Phosphatidylcholine Affects the Role of the Sorting and Assembly Machinery in the Biogenesis of Mitochondrial β-Barrel Proteins*

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Background: The role of phosphatidylcholine (PC) in the biogenesis of mitochondrial outer membrane proteins is unknown.

Results: PC is required for efficient biogenesis of mitochondrial β-barrel proteins.

Conclusion: PC promotes function of the mitochondrial protein sorting and assembly machinery in the biogenesis of outer membrane proteins.

Significance: PC and phosphatidylethanolamine affect distinct transport steps of mitochondrial β-barrel precursors.

Two protein translocases drive the import of β-barrel precursor proteins into the mitochondrial outer membrane: The translocase of the outer membrane (TOM complex) promotes transport of the precursor to the intermembrane space, whereas the sorting and assembly machinery (SAM complex) mediates subsequent folding of the β-barrel and its integration into the target membrane. The non-bilayer-forming phospholipids phosphatidylethanolamine (PE) and cardiolipin (CL) are required for the biogenesis of β-barrel proteins. Whether bilayer-forming phospholipids such as phosphatidylcholine (PC), the most abundant phospholipid of the mitochondrial outer membrane, play a role in the import of β-barrel precursors is unclear. In this study, we show that PC is required for stability and function of the SAM complex during the biogenesis of β-barrel proteins. PC further promotes the SAM-dependent assembly of the TOM complex, indicating a general role of PC for the function of the SAM complex. In contrast to PE-deficient mitochondria precursor accumulation at the TOM complex is not affected by depletion of PC. We conclude that PC and PE affect the function of distinct protein translocases in mitochondrial β-barrel biogenesis.

β-Barrel proteins are present in the outer membrane of Gram-negative bacteria and cell organelles of endosymbiotic origin like mitochondria and plastids of plant cells (1–4). In mitochondria β-barrel proteins fulfill various central functions. Tom40 forms the protein-conducting channel of the translocase of the outer membrane (TOM complex),4 which allows import of the vast majority of precursor proteins into mitochondria (5–9). The voltage-dependent anion channel (VDAC; Porin in yeast) transports metabolites across the outer membrane (10, 11). The mitochondrial distribution and morphology protein Mdm10 is a core component of the endoplasmic reticulum (ER)-mitochondria encounter structure, which forms a molecular bridge between mitochondria and the ER (12–14). Finally, Sam50 is the central subunit of the sorting and assembly machinery (SAM complex, also termed TOB complex for topogenesis of mitochondrial outer membrane β-barrel proteins), which is crucial for the biogenesis of several outer membrane proteins (3, 4, 8, 9, 15).

All β-barrel proteins are synthesized as precursors on cytosolic ribosomes and recognized by the TOM receptors Tom20, Tom22 and Tom70 on the mitochondrial surface (16–18). After passage of the outer membrane via the Tom40 channel the precursors are delivered to the SAM complex (19, 20). This transfer step is promoted by two different mechanisms. First, the TOM and SAM complexes form a supercomplex to enable efficient substrate channeling (21). Second, small TIM chaperones of the intermembrane space bind to the precursors, probably to shield hydrophobic patches (21–23). The SAM complex mediates folding and membrane integration of β-barrel precursors (21). The protein machinery consists of three subunits: The β-barrel forming Sam50 (Tob55, Omp85) is the central component of the SAM complex and conserved from bacteria

4 The abbreviations used are: TOM, translocase of the outer membrane; VDAC, voltage-dependent anion channel; ER, endoplasmic reticulum; SAM, sorting and assembly machinery; Mdm10, mitochondrial distribution and morphology protein 10; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol, PS, phosphatidylserine; Pem, phosphatidylethanolamine methyltransferase; Psd, phosphatidylycerine decarboxylase.

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to human (20, 24–26). It associates with the peripheral subunits Sam35 (Tob38, Tom38) and Sam37 (Mas37, Tom37) (27). Sam35 is crucial for initial binding to the incoming precursor via the conserved $\beta$-signal within the last $\beta$-strand of the precursor (28, 29). Sam37 was reported to be important for the stability of the SAM complex and the release of the substrate proteins into the lipid phase (19, 29–32). Sam50 and Sam35 are essential for life, indicating the central role of $\beta$-barrel biogenesis for mitochondrial function (20, 24, 26, 33, 34). Additionally, the SAM complex promotes the assembly of the $\beta$-barrel protein Tom40 with the small Tom proteins and Tom22 to form the mature TOM complex (30, 31, 35). In particular, a subpopulation of the SAM complex that additionally contains Mdm10 is important for this assembly process (35–40).

The main phospholipids of mitochondrial membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (CL). Mitochondria contribute to cellular lipid homeostasis by synthesis of CL and PE, which are not typical bilayer-forming lipids (41). The biosynthetic pathway of CL is located within mitochondria (12, 42). The PS decarboxylase (Psd1) of the mitochondrial inner membrane produces the majority of cellular PE in yeast cells (12, 42–45). Studies with yeast strains defective in CL or PE synthesis revealed that these non-bilayer-forming phospholipids affect stability and function of several mitochondrial membrane protein complexes including the respiratory chain (46–58). CL and PE play crucial roles in protein import into mitochondria (42, 59). First, CL and PE affect membrane-bound protein translocases of both mitochondrial membranes that drive protein transport processes (57, 60–68). Second, the decreased respiratory chain activity in CL and PE-depleted mitochondria leads to a reduced membrane potential, which in turn impairs protein transport into and across the inner membrane (46, 47, 57, 58, 63). Surprisingly, studies on the role of CL and PE in the import of mitochondrial $\beta$-barrel proteins revealed distinct functions of both phospholipids. Although present in low amounts, CL affects $\beta$-barrel protein biogenesis by stabilizing TOM and SAM complexes (64). In contrast, depletion of PE affects the transport of $\beta$-barrel precursors across the TOM complex, but does not lead to destabilization of TOM and SAM complexes (67). Altogether, these studies revealed that non-bilayer-forming phospholipids are important for several central processes of mitochondrial biogenesis and function.

Whereas the role of non-bilayer-forming phospholipids for mitochondrial biogenesis was studied in some detail, the role of bilayer-forming phospholipids, especially of PC in these processes has not yet been investigated. PC is the most abundant phospholipid in both mitochondrial membranes (69, 70). In yeast, two pathways localized in the ER produce PC for all cellular membranes. First, enzymes of the CDP-choline pathway (Kennedy pathway) utilize free choline to generate PC (42, 71). Second, the two PE methyltransferases Pem1 (Cho2) and Pem2 (Opi3) perform a three-step methylation reaction of PE using S-adenosylmethionine as methyl donor. Pem1 catalyzes the first methylation step, while Pem2 is capable of promoting all three steps, albeit the last two steps with higher efficiency than the first one (72–77). Biosynthesis of PC is essential for the survival of the yeast cell (75). Mice deficient in PE methyltransferase developed non-alcoholic steatohepatitis when fed with choline-deficient diet (78). In humans, defects in the synthesis of PC were found in patients suffering from congenital muscular dystrophy (79). Interestingly, the patient cells contained mitochondria with abnormal morphology, pointing to a role of PC in mitochondrial biogenesis (79). However, it was unclear whether PC may also be required for the function of specific membrane-integrated proteins.

In the present study, we investigated the role of PC in the biogenesis of mitochondrial $\beta$-barrel proteins. Upon depletion of PC, the biogenesis of $\beta$-barrel proteins was severely impaired. We found that PC is required for stability of the SAM complex and promotes its functions in the biogenesis of $\beta$-barrel proteins and in the assembly of the TOM complex. In contrast to PE-depleted mitochondria, the binding of an arrested precursor to the TOM complex is not impaired. We conclude that PC and PE differently affect the protein translocases involved in the biogenesis of $\beta$-barrel proteins in mitochondria.

Experimental Procedures

Yeast Strains, Growth Conditions, and Isolation of Mitochondria—The yeast strains pem1Δ, pem2Δ, and their corresponding wild-type BY4741 were obtained from Euroscarf. The psd1Δ strain and its corresponding wild-type were described (57). Cells were grown to mid-logarithmic growth phase at 30 °C in minimal medium containing 2% (w/v) glycerol and 0.1–0.2% (w/v) glucose as carbon source. Mitochondria were isolated by differential centrifugation as described (80), shock frozen in S.E. buffer (10 mM MOPS/KOH, pH 7.2, 1 mM EDTA, 250 mM sucrose) and stored at −80 °C until use.

Protein Import into Isolated Mitochondria—For import reactions proteins were synthesized in the presence of [35S]methionine using a cell-free coupled transcription/translation system based on reticulocyte lysate (TNT, Promega). Subsequently, 35S-labeled precursors were incubated with isolated mitochondria in import buffer (3% (w/v) BSA, 250 mM sucrose, 5 mM methionine, 80 mM KCl, 5 mM MgCl2, 10 mM MOPS/KOH pH 7.2, 10 mM KH2PO4) containing 4 mM ATP, 4 mM NADH, 5 mM creatine phosphate, and 0.1 mg/ml creatine kinase. The import reaction was stopped by transfer on ice. Mitochondria were resolated by centrifugation and washed with S.E. buffer. The mitochondrial pellet was resuspended in lysis buffer (20 mM Tris/HCl pH 7.4; 50 mM NaCl, 0.1 mM EDTA, 10% (w/v) glycerol) containing 0.4–1% (w/v) digitonin for 15 min on ice. Insoluble material was removed by centrifugation and the import was analyzed by blue native electrophoresis (80). After import of the Om45 precursor, mitochondria were treated with proteinase K to remove non-integrated precursor proteins (81). To study membrane insertion of imported precursor proteins, mitochondria were subjected to carbonate extraction as previously described (37). Aggregated 35S-labeled precursor proteins were removed by preincubation in import buffer without mitochondria prior to the import reaction. After removal of insoluble proteins by centrifugation, the supernatant was added to mitochondria to start the import reaction.

Co-immunoprecipitation—For co-immunoprecipitation antisera against Tom22 and its corresponding pre-immune
serum were covalently coupled to protein A-Sepharose (GE Healthcare) with dimethyl pimelimidate. Isolated mitochondria were resuspended in lysis buffer containing 1% (w/v) digitonin and incubated for 15 min on ice. Insoluble material was removed by centrifugation, and the supernatant was incubated with the antibody matrix for 1 h at 4 °C under constant rotation. Subsequently, the protein A-Sepharose was washed with an excess amount of lysis buffer containing 0.1% (w/v) digitonin. Bound proteins were eluted with 0.1 M glycine (pH 2.5). The eluate was immediately neutralized by addition of 1 M TRIS-base and analyzed by SDS-PAGE and Western blotting.

Results

**Phospholipid Analysis**—Lipids of isolated mitochondria were extracted with a 2:1 (v/v) mixture of chloroform/methanol as described (45, 82). Subsequently, the organic phase was washed with 0.034% (w/v) MgCl₂, a 4:1 (v/v) mixture of 2 N KCl/methanol and a mixture of methanol/water/chloroform (48:47:3, per vol.). Individual phospholipids were separated by two-dimensional thin-layer chromatography (83). For detection phospholipids were stained with iodine vapor, scrapped off, and quantified (84).

**Miscellaneous**—All antibodies used in this study have been controlled with wild-type and the corresponding mutant mitochondria as described (21, 67). After separation, proteins were transferred from the gel onto a PVDF membrane (Millipore) by semi-dry Western blotting. For immunodetection, signals of transferred proteins were controlled with wild-type and the corresponding mutant mitochondria (84).

**Proteins**—To study the role of PC in the biogenesis of mitochondrial β-barrel proteins, we used the yeast deletion strains pem1Δ and pem2Δ, which are defective in the first and the last two methylation steps of PE, respectively. To block production of PC by the CDP-choline pathway, we grew the cells on minimal medium lacking free choline. We used glycerol as the major carbon source to ensure that mitochondrial function was not decreased, which promotes transfer of β-barrel precursors to the SAM complex (21–23). Thus, we excluded the possibility that decreased steady state protein levels of translocase subunits led to impaired β-barrel protein biogenesis in the mutant mitochondria.

As the SAM complex is crucial for β-barrel protein biogenesis, we wondered whether the integrity of the SAM complex was affected upon PC-depletion. To tackle this issue, we analyzed the SAM complex in pem1Δ and pem2Δ mitochondria by blue native electrophoresis. Two main populations of the SAM complex can be resolved on blue native gels: The SAMcore complex of 200 kDa consisting of the three SAM subunits and the SAM-Mdm10 complex of 350 kDa, which additionally contains Mdm10 (Fig. 2B) (27, 36–40). Strikingly, increased amounts of a Sam35-Sam50 subcomplex were observed in PC-deficient mitochondria. In particular in pem2Δ mitochondria less Sam37 was detected in the SAM complexes (Fig. 2B), although protein levels of Sam37 were not reduced (Fig. 2A). We conclude that PC stabilizes association of Sam37 with the Sam35-Sam50 subcomplex.

To analyze whether the accumulation of precursors at the SAM complex requires PC, we imported 35S-labeled Tom40 forms three assembly stages, which can be resolved by blue native electrophoresis: The Tom40 precursor binds to the SAM complex (Fig. 1C, SAM), is subsequently released in association with Tom5 (Fig. 1C, Int. II) before assembling into the mature TOM complex of 450 kDa (Fig. 1C, TOM) (19, 31). In PC-deficient mitochondria the integration of the Tom40 precursor into intermediate II and the TOM complex was largely compromised (Fig. 1, C and D). In addition, in pem2Δ mitochondria the formation of the SAM intermediate was already decreased (Fig. 1, C and D). Similarly, the assembly of imported Porin precursor was impaired in PC-depleted mitochondria (Fig. 1, E and F). For comparison, we studied the β-barrel protein biogenesis in psd1Δ mitochondria isolated from cells grown on minimal medium. In psd1Δ mitochondria PC and PI levels were increased, whereas PE was decreased as described previously (Fig. 1B) (67, 85). The import of Tom40 and Porin was blocked in psd1Δ mitochondria (Fig. 1G) consistent with the observation with mitochondria from psd1Δ cells grown on complete medium (67). We conclude from these results that the biogenesis of β-barrel proteins depends on the presence of both PC and PE.
plex in the absence of an inner membrane potential (21). Applying this assay to PC-deficient mitochondria, we observed that the arrest of b$_2$(220)-Tom40 at the TOM-SAM supercomplex was impaired in the mutant mitochondria (Fig. 3B). We conclude that already the binding of β-barrel precursors to the TOM-SAM supercomplex depends on the presence of PC.
PC and PE Differentially Affect the Function of the TOM Complex—The import of β-barrel precursors into PE-depleted mitochondria is blocked at the TOM stage (67). We wondered whether depletion of PC affects stability and function of the TOM complex, leading to decreased binding of β-barrel precursors to the TOM-SAM supercomplex. The level of the TOM complex, which migrates at 450 kDa on blue native gels, was only mildly affected in PC-deficient mitochondria (Fig. 4A). Importantly, no subcomplex of lower molecular mass was detected, indicating that the TOM complex remained stable in the mutant mitochondria. Previously, it was reported that the association of the peripheral receptors Tom20 and Tom70 to the TOM complex is disturbed on blue native gels (86). To study the interaction of TOM core subunits with the peripheral TOM receptors in mutant mitochondria, we performed co-immunoprecipitation using antibodies against Tom22. Both Tom20 and Tom70 were efficiently co-purified with antibodies directed against Tom22, but not with the corresponding pre-immune serum (Fig. 4B, lanes 5–8). We conclude that the TOM complex remains largely intact in PC-depleted mitochondria. To analyze the capability of the TOM complex to bind precursor proteins, we imported Oxa1 into the mutant mitochondria in the absence of an inner membrane potential. Under these conditions the hydrophobic Oxa1 precursor accumulates at the TOM complex, which can be detected on blue native gels (87). Strikingly, the binding of the Oxa1 precursor to the TOM complex was not impaired in PC-deficient mitochondria (Fig. 4C, lanes 1–9). In psd1Δ mitochondria, however, the binding of Oxa1 was not observed.

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FIGURE 1. A balanced PC/PE ratio is crucial for the biogenesis of mitochondria β-barrel proteins. A, serial dilutions of WT, pem1Δ, and pem2Δ cells were spotted on minimal medium containing 2% (v/v) glycerol and 0.2% (w/v) glucose as carbon source. B, phospholipid profiles of WT, pem1Δ, pem2Δ, and psd1Δ mitochondria were analyzed by two-dimensional thin-layer chromatography. Relative amounts of the major phospholipid classes are shown. Means ± S.E. (n = 3). LP, lyso-phospholipids; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid. C, 35S-labeled Tom40 precursor was imported for the indicated time periods into WT, pem1Δ, and pem2Δ mitochondria. The import reaction was analyzed by blue native electrophoresis and autoradiography. SAM, Tom40 precursor bound to the SAM complex; Int. II, second assembly intermediate of the Tom40 precursor; TOM, Tom40 precursor assembled into the TOM complex. D, quantification of the three assembly steps of the Tom40 precursor in import assays as described in C. Means ± S.E. (n = 4). The formation of the SAM intermediate was quantified after 5 min of import; intermediate II was determined after 10 min of import and the formation of the TOM complex was quantified after 40 min of import. E, 35S-labeled Porin precursor was imported for the indicated time periods into wild-type (WT), pem1Δ and pem2Δ mitochondria. The import reaction was analyzed by blue native electrophoresis and autoradiography. F, quantification of the assembly of the Porin precursor into the 400 kDa complex in import assays as described in E. Means ± S.E. (n = 3). G, 35S-labeled Tom40 (left panel) or Porin precursor (right panel) was imported for the indicated time periods into WT and psd1Δ mitochondria. The import reaction was analyzed by blue native electrophoresis and autoradiography.

FIGURE 2. The SAM complex is destabilized in PC-depleted mitochondria. A, indicated protein amounts of WT, pem1Δ, and pem2Δ mitochondria were subjected to SDS-PAGE and immunodetection with the indicated antisera. B, WT, pem1Δ, and pem2Δ mitochondria were subjected to blue native electrophoresis and immunodetection with the indicated antisera.
accumulation of the Oxa1 precursor at the TOM complex was compromised to a great extent (Fig. 4, lanes 10–15) (67). We conclude that the stability and function of the TOM complex are not impaired in PC-deficient mitochondria. In contrast, PE promotes the interaction of precursor proteins with the TOM complex (67).

**PC Promotes the Assembly of Tom22 into the TOM Complex**—We asked whether PC is generally required for the function of the SAM complex. Previous studies have shown that in addition to the biogenesis of β-barrel proteins, the SAM complex promotes insertion and assembly of the Tom22 precursor into the mature TOM complex (32, 35, 37). Thus, we tested whether the function of the SAM complex in the assembly of Tom22 requires PC. To this end, we imported 35S-labeled Tom22 precursor into PC-deficient mitochondria. Strikingly, the assembly of Tom22 into the mature TOM complex was drastically affected in pem1Δ and pem2Δ mitochondria (Fig. 5A, lanes 1–9), substantiating a specific role of PC in the function of the SAM complex. For comparison, the integration of Tom22 into the TOM complex was only mildly delayed in psd1Δ mitochondria (Fig. 5A, lanes 10–15) (67). Similarly, the SAM-dependent integration of the Tom5 precursor into the TOM complex (31, 35) was delayed in the PC-deficient mutant mitochondria (Fig. 5B), pointing to a defective function of the SAM complex in the assembly of the TOM complex. In contrast, the assemblies of 35S-labeled Tom20 and Om45 precursors were not affected in PC-deficient mitochondria (Fig. 5, C and D). Thus, the biogenesis of outer mitochondrial membrane proteins is not generally blocked in the mutants. Interestingly, the Om45 precursor passes the TOM channel before it is
assembled into the outer membrane (81, 88). Therefore, the transport of this precursor across the TOM complex was not impaired in PC-deficient mitochondria (Fig. 5D). The precursors of Tom20 and Om45 are imported into the outer membrane in a SAM-independent manner (19, 34, 35, 81). As SAM subunits promote membrane integration of the Tom22 precursor (35), we investigated the insertion efficiency by carbonate extraction. Indeed, we found that integration of Tom22, but not of Tom20, into the outer mitochondrial membrane was compromised in pem1Δ/H9004 and pem2Δ/H9004 mitochondria (Fig. 5E). We conclude that PC promotes both functions of the SAM complex: the biogenesis of β-barrel proteins and the assembly of the TOM complex.

**Discussion**

PC is the most abundant phospholipid of cellular membranes and crucial for the formation of the phospholipid bilayer. This also applies to mitochondrial membranes whose major phospholipid is PC. However, the influence of PC on integral membrane proteins in mitochondria has not yet been analyzed. In the present study we show that PC is specifically required for the functions of the SAM complex in the biogenesis of mitochondrial β-barrel proteins and the assembly of the TOM complex. This observation may have important implications for pathological developments like non-alcoholic steatohepatitis and congenital muscular dystrophy, which were found in tissues defective in PC synthesis (78, 79).

The protein import pathway of β-barrel precursors involves the coordinated activity of two membrane-bound protein translocases and the integration of precursor proteins into a lipid bilayer. These membrane-localized steps might render the biogenesis of β-barrel proteins sensitive toward alteration of the phospholipid composition. So far, a role of the non-bilayer-forming phospholipids CL and PE in the biogenesis of β-barrel proteins was reported (64, 67). Here, we identified a novel function of PC in this process. Surprisingly, individual depletion of these three phospholipids affects different steps in the biogenesis of β-barrel proteins. First, CL is required for the stabilization of TOM and SAM complexes (64). Second, depletion of PE affects binding of precursor proteins to the TOM complex, but not the stability of the translocases (67). Third, we showed in this study that PC promotes the function of the SAM complex, whereas the precursor accumulation at the TOM complex and the transport of the Om45 precursor across the TOM channel remain largely unaffected upon depletion of PC. Interestingly, import and assembly of the Tom22 precursor is drastically
diminished in PC-deficient mitochondria. Tom22 biogenesis depends on the SAM complex, indicating that PC is generally important for SAM function in mitochondria. Supporting this view, the SAM-mediated assembly of the Tom5 precursor into the TOM complex is delayed. In contrast, the non-bilayer-forming phospholipids CL and PE are not required for the biogenesis of Tom22 (64, 67). Moreover, the SAM-independent insertion of Tom20 occurs independently of CL, PE, and PC (64, 67, 68). Thus, PC is specifically required for the SAM-dependent protein import pathways.

How do phospholipids affect TOM and SAM complexes? It was assumed that the non-bilayer-forming properties of CL and PE are important for the function of membrane-bound proteins by increasing the curvature stress within the membrane (41). Here, we found that the bilayer-forming phospholipid PC promotes the function of the SAM complex, indicating that further properties of phospholipids are crucial for the functionality of mitochondrial processes. The charge of the head group is an important feature of phospholipids. For instance, the activity of the respiratory chain depends on both CL and PE (47, 49, 57, 58). While the negatively charged head group of CL stabilizes the supercomplex of cytochrome c reductase and cytochrome c oxidase (89), depletion of PE leads to the formation of higher oligomeric structure of the respiratory chain complexes (57). PC and PE are the major phospholipids of the mitochondrial outer membrane and contain both a neutrally charged head group. Depletion of PE causes accumulation of PC and vice versa, pointing to similar structural and functional roles of both phospholipids (67, 85). Nevertheless, PE and PC differentially affect the function of TOM and SAM complexes of the mitochondrial outer membrane. As depletion of PC does not affect precursor binding to the TOM complex, one can speculate that the activity of the TOM complex specifically depends on the presence of the non-bilayer-forming character of PE. Thus, the charge of the head group and the nature of the bilayer versus non-bilayer-forming character seem to be crucial for the specific roles of phospholipids in the biogenesis of mitochondrial β-barrel proteins.

Altogether, we can conclude that the phospholipids CL, PE and PC play different roles in the biogenesis of β-barrel proteins. The individual phospholipids specifically affect the function and stability of TOM and SAM complexes. These observations reflect the central role of the intimate interplay between membrane-embedded protein translocases and the surrounding phospholipid environment. Thus, in addition to proteinaceous components phospholipids turn out more and more to be important players for mitochondrial protein biogenesis.

**References**

1. Schleif, E., and Soll, J. (2005) Membrane protein insertion: mixing eukaryotic and prokaryotic concepts. *EMBO Rep.* 6, 1023–1027

2. Walter, D. M., Rapaport, D., and Tommassen, J. (2009) Biogenesis of β-barrel membrane proteins in bacteria and eukaryotes: evolutionary conservation and divergence. *Cell Mol. Life Sci.* 66, 2789–2804

3. Webb, C. T., Heinz, E., and Lithgow, T. (2012) Evolution of the β-barrel assembly machinery. *Trends Microbiol.* 20, 612–620

4. Höhr, A. I. C., Straub, S. P., Warscheid, B., Becker, T., and Wiedemann N. (2015) Assembly of β-barrel proteins in the mitochondrial outer membrane. *Biochim. Biophys. Acta* 1853, 74–88

5. Koehler, C. M. (2004) New developments in mitochondrial assembly. *Annu. Rev. Cell Dev. Biol.* 20, 309–335

6. Baker, M. J., Frazier, A. E., Gulbis, J. M., and Ryan, M. T. (2007) Mitochondrial protein-import machinery: correlating structure with function. *Trends Cell Biol.* 17, 456–464

7. Neupert, W., and Herrmann, J. M. (2007) Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* 76, 723–749

8. Endo, T., and Yamano, K. (2010) Transport of protein across or into the mitochondrial outer membrane. *Biochim. Biophys. Acta* 1803, 706–714

9. Becker, T., Böttiger, L., and Pfanner, N. (2012) Mitochondrial protein import: from transport pathways to an integrated network. *Trends Biochem. Sci.* 37, 85–91

10. Hiller, S., Abramson, J., Mannella, C., Wagner, G., and Zeth, K. (2010) The 3D structure of VDAC represent a native conformation. *Trends Biochem. Sci.* 35, 514–521

11. Colombini, M. (2012) VDAC structure, selectivity, and dynamics. *Biochim. Biophys. Acta* 1818, 1457–1465

12. Osman, C., Voelker, D. R., and Langer, T. (2011) Making heads or tails of phospholipids in mitochondria. *J. Cell Biol.* 192, 7–16

13. Toulmay, A., and Prinz, W. A. (2011) Lipid transfer and signaling at organelle contact sites: the tip of the iceberg. *Curr. Opin. Cell Biol.* 23, 458–463

14. Lang, A., John Peter, A. T., and Kornmann, B. (2015) ER-mitochondria contact sites in yeast: beyond the myths of ERMES. *Curr. Opin. Cell Biol.* 35, 7–12

15. Dukanovic, J., and Rapaport, D. (2011) Multiple pathways in the integration of proteins into the mitochondrial outer membrane. *Biochim. Biophys. Acta* 1808, 971–980

16. Krimmer, T., Rapaport, D., Ryan, M. T., Meisinger, C., Kassenbrock, C. K., Blachly-Dyson, E., Forte, M., Douglas, M. G., Neupert, W., Nargang, F. E., and Pfanner, N. (2001) Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. *J. Cell Biol.* 152, 289–300

17. Habib, S. J., Waizenegger, T., Lech, M., Neupert, W., and Rapaport, D. (2005) Assembly of the TOB complex of mitochondria. *J. Biol. Chem.* 280, 6434–6440

18. Yamano, K., Yatsukawa, Y., Esaki, M., Hobbs, A. E., Jensen, R. E., and Endo T. (2008) Tom20 and Tom22 share the common signal recognition pathway in mitochondrial protein import. *J. Biol. Chem.* 283, 3799–3807

19. Wiedemann, N., Koizak, V., Chacinska, A., Schönfisch, B., Rospert, S., Ryan, M. T., Pfanner, N., and Meisinger, C. (2003) Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature* 424, 565–571

20. Paschen, S. A., Waizenegger, T., Stan, T., Preuss, M., Czyrklaff, M., Hell, K., Rapaport, D., and Neupert, W. (2003) Evolutionary conservation of biogenesis of β-barrel membrane proteins. *Nature* 426, 862–866

21. Qiu, J., Wenz, L. S., Zerbes, R. M., Oeljeklaus, S., Bohnert, M., Stroud, D. A., Wirth, C., Ellenrieder, L., Thornton, N., Kutik, S., Wiese, S., Schulze-Specking, A., Zufall, N., Chacinska, A., Guird, B., Hunte, C., Warscheid, B., van der Laan, M., Pfanner, N., Wiedemann, N., and Becker, T. (2013) Coupling of mitochondrial import and export translocases by receptor-mediated supercomplex formation. *Cell* 154, 596–608

22. Wiedemann, N., Truscott, K. N., Pfannschmidt, S., Guird, B., Meisinger, C., and Pfanner, N. (2004) Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: intermembrane space components are involved in an early stage of the assembly pathway. *J. Biol.
Biogenesis of the Mitochondrial Outer Membrane

van den Brink-van der Laan, E., Killian, J. A., and de Kruijff, B. (2004) Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile. Biochim. Biophys. Acta 1666, 275–288

Horvath, S. E., and Daum, G. (2013) Lipids of mitochondria. Prog. Lipid Res. 52, 590–614

Clancy, C. J., Chang, S. C., and Dowhan, W. (1993) Cloning of a gene (PSD1) encoding phosphatidylserine decarboxylase from Saccharomyces cerevisiae by complementation of an Escherichia coli mutant. J. Biol. Chem. 268, 24580–24590

Trotter, P. J., Pedretti, J., and Voelker, D. R. (1993) Phosphatidylserine decarboxylase from Saccharomyces cerevisiae: isolation of mutants, cloning of the gene, and creation of a null allele. J. Biol. Chem. 268, 21416–21424

Horvath, S. E., Böttinger, L., Vögtle, F. N., Wiedemann, N., Meisinger, C., Becker, T., and Daum, G. (2012) Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1. J. Biol. Chem. 287, 9667–9675

Jiang, F., Ryan, M. T., Schlame, M., Zhao, M., Gu, Z., Klingenberg, M., Pfanner, N., and Greenberg, M. L. (2000) Absence of cardiolipin in the crd1 null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. J. Biol. Chem. 275, 22387–22394

Pfeiffer, K., Gohil, V., Staat, R. A., Hunte, C., Brandt, U., Greenberg, M. L., and Schägger, H. (2003) Cardiolipin stabilizes respiratory chain supercomplexes. J. Biol. Chem. 278, 52873–52880

Birner, R., Nebauer, R., Schneiter, R., and Daum, G. (2003) Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine biosynthetic machinery with the prohibitin complex of Saccharomyces cerevisiae. Mol. Biol. Cell 14, 370–383

Zhang, M., Mileykovskaya, E., and Dowhan, W. (2005) Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. J. Biol. Chem. 280, 29403–29408

Claypool, S. M., Oktay, Y., Boontheung, P., Loo, J. A., and Koehler, C. M. (2008) Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. J. Biol. Chem. 283, 935–950

Gonzalez, F., Schug, Z. T., Houtkooper, R. H., Mackenzie, E. D., Brooks, D. G., Wanders, R. J., Petit, P. X., Vaz, F. M., and Gottlieb, E. (2008) Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. J. Biol. Chem. 283, 681–696

DeVay, R. M., Domínguez-Ramírez, L., Lackner, L. L., Hoppins, S., Stahlberg, H., and Nunnari, J. (2009) Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion. J. Cell Biol. 186, 793–803

Mileykovskaya, E., and Dowhan, W. (2009) Cardiolipin membrane domains in prokaryotes and eukaryotes. Biochim. Biophys. Acta 1788, 2084–2091

Kuroda, T., Tani, M., Moriguchi, A., Tokunaga, S., Higuchi, T., Kitada, S., and Kuge, O. (2011) FMP30 is required for the maintenance of a normal cardiolipin level and mitochondrial morphology in the absence of mitochondrial phosphatidylethanolamine synthesis. Mol. Microbiol. 80, 248–265

Osman, C., Haag, M., Potting, C., Rodenfels, J., Dip, P. V., Wieland, F. T., Brügger, B., Westermann, B., and Langer, T. (2009) The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. J. Cell Biol. 184, 583–596

Joshi, A. S., Thompson, M. N., Fei, N., Hüttemann, M., and Greenberg, M. L. (2012) Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in Saccharomyces cerevisiae. J. Biol. Chem. 287, 17589–17597

Böttinger, L., Horvath, S. E., Kleinschroth, T., Hunte, C., Daum, G., Pfanner, N., and Becker, T. (2012) Phosphatidylethanolamine and cardiolipin differently affect the stability of mitochondrial respiratory chain supercomplexes. J. Biol. Chem. 423, 677–686

Tasseva, G., Bai, H. D., Davidec, M., Haromy, A., Michelakis, E., and Vance, J. E. (2013) Phosphatidylethanolamine deficiency in mammalian mitochondria impairs oxidative phosphorylation and alters mitochondrial morphology. J. Biol. Chem. 288, 4158–4173
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59. Böttinger, L., Ellenrieder, L., and Becker, T. (2015) How lipids modulate mitochondrial protein import. J. Bioenerg. Biomembr. doi: 10.1007/s10166-015-9599-7

60. Tamura, Y., Harada, Y., Yamano, K., Watanabe, K., Ishikawa, D., Ohshima, C., Nishikawa, S., Yamamoto, H., and Endo, T. (2006) Identification of Tam41 maintaining integrity of the TIM23 protein translocator complex in mitochondria. J. Cell Biol. 174, 631–637

61. Gallas, M. R., Dienhart, M. K., Stuart, R. A., and Long, R. M. (2006) Characterization of Mmp37p, a Saccharomyces cerevisiae mitochondrial matrix protein with a role in mitochondrial protein import. Mol. Biol. Cell 17, 4051–4062

62. van der Laan, M., Meinecke, M., Dudek, J., Hutu, D. P., Lind, M., Persch, I., Guiard, B., Wagner, R., Pfanner, N., and Rehling, P. (2007) Motor-free mitochondrial pressequence translocase drives membrane integration of preproteins. Nat. Cell Biol. 9, 1152–1159

63. Kutik, S., Rissler, M., Guan, X. L., Guiard, B., Shui, G., Gebert, N., Heacock, N., van der Laan, M., Meinecke, M., Dudek, J., Hutu, D. P., Lind, M., Persch, I., Guiard, B., Wagner, R., Pfanner, N., and Rehling, P. (2007) The translocator maintenance protein Tam41 is required for mitochondrial cardiolipin biosynthesis. J. Cell Biol. 183, 1213–1221

64. Gebert, N., Joishi, A. S., Kutik, S., Becker, T., McKenzie, M., Guan, X. L., Mooga, V. P., Stroud, D. A., Kulkarni, G., Wenk, M. R., Rehling, P., Meisinger, C., Ryan, M. T., Wiedemann, N., Greenberg, M. L., and Pfanner, N. (2009) Mitochondrial cardiolipin involved in outer-membrane protein biogenesis: implications for Barth syndrome. Curr. Biol. 19, 2133–2139

65. Tamura, Y., Endo, T., Iijima, M., and Sasaki, H. (2009) Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. J. Cell Biol. 185, 1029–1045

66. Tamura, Y., Onguka, O., Hobbs, A. E., Jensen, R. E., Iijima, M., Claypool, S. M., and Sasaki, H. (2012) Role of two conserved intermembrane space proteins, Ups1p and Ups2p, in intra-mitochondrial phospholipid trafficking. J. Biol. Chem. 287, 15205–15218

67. Becker, T., Horvath, S. E., Böttinger, L., Gebert, N., Heacock, P. N., Rehling, P., Dowhan, W., Wenk, M. R., Pfanner, N., and Wiedemann, N. (2008) The translocator maintenance protein Tam41 is required for mitochondrial cardiolipin biosynthesis. J. Cell Biol. 183, 1213–1221

68. Sauerwald, J., Jores, T., Eisenberg-Bord, M., Churtzman, S. G., Schulner, M., and Rapaport, D. (2015) Genome-wide screens in yeast high-light a role for cardiolipin in biogenesis of mitochondrial outer membrane multispan salts proteins. Mol. Cell. Biol. 35, 3200–3211

69. Spker-a-Gottlieb, C. D. M., Hermetter, A., Paltauf, F., and Daum, G. (1988) Lipid topology and physical properties of the outer mitochondrial membrane of the yeast, Saccharomyces cerevisiae. Biochim. Biophys. Acta 946, 227–234

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

71. Cole, L. K., Vance, J. E., and Vance, D. E. (2012) Phosphatidylinositol biosynthesis and lipoprotein metabolism. Biochim. Biophys. Acta 1821, 754–761

72. Greenberg, M. L., Klug, L. S., Letts, V. A., Loewy, B. S., and Henry, S. A. (1983) Yeast mutant defective in phosphatidylinositol synthesis. J. Bacteriol. 153, 791–799

73. Kodaki, T., and Yamashita, S. (1987) Yeast phosphatidylethanolamine methylation pathways: Cloning and characterization of two distinct methyltransferase genes. J. Biol. Chem. 262, 15428–15435

74. Summers, F. E., Letts, V. A., McGraw, P., and Henry, S. A. (1988) Saccharomyces cerevisiae cho2 mutants are deficient in phospholipid methyla-

tion and cross-pathway regulation of inositol synthesis. Genetics 120, 909–922

75. Kodaki, T., and Yamashita, S. (1989) Characterization of the methyltransferases in the yeast phosphatidylethanolamine methylation pathway by selective gene disruption. Eur. J. Biochem. 183, 243–251

76. Gaynor, P. M., and Carman, G. M. (1990) Phosphatidylethanolamine and phospholipid methyltransferase activities from Saccharomyces cerevisiae. Enzymological and kinetic properties. Biochim. Biophys. Acta 1045, 156–163

77. Preitschopf, W., Lückl, H., Summers, E., Henry, S. A., Paltauf, F., and Kohlwein, S. D. (1993) Molecular cloning of the yeast OPI3 gene as a high copy number suppressor of the cho2 mutation. Curr. Genet. 23, 95–101

78. Li, Z., Agellon, L. B., Allen, T. M., Umeda, M., Jewell, L., Mason, A., and Vance, D. E. (2006) The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and stealth properties. Cell Metabol. 3, 321–331

79. Mitsuhashi, S., Ohkuma, A., Talim, B., Karahashi, M., Kouruma, T., Aoyama, C., Kurihara, M., Quinlin, R., Sewry, C., Mitsuhashi, H., Goto, K., Koksal, B., Kale, G., Ikeda, K., Taguchi, R., Noguchi, S., Hayashi, Y. K., Nonaka, I., Sher, R. B., Sugimoto, H., Nakagawa, Y., Cox, G. A., Topaloglu, H., and Nishino, I. (2011) A congenital muscular dystrophy with mitochondrial structural abnormalities caused by defective de novo phosphatidylcholine biosynthesis. Am. J. Hum. Genet. 88, 845–851

80. Stojanovski, D., Pfanner, N., and Wiedemann, N. (2007) Import of proteins into mitochondria. Methods Cell Biol. 80, 783–806

81. Wenz, L. S., Opaliński, L., Schulter, M. H., Ellenrieder, L., Ieva, R., Böttinger, L., Qiu, J., van der Laan, M., Wiedemann, N., Guiard, B., Pfanner, N., and Becker, T. (2014) The presequence pathway is involved in protein sorting to the mitochondrial outer membrane. EMBO Rep. 15, 678–685

82. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509

83. Schneider, R., and Daum, G. (2006) Analysis of yeast lipids. Methods Mol. Biol. 313, 75–84

84. Broekhuyse, R. M. (1968) Phospholipids in tissues of the eye. I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids. Biochim. Biophys. Acta 152, 307–315

85. Gohil, V. M., Thompson, M. N., and Greenberg, M. L. (2005) Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine and cardiolipin biosynthetic pathways in Saccharomyces cerevisiae. J. Biol. Chem. 280, 35410–35416

86. Dekker, P. J. T., Ryan, M. T., Brix, J., Müller, H., Höninger, A., and Pfanner, N. (1998) Preprotein translocase of the outer mitochondrial membrane: molecular dissection and assembly of the general import pore complex. Mol. Cell. Biol. 18, 6515–6524

87. Frazier, A. E., Chacinska, A., Truscott, K. N., Guiard, B., Pfanner, N., and Rehling, P. (2003) Mitochondria use different mechanisms for transport of multispanning membrane proteins through the intermembrane space. Mol. Cell. Biol. 23, 7818–7828

88. Song, J., Tamura, Y., Yoshiiisa, T., and Endo, T. (2014) A novel import route for an N-anchor mitochondrial outer membrane protein aided by the TIM23 complex. EMBO Rep. 15, 670–677

89. Wenz, T., Hielscher, R., Hellwig, P., Schägger, H., Richers, S., and Hunte, C. (2009) Role of phospholipids in respiratory cytochrome bc complex catalysis and supercomplex formation. Biochim. Biophys. Acta 1787, 609–616