

Figure S1. **Influence of LCA treatment on mitochondrial membrane proteins.** (A) Immunoblot analysis of mitochondria isolated from Ugo1<sub>HA</sub> strains that were incubated in the presence or absence of LCA. (B) Quantification of protein levels of mitochondria from LCA-treated or control Ugo1<sub>HA</sub> yeast cultures (-LCA values were set to 100%). Quantifications represent mean  $\pm$  SEM (error bars;  $n = 3$ ). (C) Import of <sup>35</sup>S-labeled Ugo1 precursor into mitochondria from cells, which were incubated in the presence or absence of LCA. Precursor was incubated for 20 min with mitochondria. Samples were washed and directly loaded on SDS-PAGE (binding; lanes 1 and 2), or carbonate-resistant fraction (lanes 3 and 4) and trypsin-protected proteins (fragment f1; lanes 5 and 6) were analyzed to monitor import. Porin immunosignal served as a loading control. For quantification, -LCA values were set to 100%. Quantifications represent mean  $\pm$  SEM (error bars;  $n = 3$ ). Carb., carbonate resistant protein. (D) Analysis of membrane protein complexes by blue native electrophoresis and immunoblotting of digitonin-solubilized mitochondria derived from yeast cultures incubated in the presence or absence of LCA. TOM, translocase of the outer membrane; SAM, sorting and assembly machinery of the outer membrane; SDH, succinate dehydrogenase complex of the inner membrane; V, complex V (ATP synthase) in the inner membrane; V<sub>2</sub>, dimer of complex V.

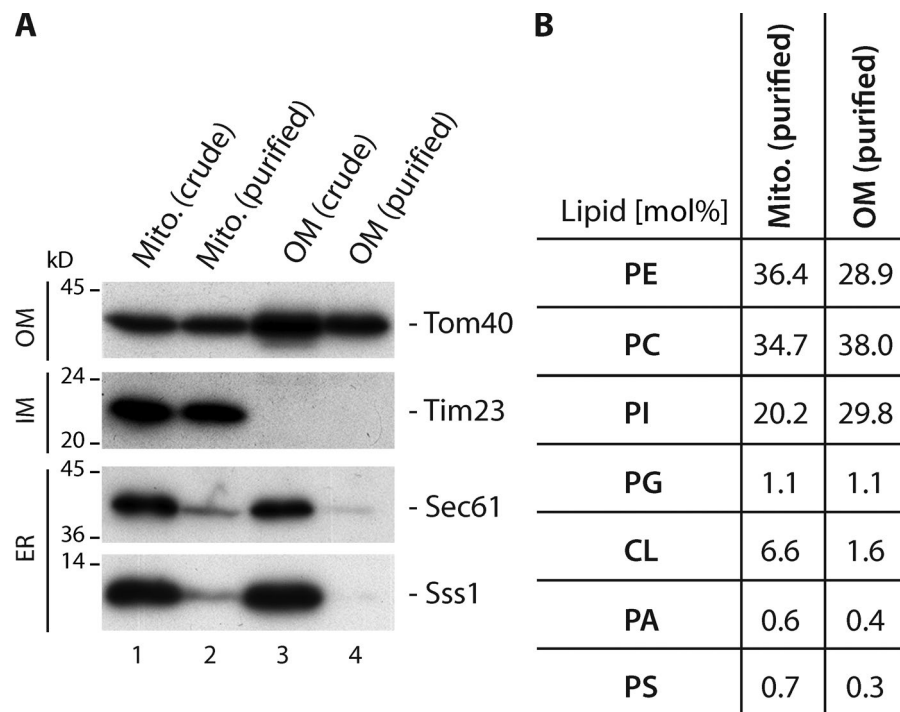


Figure S2. **Determination of mitochondrial outer membrane phospholipid composition by mass spectrometry.** (A) Enrichment of highly pure outer membrane fraction in comparison to OM fractions derived from standard mitochondrial isolations (crude). OM, outer membrane; IM, inner membrane. (B) Glycerophospholipid content (in mol%) of highly purified mitochondria (Mito.) and outer membrane vesicles (OM) analyzed by LCMS. CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

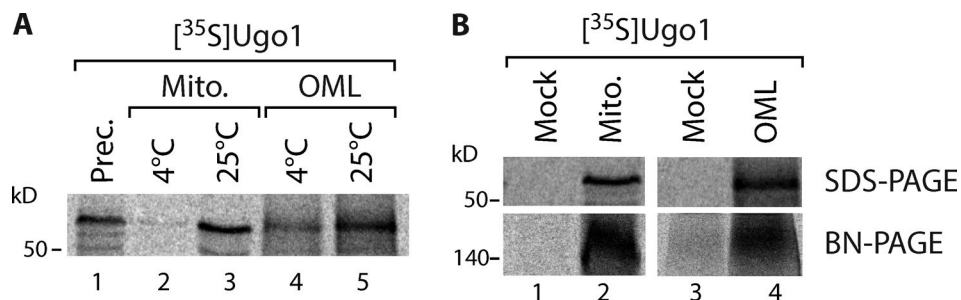


Figure S3. **Control reactions for Ugo1 import and assembly in OMLs.** (A)  $^{35}\text{S}$ -labeled Ugo1 precursor was imported into wild-type mitochondria or OMLs at 4°C or 25°C. Samples were carbonate-treated and the pellet fraction was separated by SDS-PAGE. Ugo1 was detected by autoradiography. Prec., Ugo1 precursor. (B) Mock controls for Ugo1 import reactions in mitochondria (Mito.) and OMLs. Samples were analyzed by SDS-PAGE or blue native electrophoresis after solubilization with digitonin. Membrane-integrated Ugo1 protein (top) and dimeric Ugo1 complex (bottom) were detected by autoradiography.

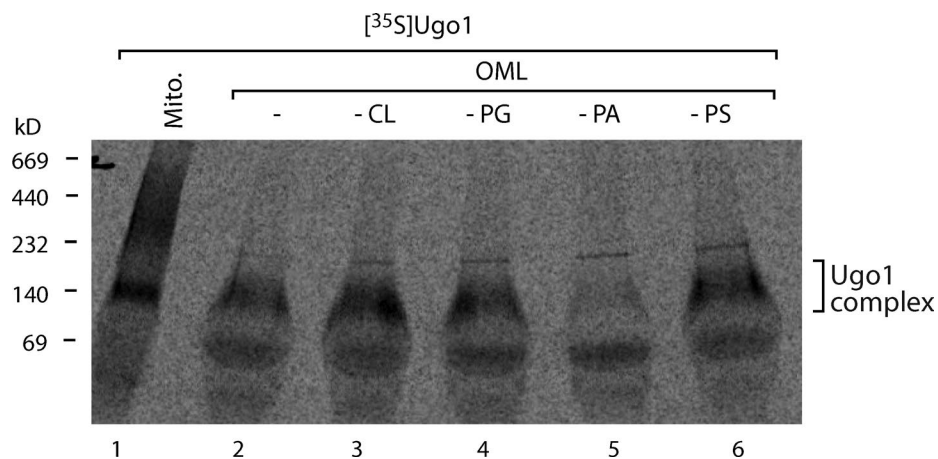


Figure S4. **Ugo1 assembly in OMLs lacking various phospholipids.** Assembly reaction of radiolabeled Ugo1 precursor in OMLs that lack CL, PG, PA, or PS. Samples were solubilized in digitonin buffer and subjected to blue native electrophoresis and autoradiography.

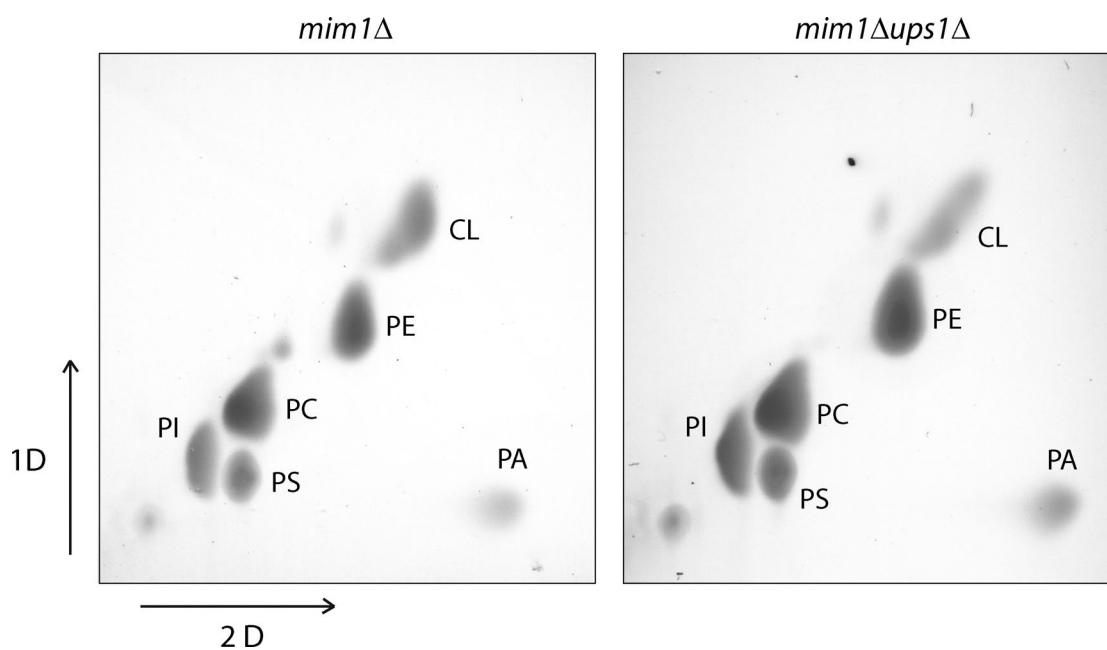


Figure S5. **TLC analysis of *mim1Δ* and *mim1Δups1Δ* mitochondria.** *mim1Δ* and *mim1Δups1Δ* mitochondria (1 mg each) were separated by two-dimensional thin-layer chromatography as described in Materials and methods. CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

Table S1. List of all antibodies used in this study

Antibodies directed against	Dilution	Number/company	Antigen	Secondary antibody
HA	1:2000 TBS	Roche		Anti-mouse
Tom20	1:5,000 TBS + 5% milk	GR 3225-7	AA 32-183	Anti-rabbit
Tom22	1:5,000 TBS + 5% milk	GR 3227-2	AA 1-97	Anti-rabbit
Tom40	1:500 TBS + 5% milk	168-5	Full-length protein	Anti-rabbit
Tom70	1:500 TBS + 5% milk	GR 657-3	AA 247-390	Anti-rabbit
Msp1	1:1,000 TBS + 5% milk	GR 1468-4	KIRPLKTKDFTKKLRMDA	Anti-rabbit
Pth2	1:500 TBS + 5% milk	GR 797-7	APKAVLDQITGDLKIY	Anti-rabbit
Mim1	1:500 TBS + 5% milk	GR 545-6	LKEISSPGTRGRVASKFL	Anti-rabbit
Scm4	1:500 TBS + 5% milk	GR 1473-2	TRDQNDLEKQKDEKLP	Anti-rabbit
Porin	1:1000 TBS + 5% milk	GR 3622-2	TRYLPDASSQVKAKVSD	Anti-rabbit
Om45	1:400 TBS-T + 5% milk	GR 1390-4	IKLKGPSKTALLSKK	Anti-rabbit
Mcr1	1:2,000 TBS + 5% milk	GR 613-7	GILNNLGYSKDQVFKF	Anti-rabbit
Mdv1 (affinity purified)	1:50 TBS-T	B 309	Full-length protein	Anti-rabbit
Fis1	1:2,000 TBS + 5% milk	B 310-4	IYKEAESRRREC	Anti-rabbit
Mpm1	1:500 TBS + 5% milk	GR 3096-2	PQVKHKVVSVDEND	Anti-rabbit
Mgm1	1:500 TBS-T + 5% milk	GR 795-4	DSILVFKSKYKGVSKNL	Anti-rabbit
Tim50	1:500 TBS + 5% milk	GR 3881-1	KIQLEKQKEVDALFEE	Anti-rabbit
Tim21	1:500 TBS + 5% milk	GR 3883-4	PVSNSKGFGLIRWGPRKD	Anti-rabbit
AAC	1:1,000 TBS + 5% milk	227-2	APKKESNFLID	Anti-rabbit
Aco1	1:2,000 TBS + 5% milk	GR 945-6	FKYGSALNKIKADEKK	Anti-rabbit
Mge1	1:500 TBS + 5% milk	23210-5	Full-length protein	Anti-rabbit
Atp2	1:1,000 TBS + 5% milk	GR 863-4	GIEDVVAKAEKL	Anti-rabbit
Atp3	1:250 TBS + 5% milk	GR 1671-3	AVITNELVDIITGASSLG	Anti-rabbit
Cox2	1:500 TBS + 5% milk	GR 1948-4	KIEAVSLPKFLEWLNEQ	Anti-rabbit
Cox3	1:500 TBS + 5% milk	MitoSciences		Anti-mouse
Rip1	1:500 TBS + 5% milk	GR 543-6	LEIPAYEFDGDKVIVG	Anti-rabbit
Sdh1	1:600 TBS + 5% milk	GR 1849-3	WQKDVAAAPVTLKYRRV	Anti-rabbit
Sam50 (affinity purified)	1:40 TBS-T	B 312-17	Full-length protein	Anti-rabbit
Tim23	1:500 TBS + 5% milk	GR 3878-4	SVKKRLEK	Anti-rabbit
Sec61	1:1,000 TBS + 5% milk	GR 760-7	AKEGGFTKNLVPGFSDLM	Anti-rabbit
Sss1	1:500 TBS + 5% milk	GR 787-6	EKLVEAPVEFVREGTQF	Anti-rabbit
Ugo1 (affinity purified)	1:100 TBS-T + 0.2% milk	Gift from J. Shaw	AA 1-111	Anti-rabbit
Ups1 (affinity purified)	1:100 TBS-T	GR 2475-2	AFVIQKLEEARNPQF	Anti-rabbit
Sdh4	1:250 TBS-T + 5% milk	GR 1854-4	KSLWDSSEKDNSQKIEAKK	Anti-rabbit
Sod1	1:250 TBS + 5% milk	GR 1080-5	QDDLKGKDTEESLKTG	Anti-rabbit

All antibodies were raised against *Saccharomyces cerevisiae* proteins except anti-HA antibody.