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Supplemental Information

The Biogenesis of Mitochondrial Outer Membrane Proteins Show Variable Dependence on Import Factors

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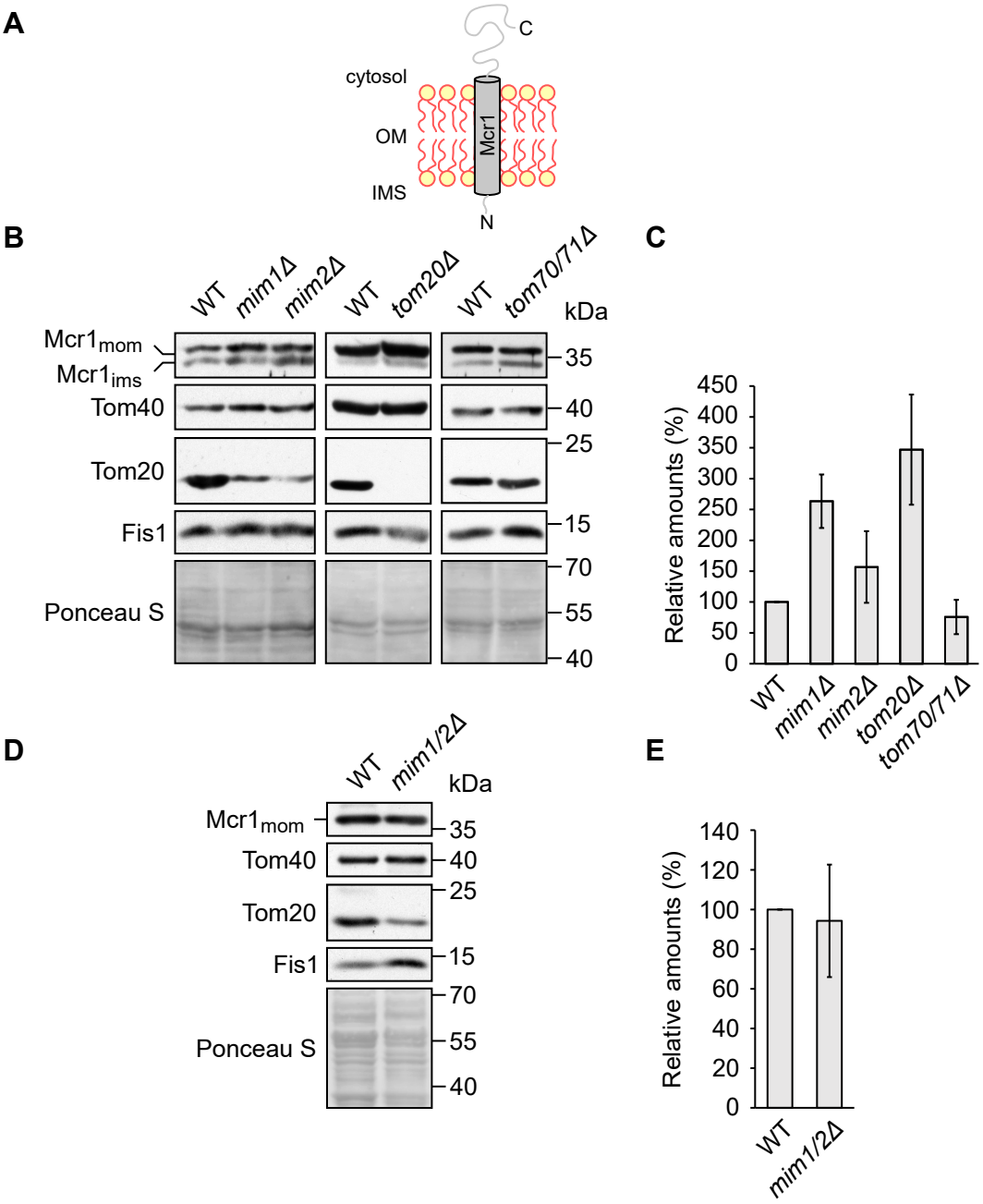

Figure S1. Related to Figure 1. Mcr1 levels are not affected by deletions of MOM import factors.

(A) Schematic representation of the topology of $Mcr1_{mom}$. IMS: intermembrane space.

(B) Crude mitochondrial fractions of *mim1Δ*, *mim2Δ*, *tom20Δ*, *tom70/71Δ*, and their respective WT strains were analysed by SDS-PAGE and immunodecoration with the indicated antibodies. The outer membrane ($Mcr1_{mom}$) and IMS ($Mcr1_{ims}$) isoforms of Mcr1 are indicated.

(C) Quantification of the band corresponding to $Mcr1_{mom}$ in experiments as in (B). The levels were normalized with the Ponceau S signal and presented as percentage of the amounts in the corresponding WT samples. The graph represents the average of at least three independent experiments ± standard deviation (SD).

(D) Isolated mitochondria were obtained from WT and *mim1/2Δ* strains and analysed by SDS-PAGE and immunodecoration with the indicated antibodies.

(E) $Mcr1_{mom}$ levels from three independent experiment as in (D) were quantified and normalized to the intensities of the Ponceau S staining. The values are presented as percentage of the amounts in WT cells. Error bars represent ± SD.

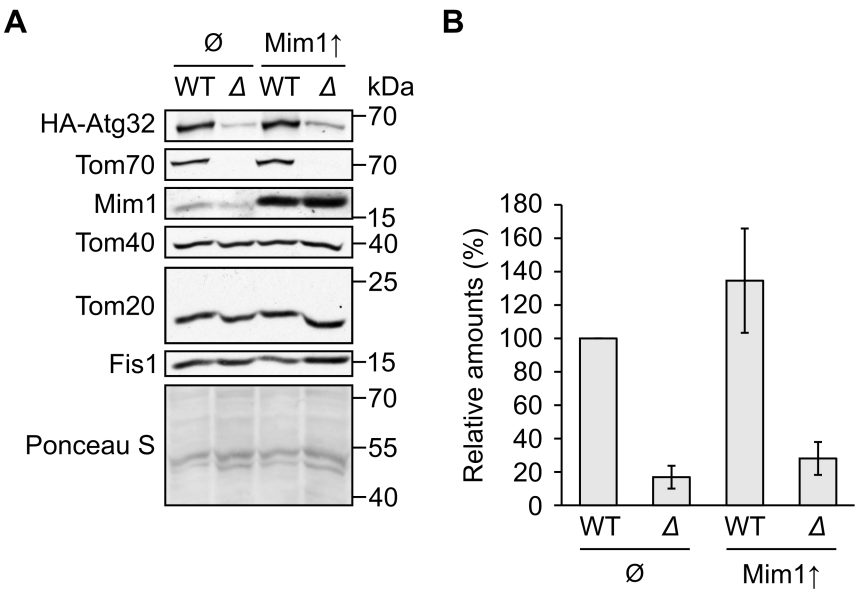

Figure S2. Related to Figure 3. Overexpression of Mim1 does not rescue the reduced levels of HA-Atg32 in cells lacking Tom70/71.

(A) Crude mitochondria fractions were isolated from either WT or tom70/71Δ (Δ) strains transformed with a plasmid expressing HA-Atg32 and either and empty plasmid (Ø) or a plasmid overexpressing MIM1 (Mim1↑). Proteins were analysed by SDS-PAGE and immunodecoration with anti-HA and the indicated antibodies.

(B) Quantification of the band corresponding to HA-Atg32 in three independent experiments as the one shown in (A). The levels were normalized to the intensity of the Ponceau S signal and are shwon as percentage of the WT transformed with the empty vector. Error bars correspond to ±SD.

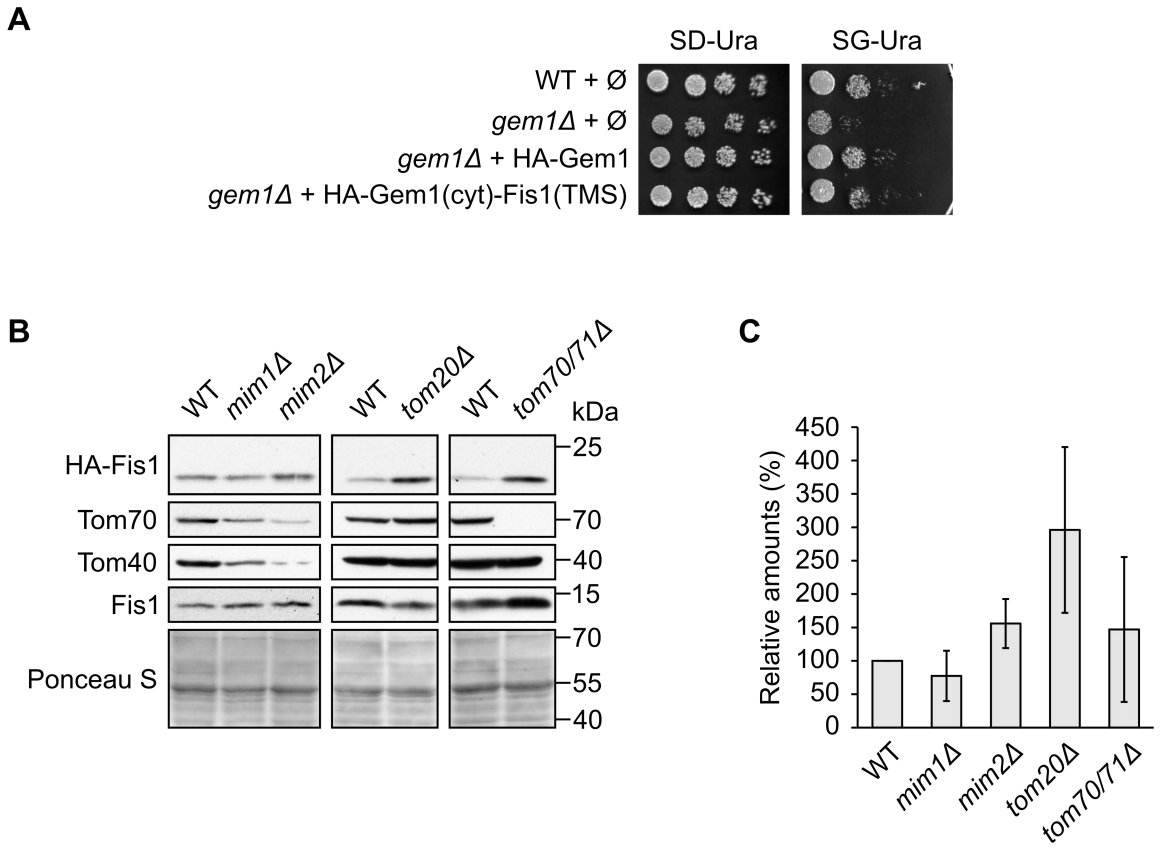

Figure S3. Related to Figure 4. N-terminally HA-tagged Fis1 and Gem1 behave as the native proteins.

(A)The indicated strains trasformed with an empty plasmid (Ø), or a plasmid expressing either HA-Gem1 or HA-Gem1(cyt)-Fis1(TMS) were tested by drop dilutions at 30°C on synthetic glucose-containing (SD-Ura) or glycerol-containing (SG-Ura) media.

(B) Crude mitochondria fractions were isolated from the indicated strains expressing HA-Fis1. Proteins were analysed by SDS-PAGE and immunodecorated with anti-HA and the indicated antibodies.

(C) Quantification of the band corresponding to HA-Fis1 in three independent experiments as the one shown in (B). The levels were normalized to the intensity of the Ponceau S signal and are shown as percentage of the corresponding WT. The graph represents the average of at least three independent experiments. Error bars correspond to ±SD.

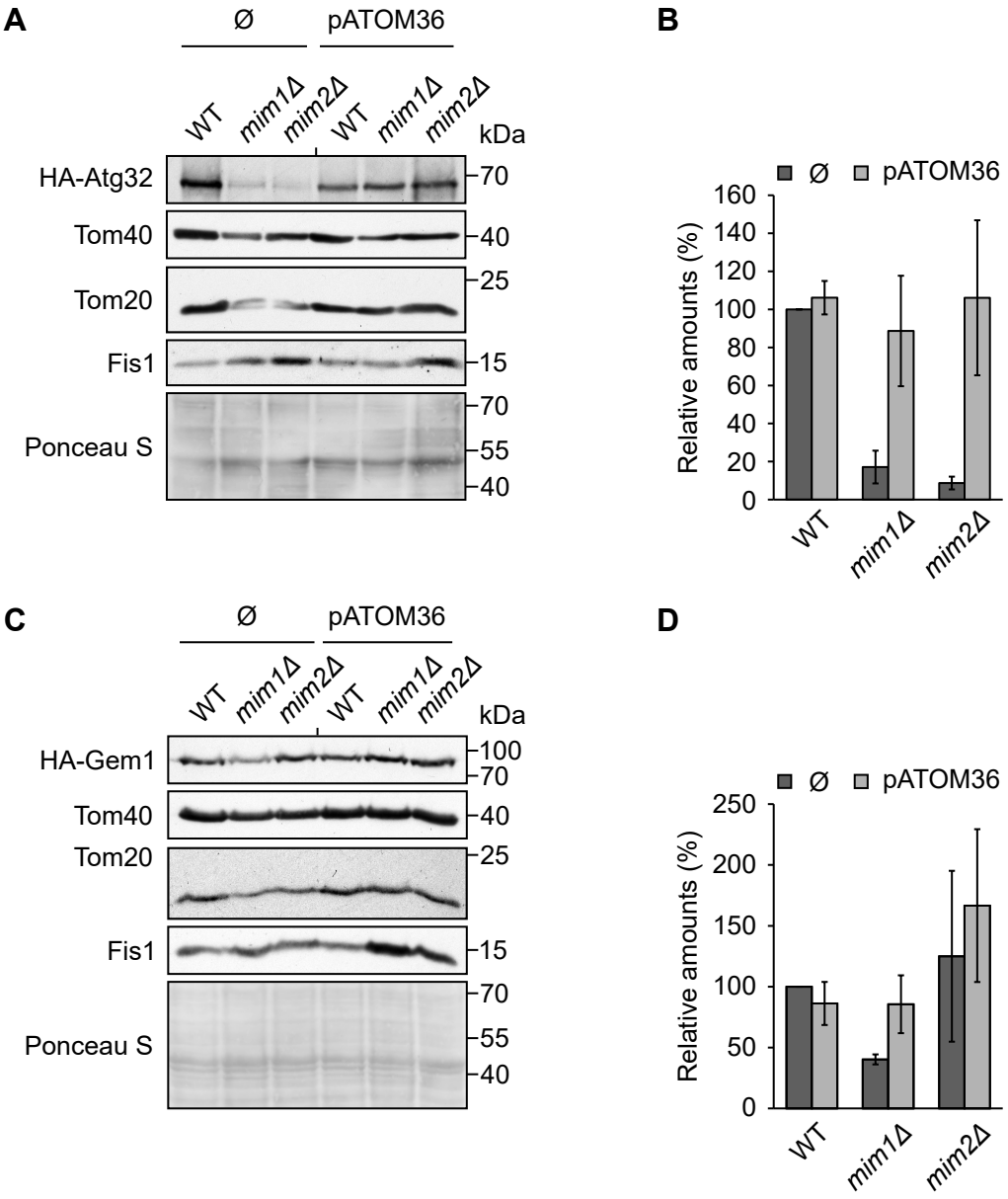

Figure S4. Related to Figures 3 and 4. The reduced levels of HA-Atg32 and HA-Gem1 in the absence of the MIM complex can be complemented by pATOM36.
(A and C) Crude mitochondrial fractions were obtained from WT, *mim1Δ*, and *mim2Δ* cells transformed with a plasmid encoding HA-Atg32 (A) or HA-Gem1 (C) and with either an empty plasmid (Ø) or a plasmid overexpressing pATOM36. Samples were analysed by SDS-PAGE and immunodecoration with anti-HA and the indicated antibodies.
(B and D) The intensity of the bands corresponding to HA-Atg32 (B) or HA-Gem1 (D) from at least three independent experiments as in (A) or (C), respectively, were quantified and normalized to the Ponceau S signal. The levels are presented as percentage of the levels in WT cells transformed with the empty plasmid. Error bars represent ± SD.

Transparent Methods

Yeast strains and growth conditions

Yeast strains used in the study were isogenic to *Saccharomyces cerevisiae* strain W303a, W303α, YPH499, or JSY7452. All strains used in this study are listed in Table S1. Standard genetic techniques were used for growth and manipulation of yeast strains. Yeast cells were grown in either rich medium YP (2% [w/v] bacto peptone, 1% [w/v] yeast extract) or synthetic medium S (0.67% [w/v] bacto-yeast nitrogen base without amino acids). Glucose (2% [w/v]), galactose (2% [w/v]), sucrose (2% [w/v]), or lactate (2% [w/v]) were used as carbon source. Transformation of yeast cells was performed by the lithium acetate method. For drop-dilution assay, cells were grown in a synthetic medium to logarithmic phase and diluted in fivefold increments followed by spotting five µl of the diluted cells on solid media.

Recombinant DNA techniques

The *MSP1* open reading frame (ORF) was amplified by PCR from yeast genomic DNA with specific primers containing BamHI and HindIII restriction sites. The yeast Kozak sequence was introduced directly upstream of the start codon via a primer. The PCR product was cloned into the plasmid pGEM4 to obtain pGEM4-yk-Msp1. The pYX142-Msp1(TMS)-GFP plasmid was obtained by PCR amplification of the DNA sequence encoding amino acid residues 1-32 of Msp1 using pYX142-Msp1-3HA as a template. Primers containing EcoRI and KpnI restriction sites were used and the PCR product was inserted upstream and in-frame with the eGFP coding region of pGEM4-eGFP plasmid. Next, the sequence encoding Msp1(TMS)-GFP was sub-cloned, using EcoRI and BamHI restriction sites, from the pGEM4 plasmid to the yeast expression pYX142 plasmid.

To insert the *FIS1* promotor (pr) and terminator (ter) into the pRS316 plasmid, DNA segments of around 500 bp upstream and downstream of the *FIS1* ORF were amplified by PCR from genomic DNA. The promotor region was cloned between SpeI and XmaI restriction sites, while the terminator sequence was inserted between HindIII and SalI sites. To obtain the pRS316-*FIS1*pr-Fis1(cyt)-*FIS1ter*, the sequence encoding for the cytosolic domain of Fis1 (amino acid residues 1-103) was amplified by PCR from genomic DNA and inserted with XmaI and NheI restriction sites between the *FIS1* promotor and terminator segments. The plasmid pRS316-3HA-Gem1 was obtained upon amplifying by PCR the *GEM1* ORF from pYX132-GFP-Gem1 (Cichocki et al., 2018) with primers containing the XmaI and HindIII restriction

sites. The obtained DNA fragment was inserted into the pRS316-*FIS1pr*-*FIS1ter* plasmid. The 3xHA tag was amplified by PCR form pYX142-3HA-YadA (Müller et al., 2011) and inserted in-frame at the N-terminus of Gem1, between two XmaI restriction sites.

The pRS316-*FIS1*pr-3HA-Fis1(cyt)-*FIS1ter* plasmid was obtained by amplifying the 3xHA tag by PCR from pYX142-3HA-YadA (Müller et al., 2011) with primers containing XmaI and EcoRI restriction sites and inserting it into the pRS316-*FIS1pr*-Fis1cyt-*FIS1ter* plasmid. To obtain the plasmid pRS316-3HA-Fis1, the *FIS1* ORF was amplified by PCR from genomic DNA with primers containing EcoRI and HindIII restriction sites and inserted into the pRS316-*FIS1pr*-3HA-*FIS1ter* plasmid. The pRS316-3HA-Gem1(cyt) plasmid, encoding the cytosolic domain of Gem1 (amino acid residues 1-634), was obtained by digesting pRS316-HA-Gem1 with SalI and XhoI, employing the SalI restriction site present in the *GEM1* ORF upstream the sequence coding for the TMS of the protein. To construct the plasmid pRS316-3HA-Gem1(cyt)-Fis1(TMS), the sequence coding for Fis1 TMS (amino acid residues 129-155) followed by *FIS1* terminator was amplified by PCR from pRS316-3HA-Fis1 with primers containing SalI and Xho1 restriction sites and inserted in-frame into pRS316-3HA-Gem1(cyt).

The pRS316-3HA-Fis1-Atg32(IMSD) plasmid was obtained upon PCR amplification of the *FIS1* ORF without the stop codon, using pRS316-3HA-Fis1 plasmid as a template, and inserting it into the pRS316-*FIS1pr*-3HA-*FIS1ter* between EcoRI and NheI restriction sites. Subsequently, the sequence coding for the IMS domain of Atg32 (amino acid residues 431-529 aa) was amplified by PCR from the pRS316-3HAn-Atg32 plasmid (Okamoto et al., 2009) with primers containing the NheI and HindIII restriction sites and inserted in-frame downstream the *FIS1* ORF. pRS316-3HA-Fis1(cyt)-Atg32(TMS+IMSD) was constructed by PCR amplification of the sequence coding for Atg32 TMS and IMS domain (amino acid residues 389-529), employing pRS316-3HAn-Atg32 (Okamoto et al., 2009) as template. The PCR product was inserted into pRS316-*FIS1pr*-3HA-Fis1(cyt)-*FIS1Ter* between NheI and HindIII restriction sites. pRS316-3HAn-Atg32(ΔC) and pRS316-3HAn-Atg32(cyt)-Fis1(TMS) were constructed as follows, a point mutation was introduced in the pRS316-3Han-Atg32(1-388)-TM^{pexo}- plasmid (Kondo-Okamoto et al., 2012) to insert the NheI restriction site directly downstream the sequence encoding for the TMS of Pex15. Afterwards, this latter sequence, which was located between two NheI sites, was excised and then the sequences coding for the TMS of either Atg32 (a.a. residues 389-430) or of Fis1 (amino acid residues 129-155), amplified from pRS316-3HAn-Atg32 and pRS316-HA-Fis1 respectively, were inserted between the two NheI restriction sites. All constructs were confirmed by DNA sequencing. Tables S2 and S3 include a full list of plasmids and primers, respectively, used in this study.

Biochemical methods

Protein samples for immunodecoration were analysed on 12.5% SDS-PAGE and subsequently transferred onto nitrocellulose membranes by semi-dry Western blotting. Before loading on the gels, the samples were heated for 10 min at either 50°C, for samples containing variants of Atg32, or at 95°C, for all other samples. Proteins were detected by incubating the membranes first with primary antibodies and then with horseradish peroxidase-conjugates of either goat anti-rabbit or goat anti-rat secondary antibodies. Band intensities were quantified with AIDA software (Raytest). See Table S4 for a list of primary antibodies used in this study.

Subcellular fractionation was performed as described before (Walther et al., 2009). Isolation of mitochondria from yeast cells was performed by differential centrifugation, as previously described (Daum et al., 1982). To obtain highly pure mitochondria, isolated organelles were layered on top of a Percoll gradient and isolated according to a published procedure (Graham, 2001).

To obtain fractions of crude mitochondria, cells were ruptured with glass beads (Ø 0.25-0.5 mm) using FastPrep-24 5G (MP Biomedicals) for 40 sec, 6.0 m/sec. The samples were then centrifuged (20000g, 10 min, 4°C) and the pellet was resuspended in a 2xLämmli solution.

Protein stability assay

For the protein stability assay, cells were grown to logarithmic phase and then treated with cycloheximide (CHX) at final concentration of 0.1 mg/ml. Samples were collected at different time points, crude mitochondria were obtained as described above and samples were analysed by SDS-PAGE and immunoblotting.

***In vitro* synthesis and mitochondrial import of radiolabelled proteins**

Cell-free transcription was performed with SP6 polymerase from pGEM4 plasmid encoding *MSP1*. The protein was then translated *in vitro* from the acquired mRNA in rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-methionine. Protein import was performed by adding 50 µl of the reticulocyte lysate (containing the translated protein) to 30 µg of isolated mitochondria diluted to a final concentration of 1 µg/µl in import buffer (250 mM sucrose, 0.25 mg/ml BSA, 80 mM KCl, 10 mM MOPS-KOH, 5 mM $MgCl_2$, 8 mM ATP and 4 mM NADH, pH 7.2). Import of Msp1 was performed at 10°C for 2, 5, 10, or 20 minutes. The import reactions were terminated by diluting the reaction with 400 µl SEM-K^{80} buffer (250 mM sucrose, 80 mM KCl, 10 mM MOPS, 1 mM EDTA, pH 7.2) and pelleting the mitochondria (13200g, 10 min, 2°C). The pellet fraction was subjected to alkaline extraction by resuspending

it in 100 µl of 0.1 M Na_2CO_3 solution and incubation for 30 min on ice. Then, the membrane fraction was isolated by centrifugation (180000g, 30 min, 2°C). The pellet was resuspended with 40 µl of 2xLaemmli buffer, heated for 10 min at 95°C, and analysed by SDS-PAGE followed by autoradiography.

Fluorescence microscopy

Microscopy images of strains expressing Msp1(TMS)-eGFP, eGFP, and mtRFP were acquired with an Axioskop 20 fluorescence microscope equipped with an Axiocam MRm camera using the 43 Cy3 filter set and the AxioVision software (Carl Zeiss).

Table S1. Yeast strains used in this study

Name	Mating type	Genetic background	Source or reference
W303a	MATa	*ade2-1 can1-100 his3-11 leu2 3_112 trp1Δ2 ura3-52*	Lab stock
W303α	MATα	*ade2-1 can1-100 his3-11 leu2 3_112 trp1Δ2 ura3-52*	Lab stock
YPH499	MATa	*ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*	Lab stock
JSY7452	MATα	*ade2-1 can1-100 his3-11,15 leu2-3 trp1-1 ura3-1*	(Kondo-Okamoto et al., 2006)
mim1Δ	MATa	W303a; *mim1Δ::KanMX*	(Dimmer et al., 2012)
mim2Δ	MATa	W303a; *mim2Δ::HIS3*	(Dimmer et al., 2012)
mim1/2Δ	MATa	W303a; *mim1Δ::KanMX mim2Δ::HIS3*	(Dimmer et al., 2012)
tom20Δ	MATα	W303α*; tom20Δ::HIS3*	(Müller et al., 2011)
tom70/71*Δ*	MATa	JSY7452; *tom70Δ::TRP1 tom71Δ::HIS3*	(Kondo-Okamoto et al., 2008)
mas37Δ	MATa	YPH499; *mas37Δ::HIS3*	(Habib et al., 2005)

Table S2. Plasmids used in this study

Plasmid	Promoter	Coding sequence (aa)	Markers	Source
pGEM4-yk-Msp1	SP6	Msp1 full length	Amp^R	This study
PYX142-Msp1-3HA	TPI	Msp1 full length	LEU2, Amp^R	This study
pGEM4-eGFP	SP6	GFP	Amp^R	Lab stock
PRS416-MTS-RFP	TPI	MTS-RFP	URA3 Amp^R	Lab stock
pYX142-Msp1(TMS)-eGFP	TPI	Msp1(1-32)	LEU2, Amp^R	This study
pRS316-3HAn-Atg32	Atg32Pr (580 bp 5'-UTR / 744 bp 3'-UTR from *ATG32*)	Atg32 full length	URA3, Amp^R	(Okamoto et al., 2009)
pRS316-3HA-Gem1	Fis1Pr (496 bp 5'-UTR / 408 bp 3'-UTR from *FIS1*)	Gem1 full length	URA3, Amp^R	This study
pYX142	TPI		LEU2, Amp^R	Lab stock
pYX142-pATOM36	TPI	pATOM36 full length	LEU2, Amp^R	(Vitali et al., 2018)
pRS316-3HAn-Atg32(ΔC)	Atg32Pr (580 bp 5'-UTR / 744 bp 3'-UTR from *ATG32*)	Atg32(1-430)	URA3, Amp^R	This study
pRS316-3HAn-Atg32(cyt)-Fis1(TMS)	Atg32Pr (580 bp 5'-UTR / 744 bp 3'-UTR from *ATG32*)	Atg32(1-388) + Fis1(129-155)	URA3, Amp^R	This study
pRS316-3HA-Gem1(cyt)-Fis1(TMS)	Fis1Pr (496 bp 5'-UTR / 408 bp 3'-UTR from *FIS1*)	Gem1(1-634) + Fis1(129-155)	URA3, Amp^R	This study
pRS316-3HA-Fis1-Atg32(IMSD)	Fis1Pr (496 bp 5'-UTR / 408 bp 3'-UTR from *FIS1*)	Fis1(1-155) + Atg32(431-529)	URA3, Amp^R	This study
pRS316-3HA-Fis1(cyt)-Atg32(TMD+IMSD)	Fis1Pr (496 bp 5'-UTR / 408 bp 3'-UTR from *FIS1*)	Fis1(1-103) + Atg32(389-529)	URA3, Amp^R	This study
pRS316-3HA-Fis1	Fis1Pr (496 bp 5'-UTR / 408 bp 3'-UTR from *FIS1*)	Fis1 full length	URA3, Amp^R	This study
pYX142-Mim1	TPI	Mim1 full length	LEU2, Amp^R	(Dimmer et al., 2012)

Table S3. Primers used in this study

Primer name	Sequence (5'-3')	Note
BamHIMsp1F	GGGGGATCCAAAAAAATGTCTCGCAAATTTGATTTAAAAACGATTACTGATCTTT	Amplification of *MSP1*, BamHI restriction site at 5'
HindIIIMsp1R	GGGAAGCTTTTAATCAAGAGGTTGAGATGACAAC	Amplification of *MSP1*, HindIII restriction site at 5'
EcoRIMsp1F	GGGGAATTCATGTCTCGCAAATTTGATTTAAAAACGATTACTG	Amplification of the sequence encoding the TMS of Msp1 (a.a. 1-32), *Eco*RI restriction site at 5'
KpnIMsp1R	GGGGGTACCGCCCGGGCCGTTGAGTAGCCGACTGACCAGGTAG	Amplification of the sequence encoding the TMS of Msp1 (a.a. 1-32), *Kpn*I restriction site at 5'
XmaIGem1F	GGGCCCGGGACTAAAGAAACGATTCGGGTAG	Amplification of *GEM1*, XmaI restriction site at 5'
HindIIIGem1R	CCCAAGCTTTTATTTTGAGAATTTTGATGATTTGAATAATTTCAT	Amplification of *GEM1*, HindIII restriction site at 5'
XmaIHAF	CCCCCCGGGATGTACCCATACGATGTTCCTG	Amplification of HA coding sequence, XmaI restriction site at 5'
XmaIHAR	GGGCCCGGGAGCGTAATCTGGAACGTCAT	Amplification of HA coding sequence, XmaI restriction site at 5'
Xma1Fis1CytF	GGGCCCGGGATGACCAAAGTAGATTTTTGGCC	Amplification of sequence encoding the cytosolic domain of Fis1, XmaI restriction site at 5'
NheIHindIIIFis1CytR	CCCAAGCTTGCTAGCTAAAGTGTCTACATATCTCTTCGCC	Amplification of sequence encoding the cytosolic domain of Fis1, NheI and HindIII restriction site at 5'
mutKOB131F	CTAGCGAGTATAGCTAGCTAACCTT	Insertion of point mutation to include NheI restriction site in pRS316-atg32(1-388)-TM^{pexo}-3HAn
mutKOB131R	AAGGTTAGCTAGCTATACTCGCTAG	Insertion of point mutation to include NheI restriction site in pRS316-atg32(1-388)-TM^{pexo}-3HAn
NheIAtg32TMDF	CCCGCTAGCAGCTGGTTCACTTGGGGCATTTC	Amplification of sequence encoding the TMS of Atg32, NheI restriction site at 5'
NheIAtg32TMD+R	GGGGCTAGCCAATATGGAGGGCCGCAAACTTAAAG	Amplification of sequence encoding the TMS of Atg32, NheI restriction site at 5'
NheIFis1TMDF	CCCGCTAGCCTCAAGGGTGTTGTCGTCG	Amplification of sequence encoding the TMS of Fis1, NheI restriction site at 5'
NheIFis1TMDR	GGGGCTAGCTTACCTTCTCTTGTTTCTTAAGAAGAAAC	Amplification of sequence encoding the TMS of Fis1, NheI restriction site at 5'
SalIFis1TMDF	GGGGTCGACTACAGACAAACGGCTCTCAAGGGTGTTGTCGTCGC	Amplification of sequence encoding the TMS of Fis1, SalI restriction site at 5'
XhoIFis1TerR	CCCCTCGAGATCTCACAATACAGTATTACGATTTAACAATAGACTATTG	Amplification of sequence encoding the TMS of Fis1, XhoI restriction site at 5'
EcoRIFis1HACytF	GGGGAATTCATGACCAAAGTAGATTTTTGGCC	Amplification of *FIS1* without stop codon, EcoRI restriction site at 5'
NheIFis1NOSTOPR	CCCGCTAGCCCTTCTCTTGTTTCTTAAGAAGAAAC	Amplification of *FIS1* without stop codon, NheI restriction site at 5'
NheIAtg32IMSDF	GGGGCTAGCGCCTCTTTACTTTCCTTAGATTCCTCTAG	Amplification of sequence encoding the IMS domain of Atg32, NheI restriction site at 5'

HindIIIAtg32R	CCCAAGCTTTTACAATAGAATATAA CCCAGTGCCAAAATC	Amplification of sequence encoding the IMS domain of Atg32, HindIII restriction site at 5’
5-Fis1Pr-SpeI	AAAACTAGTTCAAATAACATGTGTC CATTACC	Amplification of Fis1Pr, SpeI restriction site at 5’
3-Fis1pr-SmaI	AAACCCGGGGTTGTATGGCTGTG	Amplification of Fis1Pr, SmaI restriction site at 5’
5-Fis1THindIII	CCCAAGCTTATAAAAAATCAGCACA TACGTACATAC	Amplification of Fis1Term, HindIII restriction site at 5’
3-Fis1TSalI	CCCGTCGACATCTCACAATACAGTA TTACG	Amplification of Fis1Term, SalI restriction site at 5’

Table S4. Antibodies used in this study

Antibodies	**Dilution**	**Source**
polyclonal rat anti-HA	1 : 1000	11867423001 (Roche)
polyclonal rabbit anti-GFP	1: 2000	TP401 (Torrey Pines)
polyclonal rabbit anti-Mcr1	1 : 2000	Lab stocks
polyclonal rabbit anti-Tom40	1 : 4000	Lab stocks
polyclonal rabbit anti-Tom20	1 : 5000	Lab stocks
polyclonal rabbit anti-Fis1	1 : 1000	Lab stocks
polyclonal rabbit anti-Tom70	1 : 5000	Lab stocks
polyclonal rabbit anti-Mim1	1 : 500	Lab stocks
polyclonal rabbit anti-Msp1	1 : 1000	Lab of Toshiya Endo
polyclonal rabbit anti-Pic2	1 : 2000	Lab stocks
polyclonal rabbit anti-Bmh1	1 : 1500	Lab stocks
polyclonal rabbit anti-Erv2	1 : 2000	Lab of Roland Lill

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