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Accessibility
Gem1 and ERMES Do Not Directly Affect Phosphatidylserine Transport from ER to Mitochondria or Mitochondrial Inheritance

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In yeast, a protein complex termed the ER-Mitochondria Encounter Structure (ERMES) tethers mitochondria to the endoplasmic reticulum. ERMES proteins are implicated in a variety of cellular functions including phospholipid synthesis, mitochondrial protein import, mitochondrial attachment to actin, polarized mitochondrial movement into daughter cells during division, and maintenance of mitochondrial DNA (mtDNA). The mitochondrial-anchored Gem1 GTPase has been proposed to regulate ERMES functions. Here, we show that ERMES and Gem1 have no direct role in the transport of phosphatidylserine (PS) from the ER to mitochondria during the synthesis of phosphatylethanolamine (PE), as PS to PE conversion is not affected in ERMES orgem1 mutants. In addition, we report that mitochondrial inheritance defects in ERMES mutants are a secondary consequence of mitochondrial morphology defects, arguing against a primary role for ERMES in mitochondrial association with actin and mitochondrial movement. Finally, we show that ERMES complexes are long-lived, and do not depend on the presence of Gem1. Our findings suggest that the ERMES complex may have primarily a structural role in maintaining mitochondrial morphology.

Key words: ERMES, ER-mitochondria, Gem1 GTPase, Mdm10, Mdm12, Mdm34, membrane contact site (MCS), mitochondrial movement and inheritance, mitochondrial phospholipids, Mmm1, yeast Miro

The endoplasmic reticulum (ER) forms membrane contact sites (MCS) with several different organelles, including mitochondria, peroxisomes, the Golgi apparatus, endosomes and the plasma membrane (1). These sites play critical roles in cell signaling, lipid homeostasis and apoptosis (2,3). Recent studies show that contact sites play a structural role in maintaining the normal morphology of the interacting organelles. In addition, ER-mitochondrial contact sites can be maintained as organelles move within a mammalian cell (4).

Several different proteins have been implicated in tethering the ER to mitochondria in mammals and budding yeast (2,5). One of the best-characterized tethers is the ER Mitochondria Encounter Structure (ERMES), which links the ER to the outer mitochondrial membrane in Saccharomyces cerevisiae (6). ERMES is composed of four proteins that associate to form a complex. Mmm1 is anchored in the ER and Mdm10 in the outer mitochondrial membrane (6,7), whereas the cytosolic protein Mdm12 and the mitochondrial-associated protein Mdm34 bridge the interaction between Mmm1 and Mdm10 in the complex (8,9). Using fluorescent markers, ERMES appears as multiple discrete puncta (8–10) at sites of ER-mitochondrial juxtaposition (6), suggesting that these four proteins assemble into higher order structures. In the absence of any single ERMES protein, the complex falls apart and puncta are no longer visible (6); while the morphology of the ER remains intact, the normally tubular mitochondrial network is converted into one or more large spheres (7,9,11,12).

The functional role of the ERMES complex is unclear. Mdm10 and Mdm12 were originally proposed to control mitochondrial shape and mitochondrial inheritance during cell division (7,11). Additional studies suggested that Mmm1, Mdm10 and Mdm12 attach mitochondria to the actin cytoskeleton for polarized transport to the growing yeast bud (8,13). The fact that ERMES mutants lose mitochondrial DNA (mtDNA) led to the suggestion that ERMES proteins control mitochondrial genome maintenance (9,10). Consistent with this idea, Mmm1 has been shown to co-localize with replicating mtDNA nucleoids (14). In addition, a link between actin filaments, ERMES proteins and mtDNA nucleoids appears to couple inheritance of mitochondrial membranes and genomes.
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During division (8). Mdm10, Mmm1 and Mdm12 were also shown to function in the import and assembly of mitochondrial β-barrel proteins (15–17). Finally, the ERMES complex has recently been linked to the transport of phosphatidylserine (PS) from the ER into mitochondria (6,18), where it can serve as a substrate for phosphatidylethanolamine (PE) synthesis (19,20). A role for ERMES in lipid transport is also supported by the fact that three ERMES proteins (Mmm1, Mdm12 and Mdm34) have SMP (synaptotagmin-like, mitochondrial and lipid-binding proteins) domains implicated in binding hydrophobic ligands, including lipids (21).

This bewildering list of potential functions of the ERMES complex raises the question of whether some of them are indirect. For example, it is conceivable that the deletion of ERMES components causes mitochondrial morphology or mitochondrial protein import defects, which in turn cause problems in other functions. Indeed, it was recently shown that mitochondrial morphology defects in some ERMES mutants are a secondary consequence of mitochondrial protein import defects (15).

New findings raise the possibility that ERMES is regulated by Gem1, a mitochondrial outer membrane protein in the conserved Miro (mitochondrial rho-like) GTPase family. Gem1 contains two GTPase domains and two calcium-binding EF-hand motifs, all of which are required for function (22). Correlation of Gem1 and all four ERMES loci in a genetic interaction map suggested that these genes are functionally related (6,18). Similar phenotypes have been described for gem1 and ERMES mutants, including abnormal mitochondrial morphology and loss of mtDNA (22). In addition, genetic studies have implicated Gem1 in mitochondrial inheritance during cell division (23) and regulation of lipid synthesis by ERMES (24). Finally, Gem1 was recently identified as a substoichiometric component of the ERMES complex (24,25).

Here, we show that the ERMES complex or Gem1 have no direct role in the transport of PS from the ER to mitochondria during the synthesis of PE. In addition, we report that mitochondrial inheritance defects in ERMES mutants are a secondary consequence of mitochondrial morphology defects, arguing against a primary role for ERMES in mitochondrial association with actin and mitochondrial movement. Finally, we show that ERMES complexes do not depend on the presence of Gem1 and are often long-lived. Taken together, our findings suggest that the ERMES complex may have primarily a structural role in maintaining mitochondrial morphology.

Results

The ERMES complex and Gem1 do not directly affect phosphatidylserine transport from the ER to mitochondria

The ERMES complex has been proposed to facilitate the transport of lipids between the ER and mitochondria by providing a physical link between the two organelles (6,24). Specifically, ERMES may promote the transfer of ER-synthesized PS to mitochondria. Once in mitochondria, PS is converted to PE by the phosphatidylserine decarboxylase Psd1, an enzyme located in the inner membrane (see scheme in Figure S1) (19,20). The reported genetic link between ERMES components and Gem1 raises the possibility that the Gem1 GTPase may also function in lipid transport (6,18).

We first addressed whether ERMES is involved in PS transport from the ER to mitochondria by following the conversion of PS to PE in vivo. Yeast cells were incubated with radioactive serine, the phospholipids were extracted, separated by thin-layer chromatography (TLC), and analyzed by autoradiography. These studies were performed in a strain that lacks the second phosphatidylserine decarboxylase Psd2, which is localized to the Golgi and also contributes to PE synthesis. Compared with the psd2Δ control, the percentage of PS converted to PE is not significantly reduced in mutants that lack both an ERMES component and Psd2 (Figure 1A and S2 control). By contrast, the simultaneous absence of Psd1 and Psd2 resulted in a significant reduction (Figure 1A). The small decrease observed for mdm34Δ and mdm10Δ could result from insufficient Psd1 in the mitochondria of these strains (see Figure S3A). Similar results were obtained in vitro. Crude isolated mitochondria that retain ER-mitochondria connections (26) were incubated with radiolabeled serine, and the conversion of the synthesized PS to PE was determined by TLC and autoradiography. While mitochondria lacking Psd1 were totally inactive, the deletion of ERMES components had no effect (Figure 1B). Collectively, these data show that the ERMES components are not essential for the transfer of PS between the ER and mitochondria. Analysis of the total cellular phospholipid composition showed that PE and PC remained unchanged in mutants lacking both Psd2 and either Mmm1 or Mdm12, and decreased moderately in mutants lacking both Psd2 and either Mdm34 or Mdm10 (Figure 1C). PS decreased moderately in the mutant lacking both Psd2 and Mmm1, increased moderately in the mutant lacking Mdm12 and remained unchanged in the other two double mutants. These results support the conclusion that the disruption of the ERMES complex does not dramatically compromise phospholipid metabolism.

Next, we asked whether Gem1 is required for PS to PE conversion. Using the in vivo assay, the conversion of PS to PE was only modestly altered by the absence of Gem1 (Figure 2A). Of particular significance is the finding that no defect in PS conversion to PE was observed when experiments were performed directly on crude mitochondria in vitro (Figure 2B). We also compared the phospholipid composition in psd2Δ and psd2Δ gem1Δ strains. Quantification of total cellular phospholipids by TLC revealed only insignificant differences between the gem1Δ psd2Δ strain and a psd2Δ control (Figure 2C). The phospholipid compositions of mitochondrial fractions...
isolated from these strains were also similar (Figure 2D). These results argue against a specific role for Gem1 in regulating transport of PS into mitochondria, or a general role in phospholipid metabolism.

The ERMES complex does not play a direct role in mitochondrial inheritance

Next, we addressed how ERMES components affect mitochondrial inheritance (7,9,11,12). During cell division in WT yeast cells, 100% of all medium- and large-sized buds inherit mitochondria from the mother cell (Figure 3A). In contrast, in mdm12Δ or mmm1Δ single mutants, only 40 or 51% of medium and large buds contain mitochondria, respectively. This is an underestimate of the defect, since we score inheritance as successful if a large bud contains only one or a few mitochondrial fragments. Although mitochondrial inheritance defects have been reported for mdm10Δ, in our strain background, mdm10Δ derived from a newly dissected tetrad displayed essentially wild-type inheritance (though it grew slowly). The mdm10Δ strain contained short tubular mitochondria in addition to the round or globular mitochondria characteristic of mdm12Δ or mmm1Δ (unpublished observations). Owing to its lack of mitochondrial inheritance phenotype, the mdm10Δ strain was not analyzed further.

To test whether the observed mitochondrial inheritance defects are a secondary consequence of mitochondrial morphology defects, we overexpressed Ypt11 in the mmm1Δ and mdm12Δ strains. Ypt11 binds to the tail of the Myo2 motor protein, and its overexpression was previously shown to promote the transport of mitochondria out of the mother cell into the bud (23,27). When overexpressed in ERMES mutants, Ypt11 increased mitochondrial inheritance from 40 to 81% in mdm12Δ and from 59 to 89% in mmm1Δ cells (Figure 3B). Importantly, overexpression of Ypt11 did not rescue mitochondrial morphology defects in the ERMES mutants; essentially 100% of mdm12Δ and mmm1Δ cells overexpressing Ypt11 retained mutant mitochondrial morphology (Figure 3C). Thus, the morphology and inheritance defects in mdm12Δ and mmm1Δ mutants are not obligatorily linked.

To further test whether morphology defects cause inheritance defects, we used an artificial tether between ER and mitochondria (28), called ChiMERA (Construct helping in Mitochondria-ER Association) (6). As reported previously (6), ChiMERA expression restored tubular mitochondrial morphology in 73% of mdm12Δ cells (Figure 3C). It also rescued the mitochondrial inheritance defect in this strain (Figure 3B). By contrast, ChiMERA did not rescue either the mitochondrial morphology (Figure 3B and (6)) or the inheritance defects in the mmm1Δ mutant (Figure 3B). ChiMERA expression also did not rescue the mitochondrial morphology and inheritance defects observed in the gem1Δ mutant (Figure S4, and unpublished observations). When considered together, our results demonstrate that:

Figure 1: ERMES is not essential for PS transfer between the ER and mitochondria. A) The conversion of PS to PE was determined in vivo by incubating yeast cells with radioactive serine, extracting phospholipids, and analysis by TLC. The percentage of radiolabeled PS converted to PE in psd2Δ control and mutant strains is shown. Labeling of the different ERMES mutants was comparable with the exception of mdm10Δ, which consistently incorporated less label than psd2Δ control and the other mutants. B) The conversion of PS to PE was determined in vitro using crude mitochondria incubated with radioactive serine. The percentage of radiolabeled PS converted to PE in WT and mutant mitochondria is shown. Bars and error bars represent the average and SD from three independent experiments. C) Relative changes in phospholipid composition in ERMES mutants. Lipid extracts of the indicated strains were analyzed by shotgun lipidomics (35). The abundance of the indicated lipid classes are shown relative to the control strain psd2Δ. Samples were analyzed in duplicate and average values are shown. The maximal difference between two duplicate measurements was 1.2%. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol.

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Figure 2: Deletion of GEM1 does not alter cellular and mitochondrial phospholipid profiles, or the transport dependent conversion of PS to PE. A) Transport of PS and conversion into PE was assayed in vivo by growing the indicated strains in medium containing [3H]-serine and analyzing the distribution of radioactivity among phospholipid classes. B) Synthesis and transport of PS and conversion into PE was measured in vitro by pulse labeling the crude mitochondrial fraction with [3H]-serine for 15 minutes to radiolabel the PS pool, and then arresting further PS synthesis and following the conversion to [3H]-PE. The reactions were terminated by lipid extraction and the distribution of radioactivity among phospholipid classes was quantified. To assess steady state lipid content, lipids were also extracted from whole cells (C), or mitochondria isolated from psd2Δ/gem1Δ/psd2Δ strains (D). Individual lipid classes were separated by two-dimensional TLC and quantified by measuring phosphorus. Bars and error bars represent the average and SD from three independent experiments. CL, cardiolipin; PA, phosphatidic acid. Other lipid abbreviations as in Figure 1.

Figure 3: Mitochondrial inheritance defects in ERMES mutants are a secondary consequence of mitochondrial morphology defects. Quantification of mitochondrial inheritance (A) and (B) or mitochondrial morphology (C) in strains expressing a fluorescent mitochondrial marker. Genotypes of strains containing vector alone or expressing Ypt11 or ChiMERA are indicated. Bars and error bars represent the average and SD from three independent experiments.

(i) enlarged, spherical mitochondria in ERMES mutants can be transported from the mother cell to the bud during cell division, (ii) normal, tubular mitochondria are inherited more efficiently than morphologically distorted mitochondria and (iii) ERMES proteins are not essential components of the mitochondrial inheritance machinery.

We showed previously that growth defects in gem1Δ, ypt11Δ and mmr1Δ mutants are correlated with mitochondrial inheritance defects (23). (Like Ypt11, Mmr1 is a Myo2 adapter that promotes mitochondrial inheritance (29).) If Gem1 acts in the same or a parallel pathway with ERMES, we would expect growth defects and inheritance defects to correlate in ERMES mutants as
Thus, the growth defect of the mdm12 defects, but not the growth defects (Figures 3B and 4).

In contrast, neither ChiMERA nor Ypt11 overexpression rescued growth defects in strains lacking the integral membrane proteins Mmm1 or Mdm10 (Figure 4). However, we found that overexpression of Ypt11 expression rescued growth defects in mdm10Δ (middle) or mmm1Δ (bottom).

well. However, this was not the case. As shown previously (6), ChiMERA expression rescued the growth defect of mdm12Δ (Figure 4), consistent with the observation that ChiMERA rescues inheritance defects in this strain (Figure 3B). However, we found that overexpression of Ypt11 in mdm12Δ cells rescued mitochondrial inheritance defects, but not the growth defects (Figures 3B and 4).

Thus, the growth defect of the mdm12Δ ERMES mutant is not caused by a mitochondrial inheritance defect. By contrast, neither ChiMERA nor Ypt11 overexpression rescued growth defects in strains lacking the integral membrane proteins Mmm1 or Mdm10 (Figure 4).

ERMES complex formation does not require GEM1, YPT11 or MMR1, genes implicated in mitochondrial inheritance

To further test the relationship between Gem1 and ERMES, we visualized the ERMES complex by tagging its ER component Mmm1 with GFP (Mmm1-GFP). Mitochondria were simultaneously imaged using mitochondrial-targeted RFP (mtrRFP). Essentially all WT cells contained multiple ERMES puncta that colocalized with the tubular mitochondrial network (Figure 5). Although previous studies reported that yeast cells contain one to ten ERMES complexes (8–10,24), we often observed more puncta, all of which aligned along the mitochondrial surface. Our ability to visualize more ERMES puncta per cell is likely due to differences in imaging equipment and strain backgrounds used. In addition, a subset of Mmm1-GFP marked ERMES puncta underwent rapid photobleaching, and there may be additional ERMES assemblies that are too small to visualize by fluorescence microscopy. Thus, there are likely more ERMES complexes connecting the ER to mitochondria in vivo than previously appreciated. Importantly, in the absence of Gem1, 99% of the cells contained ERMES puncta despite the fact that the cells have globular and fragmented mitochondria. Thus, ERMES complexes assemble at ER-mitochondrial junctions even when mitochondrial morphology is abnormal, and the aberrant mitochondrial morphology in the gem1Δ mutant is not due to an inability to form ERMES puncta. ERMES puncta were also observed in mitochondrial inheritance mutants ypt11Δ and mmm1Δ, which were previously shown to have synthetic growth defects with gem1Δ (23). As shown in Figure 5, 96% of cells in mmm1Δ or ypt11Δ strains contained ERMES puncta. It should be noted that these cells have tubular mitochondria.

Immunoprecipitation experiments support the notion that Gem1 is not required for ERMES complex formation. When lysates from cells expressing functional Mmm1-GFP were subjected to immunoprecipitation with GFP antibodies, both Mdm10 and Mdm12 were co-immunoprecipitated (Figure 6, lane 7). As expected, in the absence of Mdm12 or Mdm34, Mdm10 was no longer immunoprecipitated (lanes 8 and 9). Importantly, Mdm10 and Mdm12 remain associated when Gem1 is absent (lane 10). Together, these data indicate that Gem1 is not a major structural component of ERMES and is not essential for formation of the ERMES complex. In contrast to a previous report (24), the number and appearance of ERMES puncta was normal in strains over-expressing Gem1 or in strains expressing Gem1 proteins that carry mutations in the GTPase domain or EF-hand (unpublished observations).

The ERMES complex is a stable structure

Because our results suggested that the ERMES complex may be primarily involved in maintaining the structural integrity of mitochondria, we tested its stability by live-cell imaging. We used fluorescence microscopy to follow Mmm1-GFP labeled ERMES complexes over time. Over the course of 40 minutes, many ERMES puncta remained stationary within a cell (Figure 7, arrows), while some appeared to migrate (Figure 7, arrowheads mark an ERMES complex moving into the growing bud). Several examples of ERMES puncta forming de novo were also documented (Figure 7, open circles).

We used fluorescence recovery after photobleaching (FRAP) to determine whether Mmm1-GFP could be incorporated into an existing ERMES puncta. As shown in Figure 8, we detected recovery of Mmm1-GFP fluorescence 15 minutes after photobleaching (compare C and D). Additional fluorescence recovery was observed 30 minutes and 45 minutes after photobleaching (Figure 8E and F). Thus, a mobile fraction of Mmm1-GFP is able to repopulate ERMES after photobleaching. No recovery of Mmm1-GFP was observed with time periods shorter than 15 minutes, indicating that the rate of recovery is
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**Figure 5:** ERMES puncta formation does not require GEM1 or genes implicated in mitochondrial inheritance. Differential interference contrast (DIC, left), Mmm1-GFP (middle), and Mmm1-GFP merged with mtRFP (right) images of the indicated strains are shown. The percentage of cells in a population containing Mmm1-GFP puncta is indicated. Bar: 5μm.

**Figure 6:** GEM1 is not required for ERMES complex formation. Mmm1-GFP (and associated proteins) was immunopurified and the integrity of the ERMES complex analyzed by immunoblotting with antibodies against GFP or the endogenous proteins Mdm10 and Mdm12.

Discussion

In this report, we provide evidence that the ERMES complex and Gem1 do not directly affect lipid transport (6,24) or mitochondrial inheritance (7,8,11,12,23) as previously suggested. In addition, ERMES and Gem1 are likely functioning in distinct pathways, despite their genetic linkage and physical association (6,24,25). Our results are more consistent with a model in which the ERMES complex serves a structural role in maintaining the morphological integrity of mitochondria.

We found that the absence of ERMES components has little or no effect on the conversion of PS to PE. Thus, there is no dramatic defect in the transport of PS from the ER into mitochondria, and the small reduction in PS to PE conversion can be explained by an indirect effect of ERMES components on the import of Psd1 into mitochondria. The absence of Gem1 had no significant effect on PS to PE conversion. Consistent with our conclusions, the cellular phospholipid composition changed only slightly in mutants lacking both Psd2 and either ERMES components or Gem1. Kornmann et al. (6) did not actually report on PS to PE conversion, but they found a moderate decrease in the conversion of PS to PC in ERMES mutants. In our experiments, ERMES mutations did not affect the incorporation of radioactivity into PC (Figure S3A). In addition, minor radiolabeling of PC can occur by other pathways (i.e. the 1-carbon pathway), making it difficult to reach conclusions based on analysis of PC alone. It should also be noted that we were unable to restore tubular mitochondrial morphology in gem1Δ and ERMES mutants by adding
lipid precursors, including ethanolamine, choline, lyso-PE or lyso-PC (Figure S5). Thus, mitochondrial morphology defects observed in gem1Δ and ERMES mutants are not simply a consequence of a deficiency of PC and PE.

The ERMES proteins Mmm1, Mdm10 and Mdm12 have been proposed to attach mitochondria to the actin cytoskeleton for polarized movement of mitochondria during cell division (8,13). Indeed, there are numerous reports in the literature that ERMES mutants produce large buds lacking mitochondria (7,9,11,12). Here, we provide evidence that mitochondrial inheritance defects in ERMES mutants are a secondary consequence of mitochondrial morphology changes. First, we showed that abnormally shaped mitochondria in ERMES mutants are transported into the bud upon overexpression of the inheritance-promoting factor Ypt11. Thus, aberrant mitochondria are able to bind and move on actin filaments in the absence of ERMES components. Second, we observed that restoration of tubular mitochondrial morphology by the artificial tether ChiMERA is correlated with the rescue of mitochondrial inheritance defects in mdm12Δ cells. The ChiMERA protein is able to restore ER-mitochondrial contact sites and tubular mitochondrial morphology but has no known actin-binding activity. Thus, mitochondrial shape is a more important determinant of mitochondrial distribution than the presence or absence of ERMES. Our combined findings argue against a model in which ERMES has a core function in mitochondrial association with, or movement along, the actin cytoskeleton.

ChiMERA expression was not able to rescue mitochondrial morphology defects in cells lacking Gem1 or the integral membrane proteins Mmm1 or Mdm10. Thus, Mdm10, Mmm1 and Gem1 carry out critical functions affecting mitochondrial shape, which cannot be bypassed by...
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Materials and Methods

Strains and plasmid construction

Standard methods were used for growth and analysis of yeast strains and plasmid construction (33). Yeast strains and plasmids are listed in Tables S1 and S2.

p415-ADH was generated by PCR amplification of the ADHpr using forward primer, 5′-CGCCTACTCTGTTGTTACATT and reverse primer 5′-GAGTGATCAGTGTAGGACTT that was cloned into p415 using the restriction sites SacI and XbaI. The PSD1 open reading frame was PCR amplified and cloned into p415-ADHpr, using restriction sites BamHl and Xhol.

Phosphatidylserine conversion assay (in vivo)

For measuring PS to PE conversion in vivo (Figure 1A), overnight cultures were grown in SC dextrose medium containing 5 mM ethanolamine (EtN) and diluted in fresh media to resume growth. Aliquots of logarithmically grown cells (A600 of 0.3 OD) were sedimented by centrifugation and resuspended in 1 mL of fresh minimal medium, containing 5 mM EtN and 1 mM L-(14C)UI-serine. Samples were incubated for 2 h at 30°C followed by lipid extraction using the Folch procedure. In brief, cells were sedimented by centrifugation, resuspended in 330 μL of methanol and disrupted by vortex mixing with 100 μL of glass beads, for 10 min. Subsequently, 660 μL of chloroform was added and samples were centrifuged for 10 min at 10000 x g to resolve insoluble material. The organic solvent supernatant was recovered and washed once with 0.9% NaCl and the lower chloroform phase was dried under a stream of nitrogen. Lipids were resuspended in 15 μL chloroform and separated by TLC on Silica60 plates using chloroform/methanol/25% ammonium hydroxide (50/25/6 v/v/v). Labeled lipids were visualized using Phosphoimagetm (BioRad, PMI) and quantified using Metamorph software. Data reported are the mean ± SD for three experiments.

For measuring PS to PE conversion in vivo in Figure 2A, the psd2Δ and psd2Δ gem1α strains were grown in synthetic complete medium plus 2 mM Etn with glucose as a carbon source (SC). Cells in mid-log phase were harvested by centrifugation and washed twice by resuspension in water and recentrifugation. The cells were suspended in SC medium, at an A600 of 0.35 in a volume of 2 mL. Radiolabeling was initiated by adding 20 μCi L-[3H]serine, and growth was continued at 30°C for 2 h with vigorous shaking. Labeled phospholipids were extracted as previously described (34) and the lipid classes were resolved by thin layer chromatography, and radioactivity was quantified by liquid scintillation spectrometry.

Phosphatidylserine conversion assay (in vitro)

For reconstituted aminoglycerophospholipid synthesis and transport (Figure 1B), yeast cells were grown to early-logarithmic phase in synthetic dextrose media at 30°C. Crude mitochondria (containing ER-mitochondrial contact sites) were purified as previously described (35). For lipid synthesis and transport, 100 μL crude mitochondria (100 μg protein) in 0.6 mM mannitol, 20 μM Tris pH 7.4 and 0.6 mM MnCl2 were incubated with 0.1 μCi L-[14C(U)]UI-serine at 30°C. After 20 min, 40 μL unlabelled serine was added and PS synthesis was arrested by addition of 5 mM EDTA. The conversion of nascent PS to PE was subsequently followed in a 45 min incubation at 30°C. The reaction was stopped by adding 1 mL chloroform:methanol 2:1 (v/v). After shaking for 1 h, lipids were purified by extracting the organic phase, washing with 100 μL 0.9% NaCl (w/v) and drying at 65°C. Lipids were resuspended in 15 μL chloroform, loaded on thin-layer chromatography plates (Silica 60), separated in chloroform/methanol/25% ammonium hydroxide (50/25/6 v/v/v) and visualized using a phosphoimagetm (BioRad, PMI). Radioactive lipids were quantified using ImageJ software. Data reported are the mean ± SD for three experiments.

For the aminoglycerophospholipid synthesis and transport studies with crude mitochondria shown in Figure 2B the process was followed using...
Phospholipid class analysis in whole cells washed in water at 4°C yeasts growing exponentially in YP-rich medium containing 2% glucose, Samples for lipidome analysis were harvested from 20 mL cultures of Lipidome analysis by mass spectrometry

Strains were grown to early log phase at 30°C washed twice with water. For mitochondrial lipid analysis (Figure 2D), in lysis buffer (50 mM HEPES-KOH pH 6.8, 150 mM potassium acetate, monitored lipid species within a FT MS scan. Samples were analyzed in negative ion mode. Lipids were quantified by intensity profiling using peak monitored in positive ion mode, and PS and PI species were monitored in Fisher Scientific) as previously described (37). PC and PE species were Biosciences, Inc.) and a LTQ Orbitrap XL mass spectrometer (Thermo analysis was performed using a Triversa NanoMate ion source (Advion Biosciences, Inc.) and a LTO Orbitrap XL mass spectrometer (Thermo Fisher Scientific) as previously described (37). PC and PE species were monitored in positive ion mode, and PS and PI species were monitored in negative ion mode. Lipids were quantified by intensity profiling using peak intensities of monitored lipid species normalized to the total intensity of all monitored lipid species within a FT MS scan. Samples were analyzed in duplicate.

Lipidome analysis by mass spectrometry

Samples for lipidome analysis were harvested from 20 mL cultures of yeast growing exponentially in YP-rich medium containing 2% glucose, washed in water at 4°C, and frozen immediately in liquid nitrogen. Lipid analysis was performed using a Triversa NanoMate ion source (Advion Biosciences, Inc.) and a LTO Orbitrap XL mass spectrometer (Thermo Fisher Scientific) as previously described (37). PC and PE species were monitored in positive ion mode, and PS and PI species were monitored in negative ion mode. Lipids were quantified by intensity profiling using peak intensities of monitored lipid species normalized to the total intensity of all monitored lipid species within a FT MS scan. Samples were analyzed in duplicate.

Phospholipid class analysis in whole cells and mitochondria

For whole cell lipid analysis (Figure 2C), strains were grown to mid-log phase at 30°C in SC dextrose medium, harvested by centrifugation, and washed twice with water. For mitochondrial lipid analysis (Figure 2D), strains were grown to early log phase at 30°C in semisynthetic lactate medium (0.3% yeast extract, 0.05% CaCl₂·2H₂O, 0.05% NaCl, 0.09% MgCl₂·6H₂O, 0.1% KH₂PO₄, 0.1% NH₄Cl, 2% lactate and 0.05% dextrose, pH 5.5) supplemented with adenine (20 μg/mL), uracil (20 μg/mL), L-arginine (100 mg/L), L-histidine (20 mg/L), and L-tryptophan (20 mg/L). Lipids were extracted (34) from crude mitochondria (38) and phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on Silica 60 plates using chloroform/methanol/ammonium hydroxide (65/35/5 v/v/v) followed by chloroform/acetic acid/methanol/water (75/25/5.2/2.5 v/v/v). Lipids were visualized with iodine vapor and quantified by measuring phosphorus (39). The results are shown as the percentage of total lipid phosphorus in each phospholipid fraction. Data reported are the mean ± SD for three experiments.

Communoprecipitation assays

Membrane fractions were prepared from logarithmically grown cells (YPD, 500 OD₆₀₀ units). Cells were collected, washed in water and resuspended in lysis buffer (50 μM HEPES-KOH pH 6.8, 150 mM potassium acetate, 2 mM MgOAc, 1 mM CaCl₂, 200 mM sorbitol, 1 mM PMSF) supplemented with protease inhibitors (Complete EDTA-free Cocktail, Roche). Samples were lysed by vortexing with glass beads for 5 × 30 s. After preclearing by centrifugation at 300 × g, membranes were pelleted at 50 000 × g for 30 min and resuspended in immunoprecipitation buffer (50 μM HEPES-KOH pH 6.8, 150 mM potassium acetate, 2 mM MgOAc, 1 mM CaCl₂, 15% glycerol). Digitonin (2%) was added and membranes were solubilized for 30 min at 4°C on a nutator. Supernatants cleared by centrifugation for 30 min at 50 000 × g were loaded on GFP-TRAP resin (ChromoTek, Planegg-Martinsried, Germany) and incubated overnight at 4°C. Samples were washed four times with IP buffer (0.1% digitonin) and bound material was eluted by addition of SDS sample buffer. Input and eluted samples were analyzed by SDS-PAGE, followed by immunoblotting using antibodies against GFP (Roche, Mannheim, Germany) or endogenous proteins. Antibodies against Mdm10 and Mdm12 were kindly provided by C. Meisinger (University of Freiburg, Germany).

Fluorescence microscopy and live cell imaging

For time-lapse studies, cells expressing Mmm1-GFP were mounted on glass-bottom dishes coated with 0.1 mg/mL concanavalin A (ConA), and visualized with a Zeiss Axiovert 200 Imaging microscope equipped with DIC optics, epifluorescence capabilities, and a 100× (NA 1.4) oil immersion objective. Optical sections (0.9 μm) were acquired at 20 min intervals for a total of four hours using a Sensicam QE cooled CCD camera. Images were analyzed with Slidebook 4.2 software using only linear adjustments of contrast and brightness. Projection view images were assembled using Adobe Photoshop and Adobe Illustrator CS5.1.

For FRAP experiments, cells expressing Mmm1-GFP mounted in 1D media containing 2% low melting point agar were visualized using a Zeiss LSM 510 ConfoCor 3 microscope with a 100×, 1.4 numerical aperture (NA) oil-immersion objective (optical sections = 0.9 μm). To obtain 100% loss of fluorescence, an Mmm1-GFP puncta defined in an oval-shaped region of interest in the equatorial plane of the cell membrane was bleached using a 488 nm laser. FRAP was monitored using low laser intensity at 15-minute intervals. Projection view images were assembled and analyzed using ImageJ, Adobe Photoshop and Adobe Illustrator CS5.1.

ERMES puncta formation was scored at 30°C in WT and mutant strains expressing integrated Mmm1-GFP grown to mid-log phase (OD₆₀₀ 0.5–1.5) in synthetic dextrose (SD; 2%) dropout media. Mitochondria were visualized as described previously with pYX142-Su9 (aa 1–69-GFP) or p414-GFP-Su9 (aa 1–69-RFP) (22), referred to as mitochondrial-targeted GFP (mtGFP) and RFP (mtRFP), respectively. Phenotypes were quantified in at least 100 cells in three or more independent experiments. Data reported are the mean ± standard deviation (SD). Images were acquired and processed as described previously (40).

Morphology and inheritance of mitochondria labeled with mtGFP (for Ypt11 expression and lipid supplementation studies) or mtRFP (for ChiMERA expression studies) were scored in log phase strains as described previously (23). For Ypt11 overexpression experiments, strains containing p416-MET72-YPT11 or p416-MET72 and the mtGFP marker were grown overnight at 30°C in SD dropout media containing 0.1 mg/mL methionine. Ypt11 expression was induced for 4 h by diluting into SD media lacking methionine and cysteine. Quantification was performed as described above.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Mitochondrial PE biosynthesis pathway. PA is converted to PE in the ER and is subsequently transferred to the mitochondrion. PE is decarboxylated by Psd1 to form PE. Mitochondrial PE can be transferred to the ER for PC synthesis.

Figure S2: Synthesis of radiolabeled PS and conversion to PE does not saturate within the time frame of conversion rate measurements. A) Yeast cells (BY4741) were incubated with radiolabeled serine and incorporation of the label into PS, PE and PC was followed for 4 h in a continuous labeling experiment. Label incorporation continued after the 2 h time point when conversion rates were measured (Figure 1A and
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Figure S3: B) Yeast cells (BY4741) were pulsed (3h) and chased (4h) with radiolabeled serine. The graph shows the percentage of radioactivity in PS, PE and PC during the chase phase. Samples were analyzed in duplicate and average values are shown. The maximal difference between two duplicate measurements was 4%.

Figure S3: Overexpression of Psd1 increases conversion of PS to PE in WT, ERMES-mutant, and psd1Δ psd2Δ strains. The small reduction in PS to PE conversion caused by the absence of mdm34Δ and mdm10Δ in Figure 1A might be indirect, since these ERMES components are involved in the insertion of β-barrel proteins into the outer mitochondrial membrane, and these in turn are required for the import of proteins, such as Psd1, into the inner membrane. To test this possibility, we measured radiolabeled serine incorporation into PS (black), PE (gray) and PC (white) in the indicated strains plus/minus a second copy of the PSD1 gene expressed from the constitutive ADH promoter. (A) Conversion of PS to PE was increased in both WT and ERMES-mutants. (B) Compromised PE synthesis in a psd1Δ psd2Δ strain lacking phosphatidylethanolamine decarboxylases is rescued by Psd1 overexpression. For reasons that are unclear, these results differ from previous reports that Psd1 is not rate-limiting for PS to PE conversion (41–43). Additional studies are required to resolve this issue.

Figure S4: Chimera does not rescue mitochondrial morphology defects in gem1Δ. Quantification of mitochondrial morphology in WT and gem1Δ strains containing vector alone or a plasmid expressing ChiMERA. Mitochondria were visualized by expressing mtGFP. Bars and error bars represent the average and SD from three independent experiments.

Figure S5: Supplemental choline does not rescue mitochondrial morphology defects in gem1Δ or ERMES mutant strains. Merged DIC and mtGFP images are shown for the indicated strains grown in the absence (+) or presence (−) of 1mM choline. pem7Δ lacks the phosphatidylethanolamine methyltransferase enzyme that catalyzes the first step in PE to PC biosynthesis. Choline supplementation rescues abnormal mitochondrial morphology in pem7Δ, but not gem1Δ and ERMES mutants. Similar results for the gem1Δ and ERMES mutant strains were obtained with 0.5mM ethanolamine, lyso-PE or lyso-PC supplementation. Bar: 5μm.

Table S1: Yeast strains (44–46)

Table S2: Plasmids (47)

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