

Cell Reports

Supplemental Information

Lam6 Regulates the Extent of Contacts between Organelles

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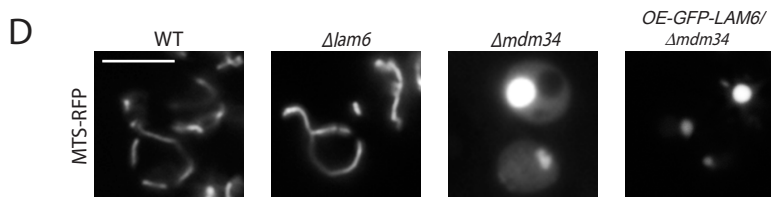
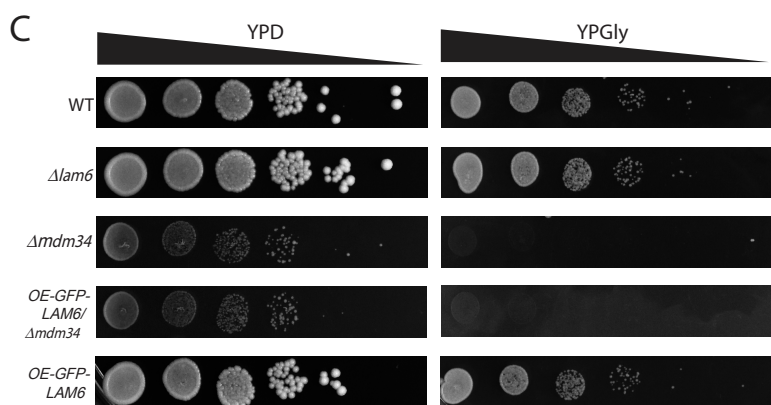
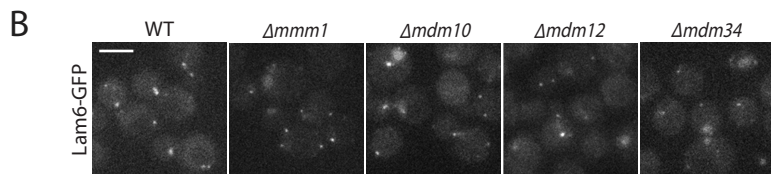
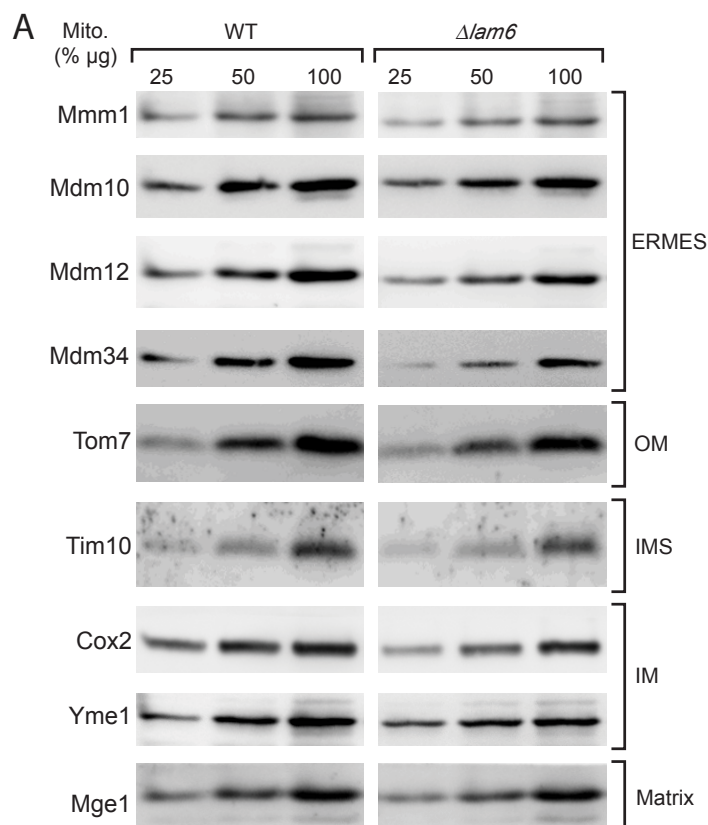


Figure S1: Lam6 is an uncharacterized binding partner of the ERMES complex. Related to figure 1.

(A) ERMES complex proteins remain in similar abundance following loss of Lam6 as do other mitochondrial proteins. Mitochondria were isolated from the yeast strains indicated. Mitochondrial proteins (% μg protein) were analyzed by SDS-PAGE and Western blotting. OM, outer membrane; IMS, intermembrane space; IM, inner membrane. (B) Deletion of any of the ERMES subunits did not result in a change in Lam6-GFP punctate structure, implying that it is not an essential part of the ERMES complex. Bar = 5 μm . (C) Serial dilutions of yeast strains demonstrate that unlike the *ERMES* mutant- Δmdm34 , *LAM6* mutants displayed normal growth on a fermentable ($\text{YPD}_{\text{extrose}}$) as well as a non-fermentable carbon source ($\text{YPGly}_{\text{cerol}}$). (D) *LAM6* mutants have a normal mitochondrial shape, in contrast to the *ERMES* mutant Δmdm34 . Overexpression of GFP-Lam6 did not affect the growth of a Δmdm34 strain (C) as well as its mitochondria's shape (D). Bar = 5 μm .

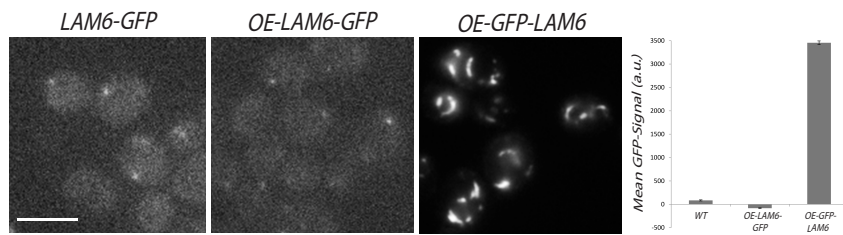


Figure S2: Tagging of Lam6 at its N' stabilizes the protein. Related to figure 2.

Replacing the endogenous promoter of C-terminally-GFP-tagged Lam6 with a constitutive promoter (*OE-LAM6-GFP*) did not affect the punctuate pattern of the protein, nor elevated the fluorescence GFP signal, implying that the protein was not overexpressed. However, replacing the promoter of an N-terminally-GFP-tagged Lam6 with a constitutive promoter (*OE-GFP-LAM6*) resulted in a robust, elevated GFP signal and the protein could be now seen clearly at the different contact sites. Flow cytometry was used in order to evaluate GFP-tagged Lam6 levels, bars are mean GFP-intensity (arbitrary units) normalized to the background signal of an untagged strain, n=3. Bar = 5 μm .

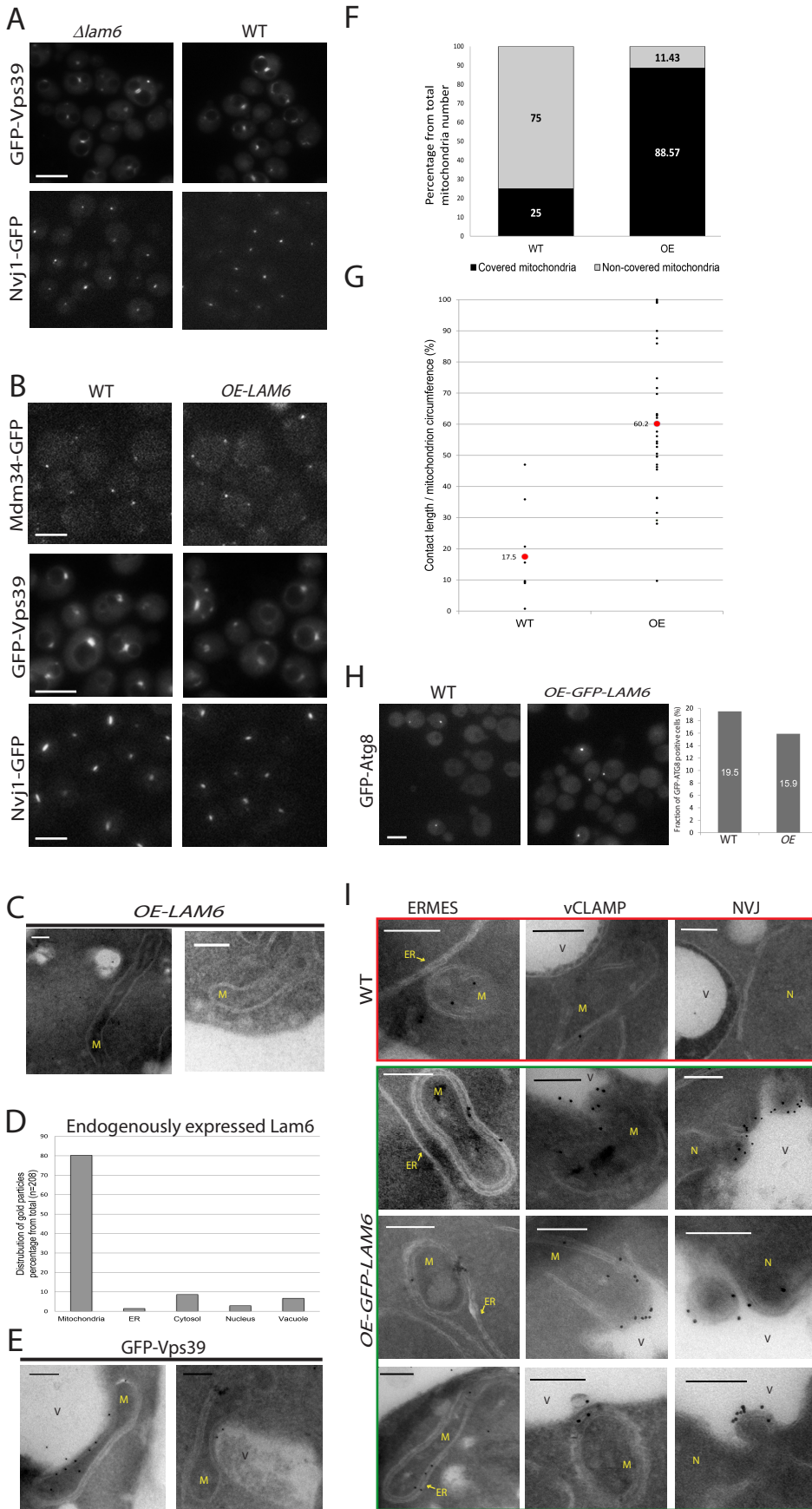


Figure S3: Overexpression of tagged Lam6 increases the extent of contact sites. Related to figure 3.

(A) Deletion of *LAM6* did not prevent the formation of NVJ (marked by Nvj1-GFP) or vCLAMP (marked by GFP-Vps39), confirming that Lam6 is not required for the formation of these contact sites. Bar = 5 μ m. (B) Overexpression of the non-tagged form of Lam6 did not affect the extent of the three contact sites: ERMES (marked by Mdm34-GFP), vCLAMP (marked by Vps39) and NVJ (marked by Nvj1-GFP). Bar = 5 μ m. (C) Replacing the endogenous promoter of Lam6 with a constitutive one alone did not result in the same expansion of the ER-mitochondria contact sites as in the case of overexpression of GFP-Lam6. Mitochondria were not surrounded with ER tubules in this condition. Bar = 200 nm. (D) Graph showing distribution analysis of gold particles in immunoelectron microscopy analysis of an Lam6-GFP strain, using an antibody against GFP. Lam6 accumulates specifically in mitochondria most probably representing ERMES contacts. (E) Upon overexpression of GFP-Lam6 instances in which vacuoles and mitochondria were found in close proximity were markedly higher. The vCLAMPs were validated by Immuno-labeling GFP-Vps39. Bar = 200 nm. (F) The number of mitochondrion that had visible contact with ER, in the endogenously expressed Lam6 strain and in the overexpressing GFP-Lam6 strain, was calculated. Bars represent the percentage of mitochondria that had contact with ER from the total mitochondria number (for WT n=36, for OE n=35). (G) The length of the contact site was measured and the percentage of the contact length out of the total circumference of the mitochondria was calculated. The average percentage is shown in red for the endogenously expressing and overexpressing GFP-Lam6 strains. (H) Fluorescence microscopy imaging of the

autophagy regulator Atg8. GFP-Atg8 levels did not increase upon overexpression of GFP-Lam6, confirming that the membranes surrounding the mitochondria are not part of autophagosomes. Bar = 5 μ m. (I) Additional images from immunoelectron microscopy of Lam6 overexpression (supplementing Figure 3B). N, nucleus, M, mitochondria, V, Vacuole. Bar = 200 nm.

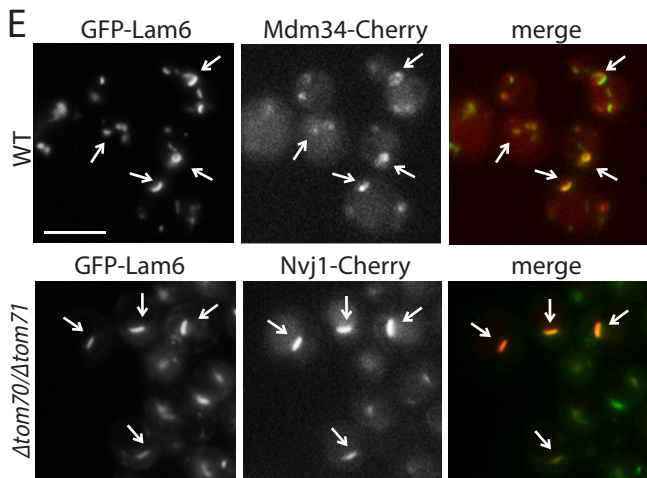
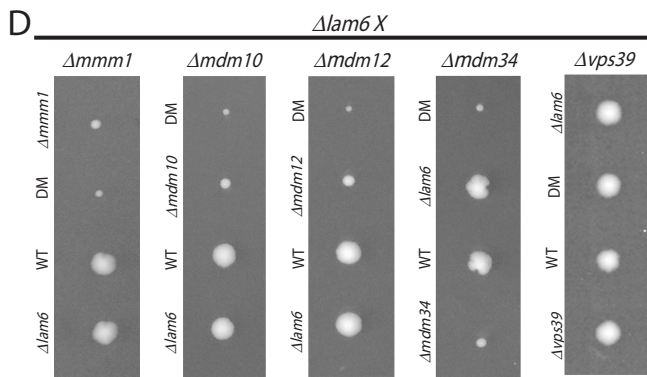
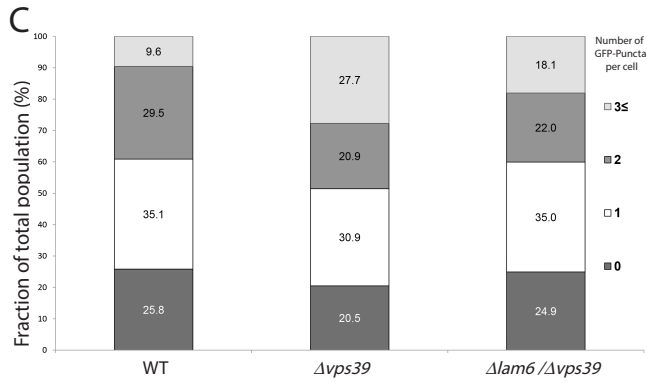
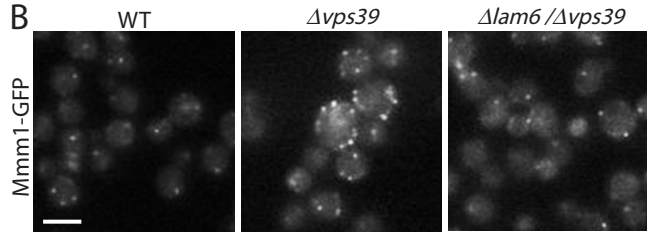
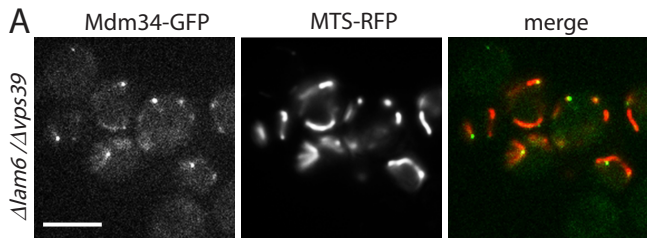


Figure S4: Lam6 is important for cross-talk between contact sites. Related to figure 4 and to the discussion.

(A) When deleting both *LAM6* and *VPS39*, the ERMES subunit Mdm34-GFP remained co-localized with mitochondria. Bar = 5 μ m. (B) Fluorescent microscopy demonstrating that the ERMES contact (as measured by the number of Mmm1-GFP puncta per cell, supplementing Figure 4A where the same experiment was performed with Mdm34-GFP) expanded in the Δ *vps39* background relative to WT. However the expansion did not occur on the background of Δ *vps39* Δ *lam6* demonstrating that Lam6 is necessary for ERMES expansion under these conditions. Bar = 5 μ m. (C) Quantitation of (B). Bars represent the percentage of cells containing specific number of puncta/cell out of total cells counted for the strain (for WT n=271 cells, for Δ *vps39* n=278, for Δ *vps39* Δ *lam6* n=277). (D) Δ *lam6* strain was crossed with strains containing a deletion in each of the ERMES subunits or Δ *vps39* to create diploids. Tetrad analysis of ascospores resulting from meiosis of those diploids demonstrate that in all cases combination of mutations in both *LAM6* and ERMES subunits resulted in decreased growth (synthetic sick phenotype) when comparing them to either single mutants. In contrast, mutants for Lam6 and Vps39 were not synthetic sick. (E) In WT cells most of the overexpressed GFP-Lam6 co-localized with ERMES (Mdm34). Deletion of Tom70 and Tom71 resulted in complete relocalization of Lam6 to the NVJ, as marked by Nvj1-Cherry. Bar = 5 μ m.

Supplemental Figure legends:

Table S1: Protein-protein interaction analysis of Mdm10, Mdm12, Mdm34 and Mmm1. Related to figure 1.

Expression levels are given as label-free quantification (LFQ) normalized intensities. Significant binders (marked with +) were extracted with a Welch's test with permutation based FDR=0.05 and S0=0.5. For each test the p-values (-log value) and the test difference are provided.

Table S2: Protein-protein interaction analysis of Lam6. Related to figure 1.

Expression levels are given as label-free quantification (LFQ) normalized intensities. Significant binders (marked with +) were extracted with a Welch's test with permutation based FDR=0.05 and S0=0.5. For each test the p-values (-log value) and the test difference are provided.

Table S3: Yeast strains used in this study. Related to figures 1-4.

Yeast strains created and used in this study are listed in the table. All yeast strains in this study are based on the BY4741 laboratory strains (Brachmann et al., 1998).

Supplemental Experimental Procedures

Electron microscopy

For immunoelectron microscopy cells were fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in 0.1M cacodylate buffer (pH=7.4), for 1 hour at room temperature and kept at 4°C during 1-2 days. The samples were soaked overnight in 2.3 M sucrose and rapidly frozen in liquid nitrogen. Frozen ultrathin (70-90 nm) sections were cut with a diamond knife at -120°C on an EM UC6 ultramicrotome (Leica Microsystems, Vienna, Austria). The sections were collected on 200-mesh formvar coated nickel grids. Sections were blocked by a blocking solution containing 0.5% BSA, 0.2% glycine, 0.5% gelatin and 0.1% Tween-20. Immuno-labeling was performed using rabbit polyclonal anti-GFP antibody (ab6556, 1:100; Abcam) during 1.5-2 hours at room temperature (RT) followed by goat anti-rabbit IgG coupled to 10-nm gold particles (1:20 dilution) 30 min at RT. Contrasting and embedding were performed as described (Tokuyasu, 1986). The embedded sections were scanned and digitally viewed on transmission electron microscopes Tecnai Spirit (FEI, Eindhoven, the Netherlands) at 120kV using a CCD Eagle camera with TIA software (FEI) or Tecnai T12 electron microscope (FEI) operating at 120 kV. Images were recorded with an ErlangshenES500W CCD camera (GATAN).

Of note, although gold particles appeared inside the mitochondria, the labeling most probably represent ERMES contact sites, reminiscent of the fluorescence microscopy pattern, and the appeared mislocalization is the result of the slicing plane as well as antibody size.

In electron micrographs, vacuoles may appear as black areas of condensed vacuolar content or as white areas where the vacuoles were before they collapsed and detached from the slide (Guthrie and Fink, 2002).

Isolation of microsomal fractions

Pull downs of individual ERMES components (Mmm1-3HA, Mdm34-3HA, Mdm12-3HA and Mdm10-3HA) for LC-MS/MS were performed from microsomal fractions. Sucrose-density-gradient purification of microsomes was performed essentially as described by (Wuestehube and Schekman, 1992). Strains were grown in YPD media to $OD_{600} \sim 1$, then harvested and washed once in 100 mM Tris-HCl pH 9.4, 10 mM dithiothreitol (DTT). Cells were then re-suspended in 0.7M sorbitol, 10mM Tris-HCl pH 7.4 buffer supplemented with 1.5% (w/v) bactopectone, 0.75% (w/v) yeast extract and 0.5% (w/v) glucose. Spheroplasts were generated by adding 3mg Zymolase (MP Biomedicals) and isolated by centrifugation through a 0.8 M sucrose cushion. Spheroplast pellet was re-suspended in lysis buffer (0.1M sorbitol, 20 mM HEPES, pH 7.4, 50 mM potassium acetate, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT and protease inhibitors (Sigma) and subjected to 25 strokes in a Dounce homogenizer. The resulting homogenate was centrifuged at 1000g and the combined low-speed supernatant was centrifuged at 27,000 g (Ti60 rotor; Beckman Instruments). The membrane fraction was then collected, re-suspended in lysis buffer and fractionated through a 2-step sucrose gradient of 1.2 M and 1.5 M sucrose by centrifugation at 100,000 g (SW41 rotor; Beckman Instruments) for 1 hour at 4°C. The microsomal fraction residing at the gradient interface was collected, washed and re-suspended in reaction buffer (20 mM HEPES pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM Sorbitol). Total protein concentration of every sample was determined using BCA reagent (Thermo Scientific).

Isolation of mitochondria-enriched fractions

Pull down of Lam6-GFP and GFP-Lam6 for LC-MS/MS analysis was performed from mitochondria-enriched preparations. Isolation of mitochondria-enriched fractions was performed

essentially as previously published (Daum et al., 1982). Briefly, strains were grown in YPD media to $OD_{600} \sim 1$, then harvested and washed once in 100 mM Tris-HCl pH 9.4, 10 mM DTT. Cells were then re-suspended in sorbitol buffer (1.2M sorbitol, 20mM KPi buffer pH 7.4). Spheroplasts were generated by adding 3 mg Zymolase, until 95% of cells were converted into spheroplasts according to OD measurement (normally takes 40-45 min) (MP Biomedicals), washed in same buffer and isolated by centrifugation 5 min at 2000g at 4°C. Spheroplast pellet was re-suspended in homogenization buffer (0.6 M sorbitol, 20 mM HEPES, pH 7.4 and protease inhibitors (Sigma)) and subjected to 15 strokes in a Dounce homogenizer. The resulting homogenate was centrifuged at 800 g and the combined low-speed supernatant was centrifuged at 12,000 g for 15 min at 4°C (SS-34 rotor; Sorvall). The membrane fraction was then collected, re-suspended in sucrose buffer (250 mM sucrose, 10 mM HEPES pH 7.4 supplemented with protease inhibitors) and aliquoted. Total protein concentration of every sample was determined using BCA reagent (Thermo Scientific).

Interaction proteomics

Pull down of individual ERMES components from microsomal fractions (Mmm1-3HA, Mdm34-3HA, Mdm12-3HA and Mdm10-3HA), or LAM6-GFP from mitochondria-enriched preparations was performed as follows: fractions with 5 mg total protein were solubilized in 15mM Tris-HCl pH 7, 150 mM NaCl, 3% digitonin and protease inhibitors (Sigma P8215) for 1 hour on ice.

Samples containing HA-tagged ERMES components were incubated with μ MACS anti-HA beads (Miltenyi Biotec, Germany), while LAM6-GFP was incubated with GFP-trap agarose beads (Chromotek, Germany) for 45' followed by three washes with 150 mM NaCl, 15 mM Tris HCl pH 7. Elution was performed through on-bead digestion, by 2h incubation of the beads with 100 μ l of 2 M urea, 50 mM Tris HCl pH 7.5, 1 mM DTT and 0.4 μ g sequencing grade trypsin,

followed by an additional wash with 2 M urea, 50 mM Tris HCl pH 7.5 and 5 mM iodoacetamide. Eluates were combined and incubated over night at room temperature. Resulting peptides were acidified with Trifluoroacetic acid (TFA) and purified on C18 StageTips (Rappsilber et al., 2007). LC-MS/MS analysis was performed on the EASY-nLC1000 UHPLC (Thermo Scientific) coupled to the Q-Exactive mass spectrometer (Thermo Scientific). Peptides were loaded onto the column with buffer A (0.5% acetic acid) and separated on a 50 cm PepMap column (75 μ m i.d. 2 μ m beads; Dionex) using a 4 h gradient of 5-30% buffer B (80% acetonitrile, 0.5% acetic acid). Raw MS files were analyzed with MaxQuant (Cox and Mann, 2008) using the label-free algorithm for protein quantification (Cox et al., 2014). Significant interactors were extracted based on the statistical difference between the label-free quantification (LFQ) intensities of the proteins in the pull down of specific proteins and the control strains without HA-tag expression. One-sided Welch's test was performed with 0.05 permutation-based false discovery rate (FDR) and $S_0=0.5$ (Tusher et al., 2001).

Isolation of mitochondrial enriched fractions followed by western blot

Yeast strains BY4741 (WT) and $\Delta lam6::Kan$, were grown at 30 °C in YPD medium (1% yeast extract; 2% bacto-peptone, 2% glucose, pH 5.0 (HCl)) to mid-logarithmic phase. Mitochondria were isolated by differential centrifugation (Meisinger et al., 2006). Mitochondrial proteins (10 - 50 μ g) were separated by Tricine-SDS-PAGE (Schägger, 2006) and transferred onto polyvinylidene difluoride (PVDF) membranes. Proteins were detected by immunodecoration with rabbit antibodies against the indicated proteins followed by incubation with goat anti-rabbit IgG peroxidase conjugate (A6154, 1:5000, Sigma, St. Louis, MO, USA) and visualized by enhanced chemiluminescence (Haan and Behrmann, 2007) using the LAS imaging system (Fujifilm).

Flow cytometry

Yeast cells grown to mid-log in liquid synthetic media (SD) and flow cytometry analysis was performed using a BD LSR II flow cytometer (BD Biosciences). Results were analyzed using BD FACSDiva software.

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