

The fusogenic lipid phosphatidic acid promotes the biogenesis of mitochondrial outer membrane protein Ugo1

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Import and assembly of mitochondrial proteins depend on a complex interplay of proteinaceous translocation machineries. The role of lipids in this process has been studied only marginally and so far no direct role for a specific lipid in mitochondrial protein biogenesis has been shown. Here we analyzed a potential role of phosphatidic acid (PA) in biogenesis of mitochondrial proteins in *Saccharomyces cerevisiae*. In vivo remodeling of the mitochondrial lipid composition by lithocholic acid treatment or by ablation of the lipid transport protein Ups1, both leading to an increase of mitochondrial PA levels, specifically stimulated the biogenesis of the outer membrane protein Ugo1, a component of the mitochondrial fusion machinery. We reconstituted the import and assembly pathway of Ugo1 in protein-free liposomes, mimicking the outer membrane phospholipid composition, and found a direct dependency of Ugo1 biogenesis on PA. Thus, PA represents the first lipid that is directly involved in the biogenesis pathway of a mitochondrial membrane protein.

Introduction

Most mitochondrial proteins are encoded in the nucleus and have to be imported as cytosolic precursors. The targeting, import, and assembly of these precursors is mediated by sophisticated proteinaceous machineries. They comprise the central preprotein translocase TOM, the sorting and assembly machinery SAM, and the MIM complex in the outer membrane, the MIA machinery in the intermembrane space, and the TIM22 and TIM23 translocases in the inner membrane (Dolezal et al., 2006; Neupert and Herrmann, 2007; Baker et al., 2007; Chacinska et al., 2009; Endo and Yamano, 2010; Dudek et al., 2013). If and how lipids might be involved in mitochondrial protein biogenesis is still unclear. For the two nonbilayer phospholipids cardiolipin (CL) and phosphatidylethanolamine (PE), a role in regulation of TOM complex stability has been proposed, thereby affecting protein biogenesis rather indirectly (Gebert et al., 2009; Becker et al., 2013; Horvath and Daum, 2013). Similarly, CL appeared to be required for the stability of the TIM23

translocation machinery (Jiang et al., 2000; Kutik et al., 2008; Tamura et al., 2009). In addition, a few precursors destined for the outer membrane were found to be imported independently of the known import machineries or to require only import receptors but not the central translocation pore. Therefore it has been speculated that a distinct lipid composition might contribute to outer membrane protein biogenesis (Otera et al., 2007; Merklinger et al., 2012). In particular, Fis1, a component of the mitochondrial fission machinery, appeared to be dependent on a distinct lipid composition of the outer membrane, and its low ergosterol content was proposed to direct the precursor to the outer membrane while the typical high ergosterol levels in other membranes prevent Fis1 import (Krumpe et al., 2012). However, so far no lipid has been identified that directly promotes biogenesis of a mitochondrial protein.

Here, we analyzed the role of phosphatidic acid (PA), a nonbilayer phospholipid, in mitochondrial protein biogenesis. We screened yeast mitochondrial proteins from strains treated with lithocholic acid (LCA) that induces changes in the glycero-

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Abbreviations used in this paper: CL, cardiolipin; LCA, lithocholic acid; OMV, OML vesicle; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

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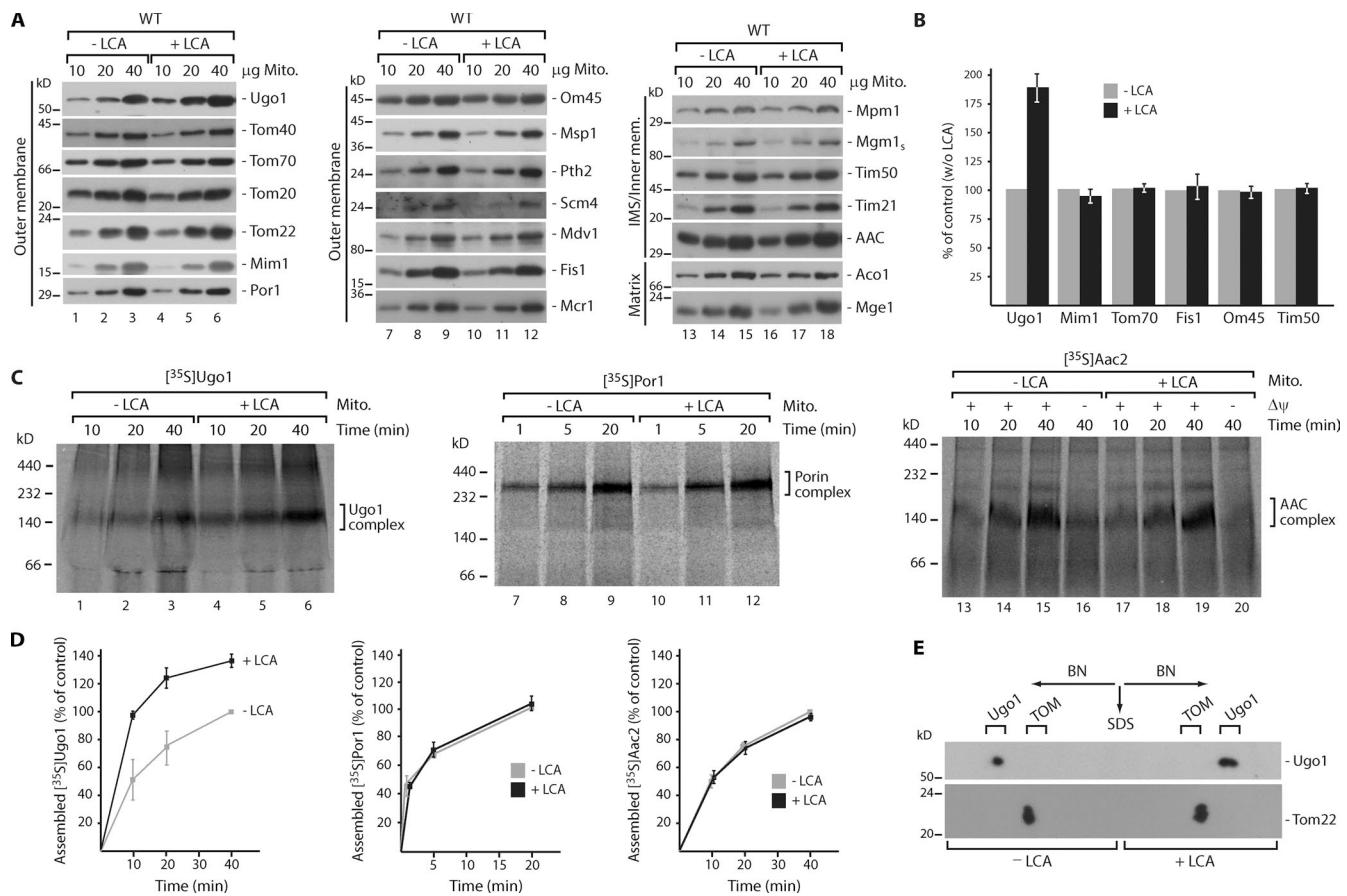


Figure 1. LCA stimulates Ugo1 biogenesis in vivo and in organello. (A) Immunoblot analysis of mitochondria isolated from wild-type yeast cultures that were incubated in the presence or absence of LCA. (B) Quantification of protein levels of mitochondria from LCA-treated or control yeast cultures (–LCA values were set to 100%). Quantifications represent mean \pm SEM (error bars; $n \geq 3$). (C) Assembly reaction of radiolabeled Ugo1, Por1, and Aac2 precursors in isolated mitochondria from yeast cultures that were incubated in the presence or absence of LCA. Samples were solubilized in digitonin buffer and separated via blue native electrophoresis. Assembly of precursor in mature complexes was visualized by autoradiography. $\Delta\psi$, membrane potential. (D) Quantification of assembly reactions from C. Values represent mean \pm SEM (error bars; $n = 3$). (E) Two-dimensional blue native/SDS-PAGE of digitonin-lysed mitochondria from a wild-type yeast strain that was cultured in the absence or presence of LCA. TOM, translocase of the outer membrane (MW \sim 400 kD); Ugo1, Ugo1 complex (MW \sim 140 kD).

phospholipid composition of mitochondrial membranes, in particular increased levels of PA (Beach et al., 2013; Burstein and Titorenko, 2014). We found a specific LCA-dependent increase of the outer membrane fusion machinery component Ugo1. A similar increase was found in *ups1Δ* mitochondria, which also exhibit increased levels of PA (Connerth et al., 2012). Using liposomes that mimic the outer membrane phospholipid composition, we show that Ugo1 assembly strictly depends on PA. The efficiency of Ugo1 assembly in protein-free liposomes directly correlates with the amounts of PA in the membranes.

Results

LCA treatment stimulates Ugo1 biogenesis in vivo and in organello

To identify mitochondrial proteins whose biogenesis might depend on a distinct membrane phospholipid composition, we grew yeast cells in the presence or absence of LCA. This bile acid leads to a remodeling of the glycerophospholipid composition of mitochondrial membranes, in particular to increased levels of PA (Beach et al., 2013; Burstein and Titorenko, 2014). We isolated mitochondria from LCA-treated and nontreated

cells and analyzed the levels of various classes of mitochondrial proteins by immunoblotting (Fig. 1 A). We found strongly increased levels of Ugo1, a 58-kD subunit of the mitochondrial fusion machinery (Wong et al., 2003; Sesaki and Jensen, 2004; Fig. 1, A and B), whereas other protein levels remained unaffected by LCA treatment. A similar LCA-dependent increase of Ugo1 was observed with a strain where Ugo1 was fused to a C-terminal triple HA affinity tag and detected with an anti-HA antibody (Fig. S1, A and B).

Ugo1 contains three transmembrane segments and exists as a homodimer, which is critical for its function in mitochondrial fusion (Hoppins et al., 2009). We asked if assembly of Ugo1 is affected by LCA treatment in organello and imported radiolabeled Ugo1 precursor into isolated mitochondria. Assembly of the Ugo1 dimer was monitored by blue native electrophoresis (Hoppins et al., 2009; Becker et al., 2011; Papic et al., 2011). Mitochondria from LCA-treated cells revealed a significantly higher capacity to assemble Ugo1 (Fig. 1, C and D). In contrast, assembly of the outer membrane protein Por1 (VDAC) that requires a complex biogenesis pathway, including the TOM and SAM machineries, and depends on CL and PE (Gebert et al., 2009; Schmidt et al., 2010; Endo and Yamano, 2010; Dukanovic and Rapaport, 2011; Becker et al., 2013), was

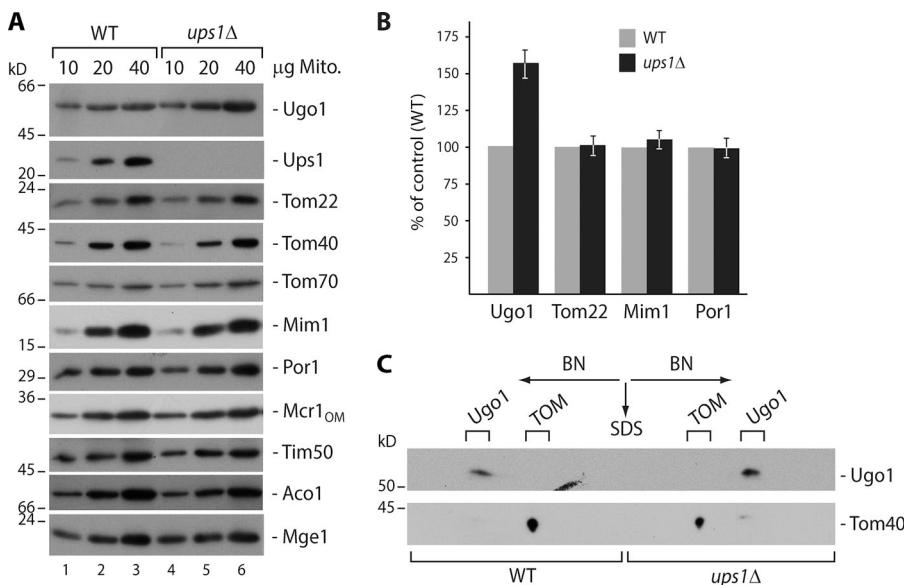


Figure 2. Analysis of Ugo1 in *ups1Δ* mitochondria. (A) Immunoblot analysis of protein levels in isolated mitochondria from wild-type (WT) and *ups1Δ* strains. (B) Quantification of Ugo1 and control protein levels of WT and *ups1Δ* mitochondria from A (WT values were set to 100%). Quantifications represent mean \pm SEM (error bars; $n \geq 3$). (C) Two-dimensional blue native/SDS-PAGE of digitonin-lysed WT and *ups1Δ* mitochondria. TOM, translocase of the outer membrane (MW \sim 400 kD); Ugo1, Ugo1 complex (MW \sim 140 kD).

not changed (Fig. 1, C and D). Similarly, the assembly pathway of the inner membrane ADP/ATP carrier was unaffected by LCA (Fig. 1, C and D). We also tested import efficiency of Ugo1 precursor in mitochondria from LCA-treated cultures via SDS-PAGE by carbonate extraction (Fujiki et al., 1982; Becker et al., 2008) or by generation of specific proteolytic fragments (Sesaki and Jensen, 2001; Coonrod et al., 2007; Hoppins et al., 2009; Papic et al., 2011). Both assays revealed stimulation upon LCA treatment similar to the import reaction monitored on blue native PAGE (Fig. S1 C). Binding of the Ugo1 precursor to mitochondria was not affected by LCA treatment (Fig. S1 C). To analyze the levels of endogenous Ugo1 complexes, we performed a two-dimensional blue native/SDS-PAGE and found increased amounts upon LCA treatment (Fig. 1 E). Further membrane protein complexes tested were not affected (Fig. S1 D). Thus, Ugo1 biogenesis appears to depend in a striking way on changes in the mitochondrial phospholipid composition induced by LCA treatment and was therefore further tested for a PA-dependent biogenesis pathway.

Deletion of *UPS1* leads to increased levels of Ugo1 in mitochondria

Mitochondrial PA is predominantly imported from the ER and serves as a major source for the synthesis of CL at the inner membrane (Horvath and Daum, 2013; Tatsuta et al., 2014; Zhang et al., 2014). While mediation of PA transfer from the ER to the mitochondrial outer membrane is still unclear, the intermembrane space protein Ups1 (Sesaki et al., 2006) has been recently identified to transport PA between the outer and inner membrane in a complex with Mdm35 (Connerth et al., 2012). Deletion of *UPS1* leads to an accumulation of PA in mitochondria while the level of CL is decreased (Connerth et al., 2012). We therefore aimed to monitor Ugo1 protein levels in *ups1Δ* mitochondria to gain further indications of a PA-specific role in Ugo1 biogenesis using an LCA-independent in vivo system. Indeed, immunoblot analysis of *ups1Δ* mitochondria revealed a specific increase of Ugo1 protein levels compared with wild type (Fig. 2, A and B). Also, assembly of the dimeric Ugo1 complex was increased in *ups1Δ* mitochondria as analyzed by two-dimensional blue native/SDS-PAGE (Fig. 2 C). Thus, deletion of *UPS1* leads to a similar stimulation of Ugo1 biogen-

esis as treatment with LCA, which points to a specific role of PA in this process in vivo.

Ugo1 assembles in protein-free liposomes that mimic outer membrane phospholipid composition

Import and assembly of Ugo1 was shown to depend on the import receptor Tom70 and the multifunctional outer membrane protein Mim1 but not the general insertion pore of the TOM complex (Becker et al., 2011; Papic et al., 2011). Deletion of either component reduced the assembly efficiency of Ugo1; however, a considerable amount of Ugo1 dimers were still formed (Becker et al., 2011). We therefore tested if Ugo1 assembly is also possible in the absence of Tom70 and Mim1 and established an Ugo1 assembly assay using protein-free liposomes that mimic the phospholipid composition of the outer membrane. This robust in vitro system would also allow us to directly assess a PA requirement for Ugo1 biogenesis. The amounts of various phospholipids in mitochondrial outer membranes from yeast were determined by two-dimensional thin-layer chromatography (Zinser et al., 1991; de Kroon et al., 1999). We generated highly pure outer membrane fractions that were largely devoid of inner membrane (Tim23) and ER marker proteins (Sec61, Sss1; Fig. S2 A) and determined the phospholipid composition by mass spectrometry (LC-MS). We found similar values for the various classes of phospholipids as obtained by phospholipid separation through thin-layer chromatography and lipid analyses (Fig. S2 B; Zinser et al., 1991; de Kroon et al., 1999). We used the phospholipid profile to generate large unilamellar vesicles (OMLs) mimicking the phospholipid composition of the mitochondrial outer membrane (Mui et al., 2003; see Materials and methods). OMLs were incubated with ³⁵S-labeled precursor proteins. Optionally, Proteinase K digestion was used to remove nonimported precursors and outer surface-exposed parts of the proteins. OMLs were reisolated using sedimentation ultracentrifugation. To separate OMLs from precursors and potentially aggregated protein species, samples were floated in a sucrose gradient (van der Laan et al., 2007). After reisolation, samples were analyzed via SDS-PAGE or blue native electrophoresis followed by autoradiography (Fig. 3 A). In parallel, all precursors were imported into iso-

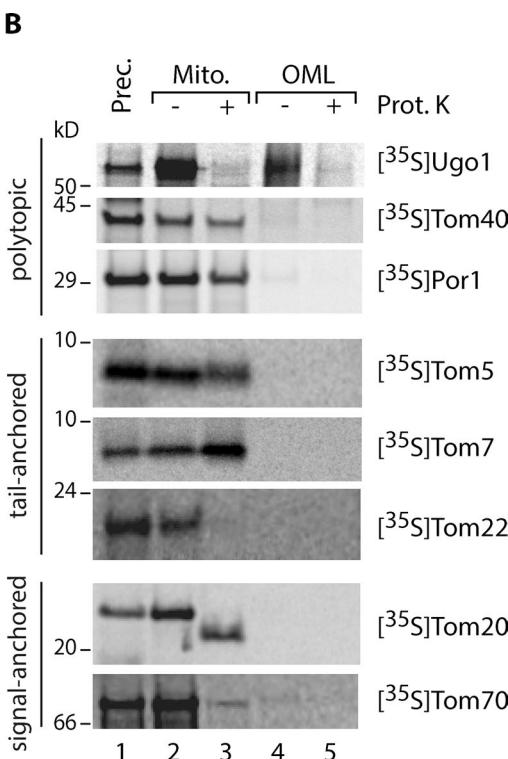
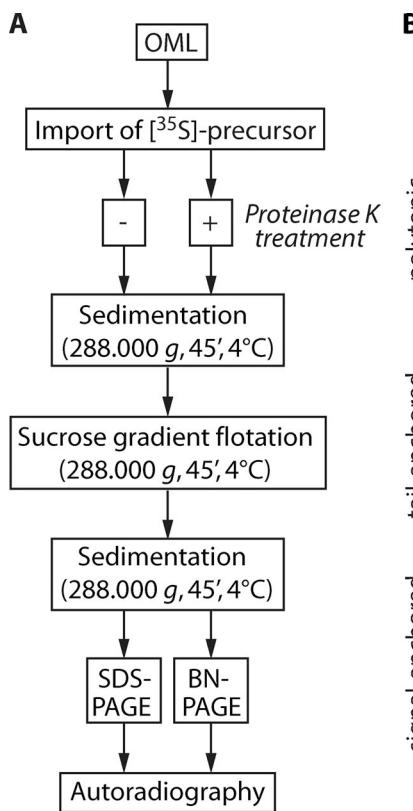


Figure 3. Assay to test protein import in liposomes mimicking the lipid composition of the mitochondrial outer membrane (OMLs). (A) Experimental setup for testing of import and assembly of radiolabeled precursor proteins into liposomes mimicking the mitochondrial outer membrane lipid composition (OMLs). (B) Import of ³⁵S-labeled precursor proteins into mitochondria and OMLs as described in A. Precursors and accessible protein parts were digested by addition of Proteinase K (Prot. K) where indicated. Samples were analyzed by SDS-PAGE and autoradiography.

lated mitochondria. As shown in Fig. 3 B, only Ugo1 precursor revealed a strong binding to OMLs while other outer membrane precursor proteins of various topological classes imported only into mitochondria but not OMLs.

The topology of Ugo1 has been analyzed by treating isolated mitochondria with Proteinase K or Trypsin and can be monitored by the appearance of specific fragments (Sesaki and Jensen, 2001; Coonrod et al., 2007; Hoppins et al., 2009; Papic et al., 2011). To analyze the topology of Ugo1 in OMLs, we compared the proteolytic pattern of imported Ugo1 in isolated mitochondria and OMLs. The generation of Trypsin protected fragments of Ugo1 was directly comparable in OMLs and mitochondria, as two dominant fragments at ~32 and 22 kD appeared (Fig. 4 A, f1 and f2), indicating a correct topological orientation of Ugo1 upon import in OMLs.

To further analyze membrane integration of Ugo1 into OMLs, we treated mitochondria and OMLs with alkaline pH (Fujiki et al., 1982) after import reactions. An ultracentrifugation step separated pellet (integral membrane proteins) and supernatant (soluble or peripherally attached proteins) fractions. Ugo1 was efficiently recovered in the pellet fraction after import into OMLs and mitochondria but not in mock-treated samples, indicating a stable integration of Ugo1 into the lipid bilayer (Fig. 4 B). Moreover, Ugo1 membrane insertion was dependent on the applied temperature (Fig. S3 A). We conclude that Ugo1 adapts its native transmembrane topology in the protein-free lipid bilayer of OMLs.

We then asked if Ugo1 precursor also forms a dimeric complex after import into OMLs. Therefore, we imported radio-labeled Ugo1 precursor into isolated mitochondria and OMLs and separated the samples after solubilization with 1% (wt/vol) digitonin buffer via blue native electrophoresis. The 140-kD mature form of Ugo1 appeared in OMLs like in mitochondria

in a time-dependent manner (Fig. 4 C). Next, we tested if the formation of the Ugo1 membrane assembly is proportional to the amount of lipids present. Indeed, increasing concentration of OMLs led to an increase in Ugo1 assembly correlating Ugo1 import and assembly with the provided surface of OMLs (Figs. 4 D and S3 B). Collectively, Ugo1 precursor imports and integrates into protein-free lipid bilayers, engaging in its correct topological orientation. Furthermore, Ugo1 matures into its native 140-kD dimeric form as shown by blue native electrophoresis, and the efficiency of this assembly correlates with the amount of lipids present.

Assembly of Ugo1 in protein-free liposomes depends on PA

PA is the major up-regulated lipid species in mitochondria upon LCA treatment (Beach et al., 2013; Burstein and Titorenko, 2014) and specifically increased upon deletion of *Ups1*, the protein that shuttles PA from the outer to the inner membrane for CL synthesis (Connerth et al., 2012). In both *in vivo* systems, we observed a specific stimulation of Ugo1 biogenesis (Figs. 1 and 2). We therefore wondered if the assembly of Ugo1 in protein-free OMLs might depend on the presence of PA. To address this, we analyzed Ugo1 assembly in PA-containing and PA-free OMLs. Ugo1 precursor was imported into these OMLs and its assembly was analyzed via blue native electrophoresis. Formation of the 140-kD Ugo1 dimer was strongly reduced in the absence of PA (Fig. 5, A and B) and could be stimulated by increasing amounts of PA present in OMLs (Fig. 5 C). Lack of other lipids, like CL, phosphatidylglycerol (PG), or phosphatidylserine (PS), the levels of which were also reported to be changed upon LCA treatment or *ups1* deletion (Connerth et al., 2012; Beach et al., 2013; Burstein and Titorenko, 2014), did not affect assembly of Ugo1 in OMLs (Fig. S4). Hence,

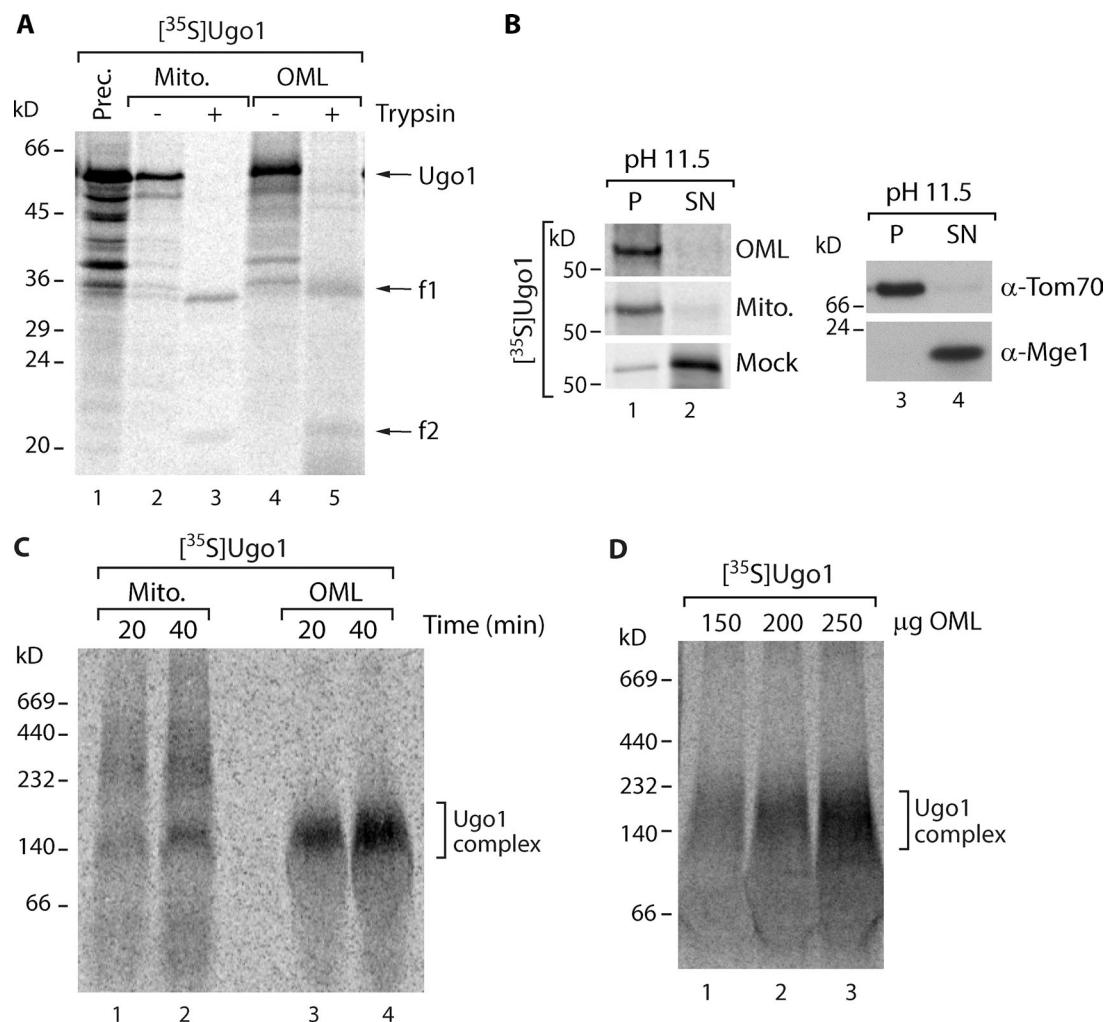


Figure 4. The fusion machinery component Ugo1 assembles into its mature complex in OMLs. (A) Membrane topology comparison of ^{35}S -labeled Ugo1 in the mitochondrial outer membrane (Mito.) and OMLs assayed by Trypsin accessibility. f1 and f2, protease-protected fragments of imported Ugo1. Prec., Ugo1 precursor. (B) Sodium carbonate extraction (pH 11.5) was performed after import of radiolabeled Ugo1 into wild-type mitochondria (Mito.) or OMLs. Mock control, radiolabeled Ugo1 precursor only. Lanes 3 and 4 show control proteins after carbonate extraction of mitochondria (Tom70, integral outer membrane protein; Mge1, soluble matrix protein). P, pellet; SN, supernatant. (C) ^{35}S -labeled Ugo1 was imported into isolated mitochondria (Mito.) or OMLs at 25°C for the indicated time periods. Mitochondria and OMLs were lysed with digitonin and analyzed by blue native electrophoresis and digital autoradiography. (D) Import of radiolabeled Ugo1 in the presence of increasing amounts of OMLs. Sample analysis was performed as in C.

PA emerges here as a major player that is directly involved in the biogenesis of Ugo1.

LCA treatment and UPS1 deletion stimulate Ugo1 biogenesis in *mim1* Δ cells

Our results indicated that proteinaceous factors, like Mim1, are dispensable for import and assembly of Ugo1 into its dimeric complex. However, this process seems to strictly depend on the phospholipid PA. We therefore wondered if increased PA levels might also stimulate Ugo1 biogenesis in the absence of Mim1 *in vivo*. We isolated mitochondria from *mim1* Δ cells, which were treated with LCA as described above (Fig. 1), and analyzed Ugo1 protein levels. We found that LCA treatment led to a similar increase of Ugo1 protein levels in *mim1* Δ mitochondria (Fig. 6, A and B) as observed previously in wild-type mitochondria (Fig. 1, A and B). Ugo1 assembly is also strongly enhanced upon LCA treatment, as revealed by two-dimensional blue native/SDS-PAGE analysis (Fig. 6 C). A very similar stimulation of Ugo1 biogenesis was observed when *ups1* was deleted in the

mim1 Δ background to increase PA levels *in vivo* (Fig. 6, D–F; and Fig. S5). These results indicate a predominant role of PA in Ugo1 biogenesis, which functions independently of Mim1.

Discussion

Our study reveals two novel insights into the biogenesis pathway of Ugo1. (1) Import and assembly of Ugo1 precursors can function in the absence of proteinaceous factors. Thus, no other outer membrane proteins are strictly required for Ugo1 biogenesis. The reported dependence of Ugo1 biogenesis on the import receptor Tom70 and Mim1 (Becker et al., 2011; Papic et al., 2011) might represent a mechanism that accelerates this process under *in vivo* conditions. (2) Ugo1 biogenesis strictly depends on the fusogenic phospholipid PA in a reconstituted system using protein-free liposomes, and the efficiency of Ugo1 import and assembly in organello and *in vivo* positively correlates with PA levels in mitochondria. With a concentration of

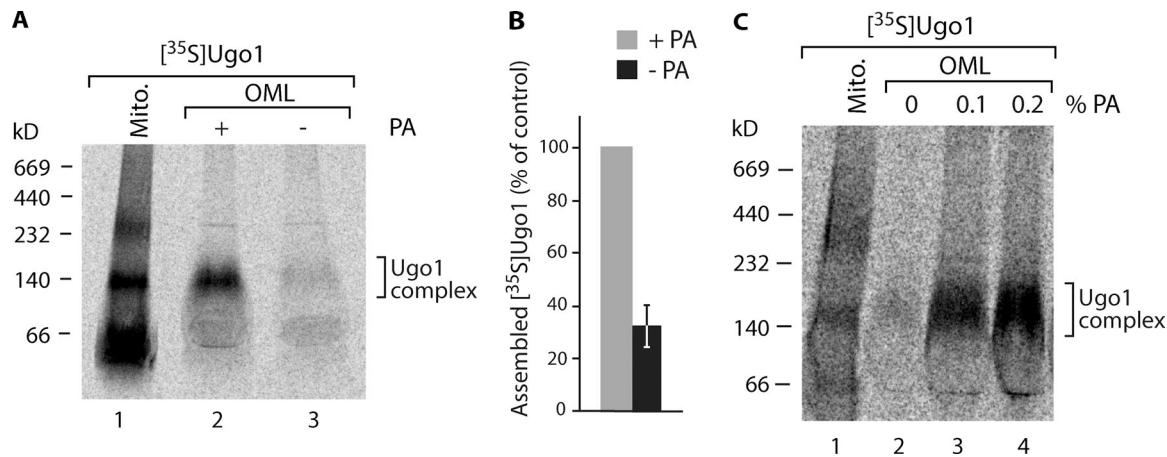


Figure 5. Assembly of Ugo1 requires PA. (A) ^{35}S -labeled Ugo1 was imported into isolated mitochondria (Mito.) and OMLs lacking or containing PA (0.4%). Samples were lysed in digitonin buffer and separated by blue native electrophoresis and analyzed by digital autoradiography. (B) Quantification of the 140 kD assembly of Ugo1 in OMLs analyzed as described in A. Values were adjusted to the lipid amount of the samples analyzed and set to 100% for OMLs containing PA. Quantifications represent mean \pm SEM (error bars; $n = 3$). (C) Radiolabeled Ugo1 was imported into mitochondria (Mito.) or OMLs with increasing concentrations of PA. Samples were solubilized in digitonin buffer and analyzed by blue native electrophoresis and digital autoradiography.

~ 0.4 mol%, PA is present in only small amounts in the outer membrane (Fig. S2 B). However, a slight reduction of this concentration in OMLs is sufficient to dramatically decrease Ugo1 assembly efficiency, reflecting a direct dependency on this lipid (Fig. 5). While CL and PE were shown to influence mitochondrial protein biogenesis indirectly by stabilizing structure and function of preprotein translocases (Jiang et al., 2000; Kutik et al., 2008; Gebert et al., 2009; Tamura et al., 2009; Becker et al., 2013), our results uncovered PA as the first lipid that directly promotes biogenesis of a mitochondrial membrane protein.

PA was the major up-regulated lipid species upon LCA treatment (Beach et al., 2013; Burstein and Titorenko, 2014) and has been shown to facilitate mitochondrial fusion in mammals by a so-far unknown mechanism (Choi et al., 2006; Yang and Frohman, 2012). Notably, LCA treatment of yeast also leads to increased mitochondrial fusion activity that is likely caused by increased PA level (Beach et al., 2013; Burstein and Titorenko, 2014). It has been proposed that PA, which possesses a small, negatively charged head group, influences the negative curvature of mitochondrial membranes (Yang and Frohman, 2012; Zhang et al., 2014), thereby promoting membrane fusion. Given the very low content in the outer mitochondrial membrane, it is difficult to envision how PA may control the overall phospholipid organization, leading to membrane bending (Fig. S2 B; Zinser et al., 1991; de Kroon et al., 1999; Mejia and Hatch, 2015). It is therefore tempting to speculate that PA might exert its fusogenic potential by supporting assembly of Ugo1 that is required in yeast to activate conserved GTPases/mitofusins, mediating membrane fusion (Wong et al., 2003; Sesaki and Jensen, 2004; Anton et al., 2011). (A mammalian Ugo1 homologue has not yet been found.) A possible role of Mim1 in such a scenario might be to concentrate PA into foci to stimulate Ugo1 biogenesis at the outer membrane *in vivo*. Additionally, proteinaceous factors like Mim1 and the import receptor Tom70 may play a role in the targeting of Ugo1 precursor to mitochondria *in vivo*. Due to the presence of PA in other cellular compartments, a specific mitochondrial recognition mechanism for the Ugo1 precursor will be required. In line with this, our results revealed that in contrast to membrane insertion and assembly, the mitochondrial targeting of Ugo1 precursor does indeed not

depend on PA (Fig. S1 C). However, the subsequent steps in Ugo1 biogenesis including membrane insertion and assembly into homodimeric complexes occur in a protein-independent manner and require the phospholipid PA.

Materials and methods

Yeast strains and growth conditions

The following yeast strains were used in this study: YPH499 (*MATA*, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*), BY4741 (*MATA*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*), and *mim1Δ* (*MATA*, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, *mim1Δ::ADE2*; Becker et al., 2008). Ugo1 was chromosomally tagged with a C-terminal 3HA tag using as a selectable marker the *His3MX6* module (*MATA*, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, *ugo1Δ::UGO1HA3-HIS3*; Longtine et al., 1998). *UPS1* was deleted in the *mim1Δ* background by homologous recombination using a *HIS3* cassette (*MATA*, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, *mim1Δ::ADE2*, *ups1Δ::HIS3*; Longtine et al., 1998). Yeast cells were grown on YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, and 3% [wt/vol] glycerol) at 21°C to an OD of 1.5. For LCA treatment, yeast cells were grown to mid-logarithmic growth phase in medium containing 0.2% (wt/vol) glucose, 1% (wt/vol) yeast extract, and 2% (wt/vol) bacto-peptone, and supplemented with 50 μM LCA in DMSO (final concentration of DMSO, 1% [vol/vol]). Non-treated cells received DMSO only. Cells were harvested after growth at 30°C for 48 h (Beach et al., 2013).

Isolation of highly purified mitochondrial outer membrane vesicles (OMVs)

Wild-type yeast cells (YPH499) were grown on YPG medium at 24°C to an OD of 1.5. Cells were harvested and mitochondria were isolated using differential centrifugation (Meisinger et al., 2000). Highly purified mitochondria were obtained by further sucrose gradient purification (Meisinger et al., 2000). For isolation of OMVs, 50 mg of highly purified mitochondria were resuspended in swelling buffer containing 5 mM potassium phosphate, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride (4 mg/ml protein concentration) and incubated for 20 min on ice. The sample was treated with a Teflon glass potter with 20 strokes

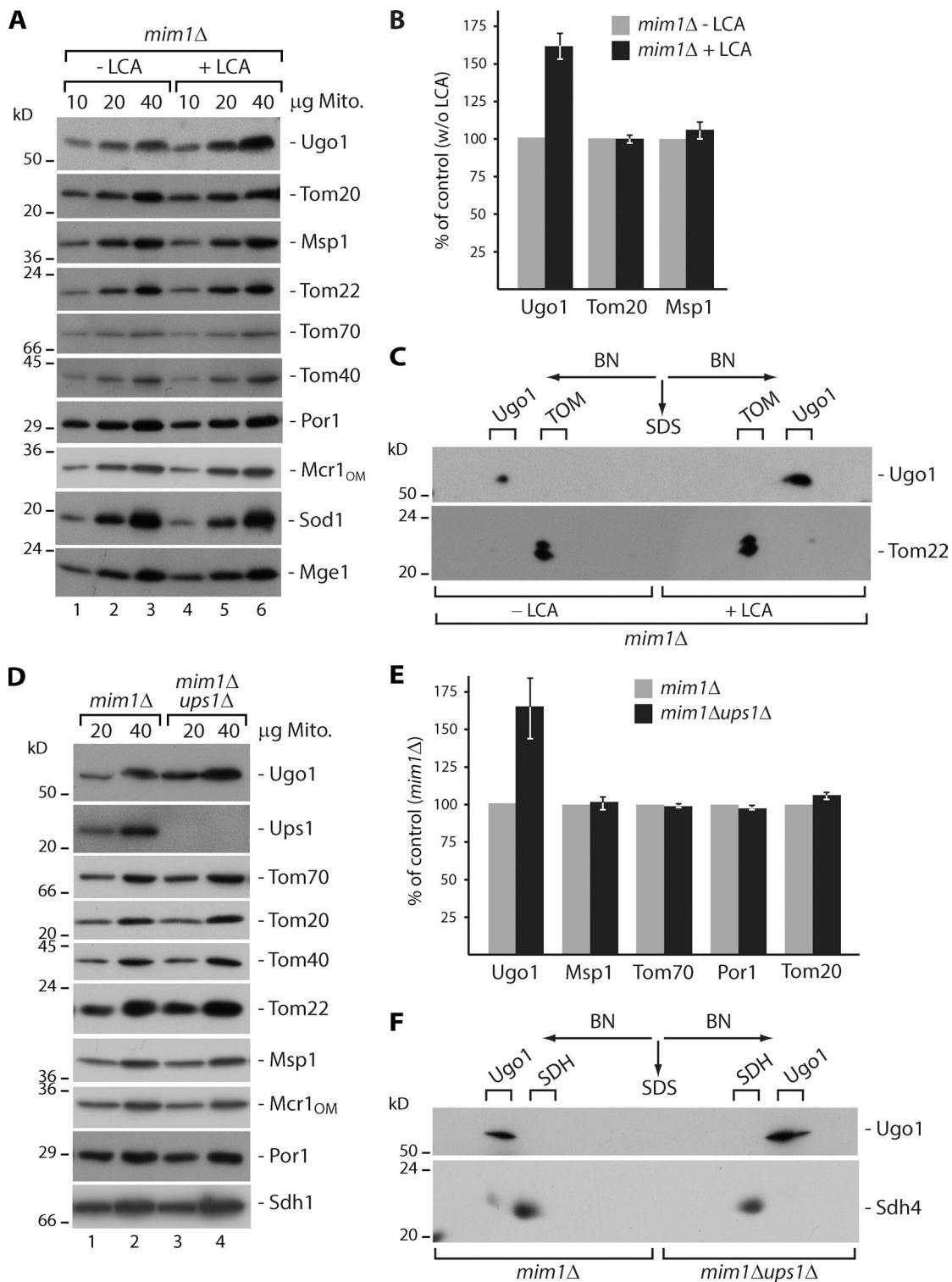


Figure 6. In vivo stimulation of Ugo1 biogenesis in *mim1 Δ* cells by LCA treatment or *UPS1* deletion. (A) Immunoblot analysis of *mim1 Δ* mitochondria from cells that were cultured in the absence or presence of LCA. (B) Quantification of Ugo1 and control protein levels from A. Quantifications represent mean \pm SEM (error bars; $n = 3$). (C) Two-dimensional blue native/SDS-PAGE of digitonin-lysed mitochondria from *mim1 Δ* yeast that was cultured in the absence or presence of LCA. TOM, translocase of the outer membrane (MW \sim 400 kD); Ugo1, Ugo1 complex (MW \sim 140 kD). (D) Immunoblot analysis of mitochondria from *mim1 Δ* and *mim1 Δ ups1 Δ* strains. (E) Quantification of Ugo1 and control proteins from D as described in B ($n \geq 3$). (F) Two-dimensional blue native/SDS-PAGE of digitonin-lysed mitochondria from *mim1 Δ* and *mim1 Δ ups1 Δ* strains. SDH, succinate dehydrogenase complex (MW \sim 230 kD).

and loaded on a three-step sucrose gradient (1 ml 60%, 4 ml 32%, and 1 ml 15% sucrose (wt/vol) in EM buffer (10 mM MOPS/KOH, pH 7.2, and 1 mM EDTA). Centrifugation was performed in a SW41 swing-out

rotor for 1 h at 134,000 g). OMVs were collected from the 15–32% interface and after adjustment of sucrose concentration to 50% (wt/vol), floated (from the bottom) on a sucrose gradient (5 ml 32% [wt/vol]

vol] sucrose in EM buffer and 1.5 ml EM buffer) at 240,000 *g* overnight. Highly pure OMVs were obtained from the 0–32% interface and resuspended after pelleting in EM buffer (Zahedi et al., 2006).

Lipid analysis

For lipid profiling by mass spectrometry, highly purified mitochondria and outer membrane vesicles were extracted using the Bligh-Dyer method (Bligh and Dyer, 1959). Di-C19:0 phosphatidylcholine (PC), di-C12:0 PE, di-C10:0 PG, di-C10:0 PS, tetra-C14:0 CL, C12:0-C13:0 PA (Avanti Polar Lipids), and di-C8:0 GPIs (Echelon Biosciences) were added as internal lipid standards. Quantification of individual molecular species was performed using multiple reaction monitoring (MRM) with an Applied Biosystems 4000 Q-Trap mass spectrometer (Applied Biosystems), with upfront normal phase liquid chromatography separation (Agilent 1100 LC), as described previously (Guan et al., 2010; Shui et al., 2010). Each lipid species (defined by the sum of carbons and double bonds of the acyl chains) were quantified using the parent >headgroup fragment transitions. Each individual ion dissociation pathway was optimized with regard to collision energy and fragmentor voltage to minimize variations in relative ion abundance due to differences in dissociations. Lipid levels were normalized to the internal standards and calculated as mol% of total lipid measured. For analysis of the lipid composition by two-dimensional thin-layer chromatography, lipids were isolated from 1 mg mitochondria with chloroform/methanol (2:1 [vol/vol]; Folch et al., 1957). Non-lipid contaminants were removed from the extracts by several washing steps including a 0.034% (wt/vol) $MgCl_2$ solution, 2 M KCl/methanol (4:1 [vol/vol]), and methanol/water/chloroform (48:47:3 [vol/vol/vol]). Lipid extracts were applied to thin-layer chromatography plates (Silica gel 60; Merck) and developed in an ascending manner using the solvent systems chloroform/methanol/25% NH_3 (68:35:5 [vol/vol/vol]) in the first and chloroform/acetone/methanol/acetic acid/water (53:20:10:10:5 per volume) in the second dimension (Schneiter and Daum, 2006). Individual phospholipids were stained with 0.8 g $MnCl_2 \cdot 4H_2O$, 120 ml water, 120 ml methanol, and 9 ml concentrated sulfuric acid and visualized after charring at 100°C for 30 min.

In organello protein import

Radio-labeled precursor proteins were synthesized in the presence of [^{35}S]methionine (PerkinElmer) in rabbit reticulocyte lysate (Promega). For in organello import assays, [^{35}S] precursors were incubated with 30 μ g of isolated yeast mitochondria in BSA buffer (10 mM MOPS/KOH, pH 7.2, 3% [wt/vol] BSA, 250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl, and 5 mM KP_i) and supplemented with 2 mM ATP and 2 mM NADH. Samples were incubated for the indicated time periods at 25°C. Reactions were stopped on ice and treated with 50 μ g/ml Proteinase K for 10 min where indicated. After addition of 2 mM PMSF (phenylmethylsulfonyl fluoride in isopropanol) and additional incubation for 10 min, samples were centrifuged at 16,000 *g* for 10 min at 4°C. Samples were suspended in 200 μ l SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH, pH 7.2) and centrifuged as before. Samples were analyzed by SDS-PAGE or blue native electrophoresis followed by autoradiography and immunodecoration (Table S1).

Blue native electrophoresis

50 μ g mitochondria or 150–250 μ g OMLs were solubilized in 1% (vol/vol) digitonin solubilization buffer after import reaction with radio-labeled precursor protein was completed (Becker et al., 2011). Samples were centrifuged at 16,000 *g* for 10 min at 4°C, and supernatant was loaded on a 4–16.5% blue native gradient gel followed by autoradiography. For two-dimensional blue native/SDS-PAGE, gel lanes from

1D blue native electrophoresis were cut and polymerized on top of a 12.5% SDS-polyacrylamide gel.

Generation of large unilamellar vesicles mimicking the outer membrane lipid composition (OMLs)

Lipids dissolved in a mixture of chloroform/methanol (9:1 [vol/vol]) were mixed in order to obtain a final lipid composition as determined by the analysis of the outer mitochondrial membrane by liquid chromatography–mass spectrometry (LC-MS; Fig. S2): PC (37.9%), CL (1.6%), PA (0.4%), PE (28.9%), PG (1.1%), phosphatidylinositol (PI, 29.8%), and PS (0.1%). Lipids were purchased from Sigma-Aldrich, except for PC, which was provided by Lipoid. Values are given in percentage of weight. The lipid mixture was labeled with 0.2% (wt/wt) rhodamine PE (MoBiTec) and dried under a vacuum. By addition of buffer (135 mM KCl, 10 mM Hepes, and 1 mM $MgCl_2$, pH 7.0) to the lipid film, a crude liposomal suspension with a lipid concentration of 10 mg/ml was obtained. By extruding the suspension 51 times with a LiposoFast extruder (Avestin) through a nuclepore polycarbonate track-etched membrane (Whatman; GE Healthcare) of 100 nm pore size, a suspension of unilamellar vesicles with a narrow size distribution was prepared. Vesicles of a diameter <30 nm were removed by centrifugation at 288,000 *g* for 60 min. Thus, only vesicles with a diameter of ~100 nm were used in further studies. Quality control measurements to confirm size and unilamellarity were performed by means of dynamic light scattering (mean hydrophobic radius [Z average]: 113 nm \pm 9.2 nm; mean polydispersity index: 0.0627 \pm 0.013). In addition, quality was controlled by cryotransmission electron microscopy as described previously (Holzer et al., 2009). In brief, 3 μ l of the diluted liposome dispersion (5–10 mM) were applied on a 400 \times 100 mesh Quantifoil S7/2 holey carbon film on copper grids (Quantifoil Micro Tools GmbH). Excess liquid was removed from the grid with filter paper, and the sample was immediately shock-frozen by injection into liquid ethane. Sample preparation steps were performed in a climate-controlled room using a CryoBox 340719 (Carl Zeiss). The subsequent fixation of the grid on the sample rod (626-DH; Gatan, Inc.) and transfer of the rod into the transmission electron microscope (Leo 912 Ω -mega; Leo) were performed under a nitrogen atmosphere at a temperature of 90 K (−183°C). The instrument was operated at 120 kV. Finally, the total lipid concentration was measured using the Bartlett method for phosphate quantification (Bartlett, 1959).

Protein import in OMLs

Radio-labeled precursors were mixed with 150–250 μ g OMLs in reaction buffer (3 mM Na_2HPO_4 , 2 mM KH_2PO_4 , and 50 mM NaCl, pH 7.0). Samples were incubated at 25°C (or 4°C as controls) for 20–40 min and reactions were stopped by placing the samples on ice. Non-imported precursors were removed by Proteinase K digestion (50 μ g/ml) for 10 min on ice. Digestion was stopped by the addition of 2 mM PMSF, and OMLs were reisolated by centrifugation at 288,000 *g* for 45 min at 4°C. OMLs were washed in 400 μ l of reaction buffer and centrifuged. The obtained pellet was resuspended in 150 μ l of 1.4 M sucrose and overlaid with 400 μ l of 1 M sucrose. The sucrose gradient was overlaid with 200 μ l of reaction buffer. Samples were centrifuged as before and OMLs were recovered in the interface between 1 M sucrose and reaction buffer. OMLs were washed in reaction buffer to remove residual sucrose and dissolved in Laemmli buffer for analysis on SDS-PAGE or in 1% (vol/vol) digitonin buffer for blue native electrophoresis (van der Laan et al., 2007). For separation of integral and peripheral or soluble proteins, mitochondria or OMLs were reisolated after import and suspended in 400 μ l of 100 mM Na_2CO_3 . Samples were mixed and incubated on ice for 30 min. Pellet and supernatant fractions were

separated by centrifugation at 100,000 g. Supernatant was precipitated using StrataClean beads (Agilent Technologies).

To quantify the assembly efficiency of Ugo1, the radioactive intensity of the 140-kD band on blue native gels was set in relation to the loaded lipid amount determined by thin layer chromatography. For this, 15% of the sample volume (before lysis in digitonin) was dissolved in methanol and spotted on HPTLC Silica gel 60 F254 plates (Merck) using a Linomat IV sample applicator (CAMAG). Lipid spots were visualized without further development using 8-anilinonaphthalene-1-sulfonic acid and fluorescence was measured in a TLC Scanner 3 (CAMAG; Jork et al., 1990).

Statistical analysis

Quantifications were obtained from at least three independent experiments and are shown as mean \pm SEM.

Online supplemental material

Fig. S1 shows further results and controls on the analysis of Ugo1 biogenesis upon LCA treatment. Fig. S2 shows purity of the highly enriched outer membrane vesicle preparation and the determined phospholipid composition. Fig. S3 shows further controls for Ugo1 import into OMLs. Fig. S4 shows Ugo1 assembly reactions in OMLs lacking different phospholipids. Fig. S5 shows lipid profiling from *mim1Δ* and *mim1Δups1Δ* mitochondria. Table S1 lists all antibodies used in this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201506085/DC1>.

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References

Anton, F., J.M. Fres, A. Schauss, B. Pinson, G.J. Praefcke, T. Langer, and M. Escobar-Henriques. 2011. Ugo1 and Mdm30 act sequentially during Fzo1-mediated mitochondrial outer membrane fusion. *J. Cell Sci.* 124:1126–1135. <http://dx.doi.org/10.1242/jcs.073080>

Baker, M.J., A.E. Frazier, J.M. Gulbis, and M.T. Ryan. 2007. Mitochondrial protein-import machinery: correlating structure with function. *Trends Cell Biol.* 17:456–464. <http://dx.doi.org/10.1016/j.tcb.2007.07.010>

Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.

Beach, A., V.R. Richard, A. Leonov, M.T. Burstein, S.D. Bourque, O. Koupaki, M. Juneau, R. Feldman, T. Iouk, and V.I. Titorenko. 2013. Mitochondrial membrane lipidome defines yeast longevity. *Aging (Albany NY)*. 5:551–574.

Becker, T., S. Pfannschmidt, B. Guiard, D. Stojanovski, D. Milenovic, S. Kutik, N. Pfanner, C. Meisinger, and N. Wiedemann. 2008. Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. *J. Biol. Chem.* 283:120–127. <http://dx.doi.org/10.1074/jbc.M706997200>

Becker, T., L.S. Wenz, V. Krüger, W. Lehmann, J.M. Müller, L. Goroncy, N. Zufall, T. Lithgow, B. Guiard, A. Chacinska, et al. 2011. The mitochondrial import protein Mim1 promotes biogenesis of multispanning outer membrane proteins. *J. Cell Biol.* 194:387–395. <http://dx.doi.org/10.1083/jcb.201102044>

Becker, T., S.E. Horvath, L. Böttlinger, N. Gebert, G. Daum, and N. Pfanner. 2013. Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins. *J. Biol. Chem.* 288:16451–16459. <http://dx.doi.org/10.1074/jbc.M112.442392>

Bligh, E.G., and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917. <http://dx.doi.org/10.1139/o59-099>

Burstein, M.T., and V.I. Titorenko. 2014. A mitochondrially targeted compound delays aging in yeast through a mechanism linking mitochondrial membrane lipid metabolism to mitochondrial redox biology. *Redox Biol.* 2:305–307. <http://dx.doi.org/10.1016/j.redox.2014.01.011>

Chacinska, A., C.M. Koehler, D. Milenovic, T. Lithgow, and N. Pfanner. 2009. Importing mitochondrial proteins: machineries and mechanisms. *Cell*. 138:628–644. <http://dx.doi.org/10.1016/j.cell.2009.08.005>

Choi, S.Y., P. Huang, G.M. Jenkins, D.C. Chan, J. Schiller, and M.A. Frohman. 2006. A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. *Nat. Cell Biol.* 8:1255–1262. <http://dx.doi.org/10.1038/ncb1487>

Connerth, M., T. Tatsuta, M. Haag, T. Klecker, B. Westermann, and T. Langer. 2012. Intramitochondrial transport of phosphatidic acid in yeast by a lipid transfer protein. *Science*. 338:815–818. <http://dx.doi.org/10.1126/science.1225625>

Coonrod, E.M., M.A. Karren, and J.M. Shaw. 2007. Ugo1p is a multi-pass transmembrane protein with a single carrier domain required for mitochondrial fusion. *Traffic*. 8:500–511. <http://dx.doi.org/10.1111/j.1600-0854.2007.00550.x>

de Kroon, A.I.P.M., M.C. Koorenevel, S.S. Goerdayal, P.C. Mulders, M.J.F.W. Janssen, and B. de Kruijff. 1999. Isolation and characterization of highly purified mitochondrial outer membranes of the yeast *Saccharomyces cerevisiae* (method). *Mol. Membr. Biol.* 16:205–211. <http://dx.doi.org/10.1080/096876899294670>

Dolezal, P., V. Likic, J. Tachezy, and T. Lithgow. 2006. Evolution of the molecular machines for protein import into mitochondria. *Science*. 313:314–318. <http://dx.doi.org/10.1126/science.1127895>

Dudek, J., P. Rehling, and M. van der Laan. 2013. Mitochondrial protein import: common principles and physiological networks. *Biochim. Biophys. Acta*. 1833:274–285. <http://dx.doi.org/10.1016/j.bbapap.2012.05.028>

Dukanovic, J., and D. Rapaport. 2011. Multiple pathways in the integration of proteins into the mitochondrial outer membrane. *Biochim. Biophys. Acta*. 1808:971–980. <http://dx.doi.org/10.1016/j.bbapap.2010.06.021>

Endo, T., and K. Yamano. 2010. Transport of proteins across or into the mitochondrial outer membrane. *Biochim. Biophys. Acta*. 1803:706–714. <http://dx.doi.org/10.1016/j.bbapap.2009.11.007>

Folch, J., M. Lees, and G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226:497–509.

Fujiki, Y., A.L. Hubbard, S. Fowler, and P.B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97–102. <http://dx.doi.org/10.1083/jcb.93.1.97>

Gebert, N., A.S. Joshi, S. Kutik, T. Becker, M. McKenzie, X.L. Guan, V.P. Mooga, D.A. Stroud, G. Kulkarni, M.R. Wenk, et al. 2009. Mitochondrial cardiolipin involved in outer-membrane protein biogenesis: implications for Barth syndrome. *Curr. Biol.* 19:2133–2139. <http://dx.doi.org/10.1016/j.cub.2009.10.074>

Guan, X.L., I. Riezman, M.R. Wenk, and H. Riezman. 2010. Yeast lipid analysis and quantification by mass spectrometry. *Methods Enzymol.* 470:369–391. [http://dx.doi.org/10.1016/S0076-6879\(10\)70015-X](http://dx.doi.org/10.1016/S0076-6879(10)70015-X)

Holzer, M., S. Barnert, J. Momm, and R. Schubert. 2009. Preparative size exclusion chromatography combined with detergent removal as a versatile tool to prepare unilamellar and spherical liposomes of highly uniform size distribution. *J. Chromatogr. A*. 1216:5838–5848. <http://dx.doi.org/10.1016/j.chroma.2009.06.023>

Hoppins, S., J. Horner, C. Song, J.M. McCaffery, and J. Nunnari. 2009. Mitochondrial outer and inner membrane fusion requires a modified carrier protein. *J. Cell Biol.* 184:569–581. <http://dx.doi.org/10.1083/jcb.200809099>

Horvath, S.E., and G. Daum. 2013. Lipids of mitochondria. *Prog. Lipid Res.* 52:590–614. <http://dx.doi.org/10.1016/j.plipres.2013.07.002>

Jiang, F., M.T. Ryan, M. Schlame, M. Zhao, Z. Gu, M. Klingenberg, N. Pfanner, and M.L. Greenberg. 2000. Absence of cardiolipin in the *crdl* null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. *J. Biol. Chem.* 275:22387–22394. <http://dx.doi.org/10.1074/jbc.M909868199>

Jork, H., W. Funk, W. Fischer, and H. Wimmer. 1990. 8-Anilinonaphthalene-1-sulfonic Acid Ammonium Salt Reagent (ANSReagent). In *Thin-Layer Chromatography. Volume 1a. Physical and Chemical Detection Methods: Fundamentals, Reagents I*. VCH Verlagsgesellschaft, Weinheim, Germany. 191–194.

Krumpe, K., I. Frumkin, Y. Herzig, N. Rimon, C. Özbalci, B. Brügger, D. Rapaport, and M. Schuldiner. 2012. Ergosterol content specifies targeting of tail-anchored proteins to mitochondrial outer membranes. *Mol. Biol. Cell.* 23:3927–3935. <http://dx.doi.org/10.1091/mbc.E11-12-0994>

Kutik, S., M. Rissler, X.L. Guan, B. Guiard, G. Shui, N. Gebert, P.N. Heacock, P. Rehling, W. Dowhan, M.R. Wenk, et al. 2008. The translocator maintenance protein Tam41 is required for mitochondrial cardiolipin biosynthesis. *J. Cell Biol.* 183:1213–1221. <http://dx.doi.org/10.1083/jcb.200806048>

Longtine, M.S., A. McKenzie III, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philipsen, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast.* 14:953–961. [http://dx.doi.org/10.1002/\(SICI\)1097-0061\(199807\)14:10<953::AID-YEA293>3.0.CO;2-U](http://dx.doi.org/10.1002/(SICI)1097-0061(199807)14:10<953::AID-YEA293>3.0.CO;2-U)

Meisinger, C., T. Sommer, and N. Pfanner. 2000. Purification of *Saccharomyces cerevisiae* mitochondria devoid of microsomal and cytosolic contaminants. *Anal. Biochem.* 287:339–342. <http://dx.doi.org/10.1006/abio.2000.4868>

Mejia, E.M., and G.M. Hatch. 2015. Mitochondrial phospholipids: role in mitochondrial function. *J. Bioenerg. Biomembr.* In press. <http://dx.doi.org/10.1007/s10863-015-9601-4>

Merklinger, E., Y. Gofman, A. Kedrov, A.J. Driessens, N. Ben-Tal, Y. Shai, and D. Rapaport. 2012. Membrane integration of a mitochondrial signal-anchored protein does not require additional proteinaceous factors. *Biochem. J.* 442:381–389. <http://dx.doi.org/10.1042/BJ20111363>

Mui, B., L. Chow, and M.J. Hope. 2003. Extrusion technique to generate liposomes of defined size. *Methods Enzymol.* 367:3–14. [http://dx.doi.org/10.1016/S0076-6879\(03\)67001-1](http://dx.doi.org/10.1016/S0076-6879(03)67001-1)

Neupert, W., and J.M. Herrmann. 2007. Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* 76:723–749. <http://dx.doi.org/10.1146/annurev.biochem.76.052705.163409>

Otera, H., Y. Taira, C. Horie, Y. Suzuki, H. Suzuki, K. Setoguchi, H. Kato, T. Oka, and K. Miura. 2007. A novel insertion pathway of mitochondrial outer membrane proteins with multiple transmembrane segments. *J. Cell Biol.* 179:1355–1363. <http://dx.doi.org/10.1083/jcb.200702143>

Papic, D., K. Krumpe, J. Dukanovic, K.S. Dimmer, and D. Rapaport. 2011. Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent import pathway. *J. Cell Biol.* 194:397–405. <http://dx.doi.org/10.1083/jcb.201102041>

Schmidt, O., N. Pfanner, and C. Meisinger. 2010. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat. Rev. Mol. Cell Biol.* 11:655–667. <http://dx.doi.org/10.1038/nrm2959>

Schneiter, R., and G. Daum. 2006. Analysis of yeast lipids. *Methods Mol. Biol.* 313:75–84.

Sesaki, H., and R.E. Jensen. 2001. *UGO1* encodes an outer membrane protein required for mitochondrial fusion. *J. Cell Biol.* 152:1123–1134. <http://dx.doi.org/10.1083/jcb.152.6.1123>

Sesaki, H., and R.E. Jensen. 2004. Ugo1p links the Fzo1p and Mgm1p GTPases for mitochondrial fusion. *J. Biol. Chem.* 279:28298–28303. <http://dx.doi.org/10.1074/jbc.M401363200>

Sesaki, H., C.D. Dunn, M. Iijima, K.A. Shepard, M.P. Yaffe, C.E. Machamer, and R.E. Jensen. 2006. Ups1p, a conserved intermembrane space protein, regulates mitochondrial shape and alternative topogenesis of Mgm1p. *J. Cell Biol.* 173:651–658. <http://dx.doi.org/10.1083/jcb.200603092>

Shui, G., X.L. Guan, C.P. Low, G.H. Chua, J.S. Goh, H. Yang, and M.R. Wenk. 2010. Toward one step analysis of cellular lipidomes using liquid chromatography coupled with mass spectrometry: application to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lipidomics. *Mol. Biosyst.* 6:1008–1017. <http://dx.doi.org/10.1039/b913353d>

Tamura, Y., T. Endo, M. Iijima, and H. Sesaki. 2009. Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. *J. Cell Biol.* 185:1029–1045. <http://dx.doi.org/10.1083/jcb.200812018>

Tatsuta, T., M. Scharwey, and T. Langer. 2014. Mitochondrial lipid trafficking. *Trends Cell Biol.* 24:44–52. <http://dx.doi.org/10.1016/j.tcb.2013.07.011>

van der Laan, M., M. Meinecke, J. Dudek, D.P. Hutu, M. Lind, I. Perschil, B. Guiard, R. Wagner, N. Pfanner, and P. Rehling. 2007. Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. *Nat. Cell Biol.* 9:1152–1159. <http://dx.doi.org/10.1038/ncb1635>

Wong, E.D., J.A. Wagner, S.V. Scott, V. Okreglak, T.J. Holewinski, A. Cassidy-Stone, and J. Nunnari. 2003. The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion. *J. Cell Biol.* 160:303–311. <http://dx.doi.org/10.1083/jcb.200209015>

Yang, C.Y., and M.A. Frohman. 2012. Mitochondria: signaling with phosphatidic acid. *Int. J. Biochem. Cell Biol.* 44:1346–1350. <http://dx.doi.org/10.1016/j.biocel.2012.05.006>

Zahedi, R.P., A. Sickmann, A.M. Boehm, C. Winkler, N. Zufall, B. Schönfisch, B. Guiard, N. Pfanner, and C. Meisinger. 2006. Proteomic analysis of the yeast mitochondrial outer membrane reveals accumulation of a subclass of preproteins. *Mol. Biol. Cell.* 17:1436–1450. <http://dx.doi.org/10.1091/mbc.E05-08-0740>

Zhang, Q., Y. Tamura, M. Roy, Y. Adachi, M. Iijima, and H. Sesaki. 2014. Biosynthesis and roles of phospholipids in mitochondrial fusion, division and mitophagy. *Cell. Mol. Life Sci.* 71:3767–3778. <http://dx.doi.org/10.1007/s00018-014-1648-6>

Zinser, E., C.D. Sperka-Gottlieb, E.V. Fasch, S.D. Kohlwein, F. Paltauf, and G. Daum. 1991. Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. *J. Bacteriol.* 173:2026–2034.