing 0.5% protease inhibitor cocktail (PIC) (Calbiochem, 539134), incubated at 4ºC for 15 min with gentle agitation, and subjected to centrifugation (18,000 *g*) at 4ºC for 10 min. The supernatant fractions were transferred to a new tube containing 20 μl Anti-HA Agarose Conjugate (Sigma, A2095), and incubated at 4ºC for 2 h with gentle agitation. The beads were washed twice with 0.6 ml lysis buffer, and once with 0.6 ml PBS, and collected by centrifugation (12,000 *g*) at 4ºC for 1 min. The proteins were eluted with SDS-sample buffer lacking β-mercaptoethanol (BME) at 65ºC for 8 min followed by addition of BME (0.9 M), and analyzed by Western blotting. The mitochondrial extract inputs (I) and eluted immunoprecipitates (E) loaded per lane were 2% and 50% (Fig 1A), or 2% and 33% (Fig 1B) of the total extracts subjected to incubation with anti-HA-conjugated agarose, respectively.

***In vitro* binding assays.** Binding of Mfb1 to isolated mitochondria was investigated by sedimentation as described previously (Kondo-Okamoto *et al*, 2006). Mitochondria (60 μg of protein) isolated from *mfb1Δ tom70Δ tom71Δ* cells expressing Tom70 or Tom70(HCS), a variant with a HRV 3C processing site (Fig 5A), *mfb1Δ tom70Δ* cells expressing Tom70 or Tom70(HCS) (Fig 5B), or *mfb1Δ tom70Δ* cells expressing Tom70 or Tom71 at 2-3 (Tom71↑) and 10-15 (Tom71↑↑) fold higher levels (Fig 5C) were pretreated with or without HRV 3C Protease (80 U/ml) (Novagen) in 0.5 ml HRV 3C Protease Cleavage Buffer (50 mM Tris-HCl, [pH 7.5], 150 mM NaCl, 0.6 M sorbitol) at 4ºC for 30 min or proteinase K (PK) (50 μg/ml) in 0.5 ml HS buffer (20 mM HEPES-KOH [pH 7.4], 0.6 M sorbitol) on ice for 15 min followed by the addition of PMSF (1 mM) on ice for 5 min. Samples were subjected to centrifugation (18,000 *g*) at 4ºC for 10 min. The mitochondrial pellet was washed twice in 0.5 ml HS buffer containing 1 mM PMSF and 0.4% PIC, and collected by centrifugation (18,000 *g*) at 4ºC for 10 min. Mitochondria were resuspended in 0.4 ml of a PMS (1.6 mg of protein) fraction isolated from *mfb1Δ tom70Δ tom71Δ* cells overexpressing Mfb1-3HA, and incubated at 4ºC for 20 min. 0.1 ml of the reaction mixture was layered onto a 0.5 ml sucrose cushion (25% sucrose, 20 mM HEPES-KOH [pH 7.4], 1mM PMSF, 0.4% PIC), and subjected to centrifugation (12,500 *g*) at 4ºC for 10 min. After collecting the supernatant, the sucrose

cushion was discarded, and the mitochondrial pellet was washed once in 0.2 ml HS buffer containing 1 mM PMSF and collected by centrifugation (12,500 *g*) at 4ºC for 10 min. The mitochondrial pellet fractions (20 μg of protein per lane) were analyzed.

Immunoblot analysis. Proteins were analyzed by immunoblotting with antibodies specific for: Porin (1:20,000) and 3-PGK (1:10,000) from Molecular Probes (1 mg/ml); HA (1:1,000) from the University of Utah Core Facility; Mge1 (1:5,000), Tom40 (1:20,000) and Tom70 (1:10,000), gifts from T. Endo (Nagoya University, Nagoya, Japan); Tom20 (1:10,000) and Tim44 (1:10,000), gifts from W. Neupert and K. Hell (Ludwig-Maximilians-Universität München, Munich, Germany); Tom71 (1:500), a gift from D. Rapaport (Universität Tübingen, Tübingen, Germany); and Tom22 (1:1,000), a gift from N. Pfanner (Universität Freiburg, Freiburg, Germany).

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Nakai M, Kinoshita K, Endo T (1995) Mitochondrial receptor complex protein. The intermembrane space domain of yeast MAS17 is not essential for its targeting or function. *J Biol Chem* **270:** 30571-30575

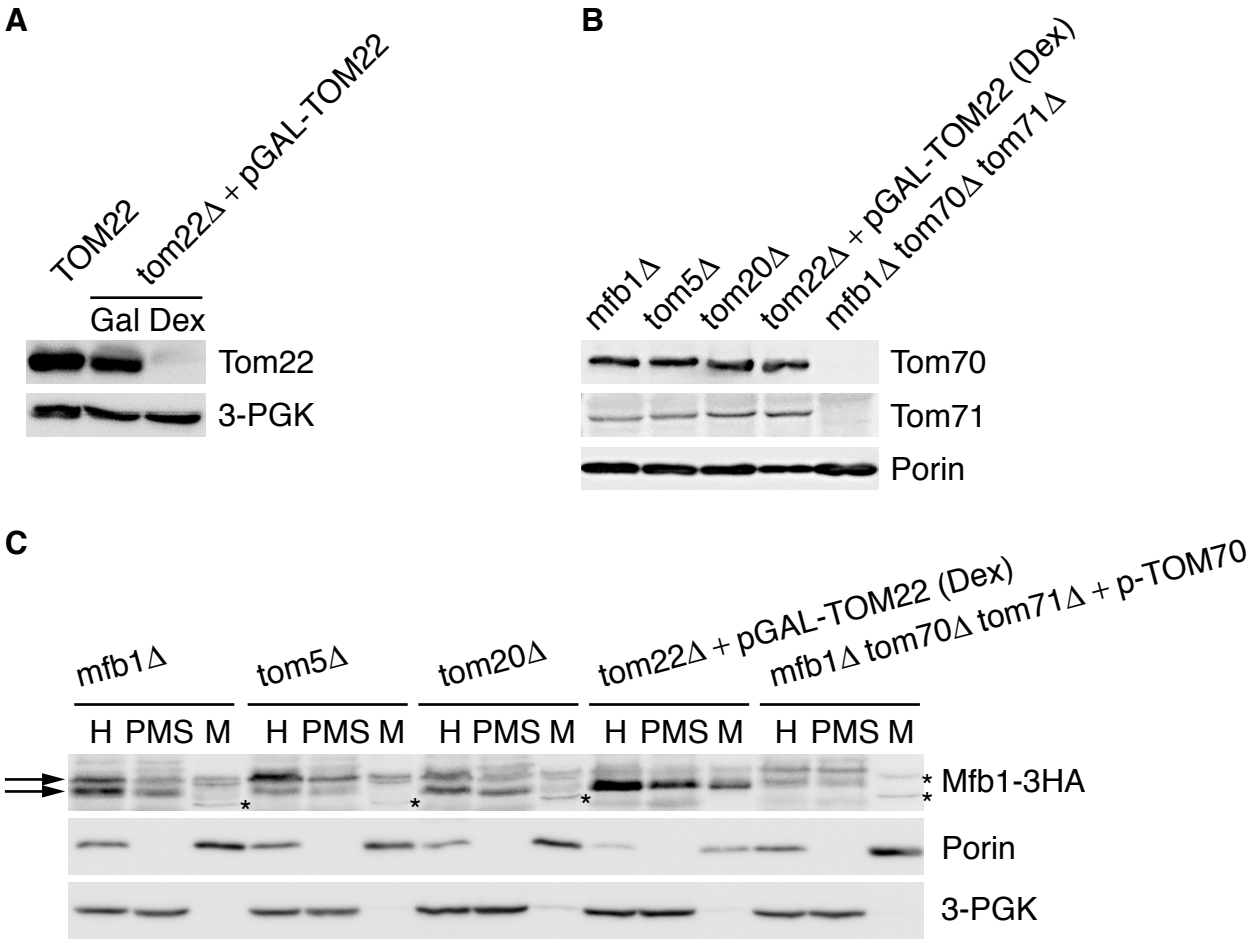

Fig S1 | Subcellular fractionation assays for Mfb1-3HA localization in cells lacking Tom5 or Tom20, and depleting Tom22. (**A**) Depletion of Tom22. Steady-state levels of Tom22 in total extracts of *mfb1*Δ cells containing pRS416-MFB1-3HA (TOM22) grown on dextrose media, or *tom22*Δ cells containing pRS314-MFB1-3HA and pGAL-TOM22 grown on galactose (Gal for expression) or dextrose media for 18 h (Dex for depletion). 3-PGK was monitored as a loading control. (**B**) Steady-state levels of Tom70 and Tom71 in mitochondria isolated from strains expressing Mfb1-3HA. Porin was monitored as a loading control. The Tom70 and Tom71 levels are not significantly altered in cells lacking Tom5 or Tom20, and depleting Tom22. (**C**) The cell homogenate (H) was separated into post-mitochondrial supernatant (PMS) and mitochondrial pellet (M). Porin and 3-PGK were monitored as mitochondria and cytoplasm markers, respectively. Arrows and asterisks indicate Mfb1 and non-specific bands, respectively. The upper bands of Mfb1 are posttranslationally modified species (Kondo-Okamoto *et al*, 2006).

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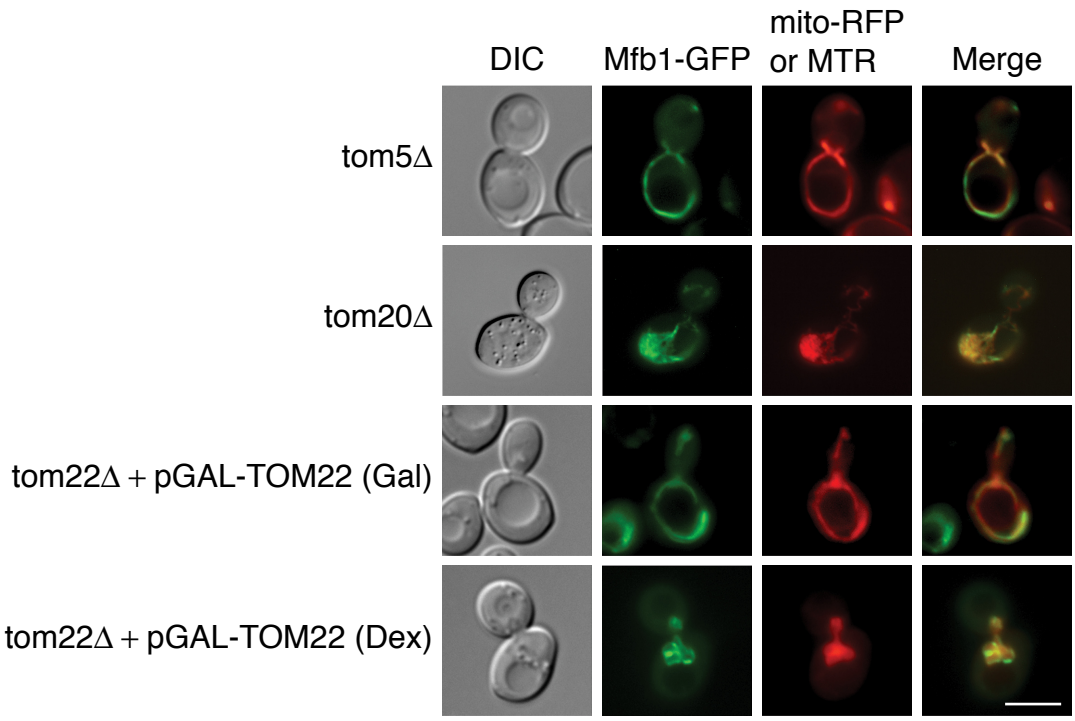

Fig S2 | Mfb1 mitochondrial targeting is not disrupted in cells lacking Tom5 or Tom20, and depleting Tom22. Mfb1-GFP localization and mitochondria visualized by mito-RFP or MitoTracker Red (MTR) in *tom5*Δ or *tom20*Δ cells grown on dextrose media, and *tom22*Δ cells containing pGAL-TOM22 grown on galactose (Gal for expression) or dextrose media for 18 h (Dex for depletion). Representative images are shown. Bar, 5 μm.

Kondo-Okamoto et al, Fig S2
EMBOR-2007-31150V1

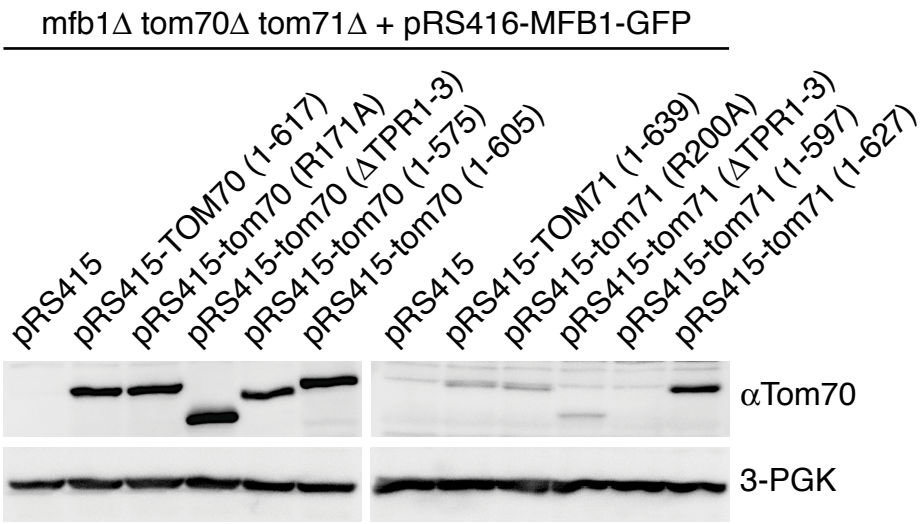

Fig S3 | Steady-state levels of Tom70 and Tom71 wild-type and mutant proteins in total extracts of *mfb1Δ tom70Δ tom71Δ* cells containing pRS416-MFB1-GFP. 3-PGK was monitored as a loading control. Note that the Tom70 antibody cross-reacts with Tom71 wild-type and mutant proteins (two non-specific bands were seen due to long exposure for ECL detection). Tom71(1-597) was not stably expressed.

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