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COMMUNICATION

Phosphatidylethanolamine and Cardiolipin Differentially Affect the Stability of Mitochondrial Respiratory Chain Supercomplexes

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The mitochondrial inner membrane contains two non-bilayer-forming phospholipids, phosphatidylethanolamine (PE) and cardiolipin (CL). Lack of CL leads to destabilization of respiratory chain supercomplexes, a reduced activity of cytochrome *c* oxidase, and a reduced inner membrane potential $\Delta\psi$. Although PE is more abundant than CL in the mitochondrial inner membrane, its role in biogenesis and assembly of inner membrane complexes is unknown. We report that similar to the lack of CL, PE depletion resulted in a decrease of $\Delta\psi$ and thus in an impaired import of preproteins into and across the inner membrane. The respiratory capacity and in particular the activity of cytochrome *c* oxidase were impaired in PE-depleted mitochondria, leading to the decrease of $\Delta\psi$. In contrast to depletion of CL, depletion of PE did not destabilize respiratory chain supercomplexes but favored the formation of larger supercomplexes (megacomplexes) between the cytochrome bc_1 complex and the cytochrome *c* oxidase. We conclude that both PE and CL are required for a full activity of the mitochondrial respiratory chain and the efficient generation of the inner membrane potential. The mechanisms, however, are different since these non-bilayer-forming phospholipids exert opposite effects on the stability of respiratory chain supercomplexes.

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Abbreviations used: AAC, ADP/ATP carrier; CL, cardiolipin; Crd1, cardiolipin synthase; $F_1\beta$, β subunit of F_1F_o-ATP synthase; PE, phosphatidylethanolamine; Psd, phosphatidylserine decarboxylase; TIM22, carrier translocase of the inner mitochondrial membrane; TIM23, presequence translocase of the inner mitochondrial membrane.

Mitochondria are crucial for the synthesis of the major non-bilayer-forming phospholipids, phosphatidylethanolamine (PE) and cardiolipin (CL).[1–3] Non-bilayer lipids have a comparably small head group and a bulky fatty acid moiety, which results in a conical shape of the phospholipid. These phospholipids have the tendency to form hexagonal-phase structures and thus to increase the tension within a bilayer, which is important for the function of membrane proteins.[2] PE is an abundant phospholipid present in all cellular membranes

and essential for cell survival,[2,4–8] whereas CL is specific for mitochondria.[2,4,8,9] The synthesis of CL takes place in the inner mitochondrial membrane, where the CL synthase (Crd1) catalyzes the formation of CL from phosphatidylglycerol and CDP-diacylglycerol.[2,3,10–12] In yeast, the majority of PE is generated by decarboxylation of phosphatidylserine catalyzed by phosphatidylserine decarboxylases (Psd). Two Psd enzymes have been described. Psd1 plays the major role in PE synthesis and is located in the mitochondrial inner membrane/intermembrane space.[13–16] Smaller amounts of PE are generated by the Psd2 activity of the Golgi apparatus/vacuole membrane, as well as by the CDP-ethanolamine pathway and the acyltransferases Tgl3 and Ale1.[1,4,13,17–26] Little is known of how PE synthesized in mitochondria is transported to other cellular membranes. The efficient transfer of phospholipids between the mitochondrial membranes and the endoplasmic reticulum might occur at contact sites between the membranes.[2]

CL and PE are of particular importance for mitochondrial functions. Lack of either Crd1 or Psd1 impairs growth of cells on non-fermentable carbon sources and leads to an altered mitochondrial morphology.[6,27–29] CL and PE are required for mitochondrial fusion,[30,31] and Crd1 as well as Psd1 show genetic interactions with prohibitins, which have been proposed to function as scaffolds that enrich CL and PE in membrane domains.[2,15,32] Deletion of both genes, *CRD1* and *PSD1*, is synthetically lethal for yeast cells.[27] Based on these observations, it was proposed that CL and PE perform overlapping functions, which might be partially attributed to their non-bilayer-forming character.[33]

The role of CL in the mitochondrial inner membrane has been analyzed on a molecular level. CL is required for function and stability of several protein complexes. It binds to the ADP/ATP carrier (AAC) and is crucial for the formation of AAC oligomers.[34–41] CL also plays a central role for the activity and organization of the mitochondrial respiratory chain. It binds to the cytochrome bc_1 complex (complex III) and cytochrome *c* oxidase (complex IV)[40,42,43] that form high-molecular-weight supercomplexes.[44–46] In the absence of CL, the III–IV supercomplexes are destabilized, the activity of the respiratory chain, particularly of cytochrome *c* oxidase, is decreased, and thus the inner membrane potential $\Delta\psi$ is reduced.[38,47–49] CL-deficient mitochondria are impaired in the import and assembly of inner membrane proteins.[35,39] Precursor proteins are transported to the inner membrane by two routes.[50–56] In the presequence pathway, preproteins with a cleavable presequence are transported by the general translocase of the outer membrane and the presequence translocase of the inner membrane (TIM23 complex). Precursors of carrier proteins contain internal targeting signals and are integrated into the inner membrane by the carrier translocase (TIM22 complex). Since both import routes into the inner membrane depend on a $\Delta\psi$, the decrease of $\Delta\psi$ in CL-deficient mitochondria is a main reason for disturbed protein import.[35,39,57] In addition, the stability and function of protein translocases such as the TIM23 complex are affected when CL is absent, and also the assembly of AAC into oligomers depends on the presence of CL.[39,57–61]

PE is the most abundant non-bilayer-forming phospholipid in the mitochondrial inner membrane.[4,62,63] PE binds to respiratory chain complexes[43,64] and *in vivo* data indicate an important role of PE for mitochondrial functions.[6,29,31] Studies with lactose permease revealed a role of PE in folding and activity of membrane proteins in *Escherichia coli*.[65–68] The effect of PE depletion on mitochondrial processes, however, has not been studied on a molecular level.

Here, we report that protein transport into and across the inner membrane is impaired in PE-depleted mitochondria. The protein translocases and inner membrane complexes are not dissociated upon lack of PE, but the activity of the respiratory chain, in particular of cytochrome *c* oxidase, is impaired, leading to a reduction of $\Delta\psi$. Thus, the reduced $\Delta\psi$ leads to an impairment of protein import into the inner membrane. In contrast to the lack of CL, lack of PE stabilizes supercomplexes of the respiratory chain and does not block the formation of AAC oligomers. Though both PE and CL are required for respiratory activity and efficient generation of a $\Delta\psi$ by mitochondria, they play opposing roles in the stabilization of protein complexes.

PE-depleted mitochondria are impaired in preprotein transport to the inner membrane

To study the role of PE in mitochondrial protein biogenesis, we used a *Saccharomyces cerevisiae* strain lacking Psd1 and a double deletion strain lacking Psd1 and Psd2.[69] Both yeast strains exhibited a poor growth on non-fermentable carbon sources and were sensitive to growth at high and low temperatures (Fig. S1a). For further analysis, we grew the cells at an intermediate temperature (30 °C, early logarithmic growth phase) on non-fermentable carbon sources and analyzed the phospholipid profiles of cell extracts from *psd1*Δ and *psd1*Δ *psd2*Δ strains.[6] The level of PE (29 mol% of total phospholipids in wild-type cells) was considerably decreased in *psd1*Δ cells (7%) and strongly reduced in the double deletion mutant (1%) (Fig. S1b).[6] We also determined the content of total phospholipids in purified inner membrane vesicles from these mutants and observed a moderate reduction of the phospholipid-to-protein ratio compared to wild type (Fig. S1c). Moreover, we determined the

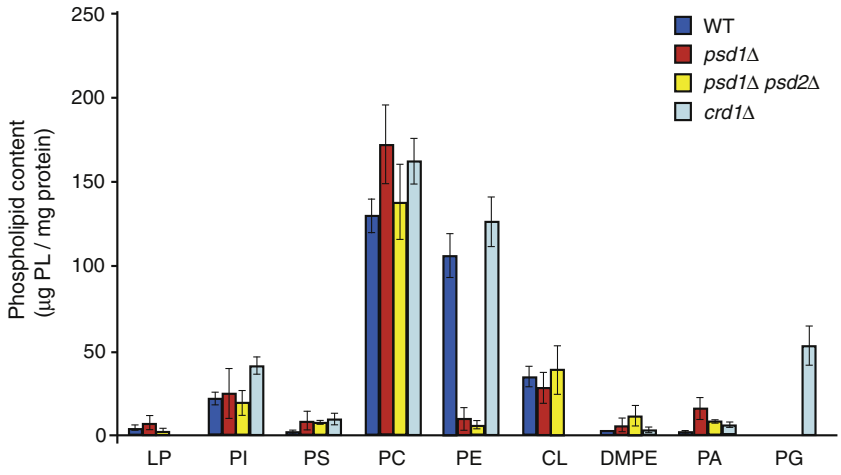

Fig. 1. PE is selectively depleted in inner membrane vesicles from *psd1*Δ and *psd1*Δ *psd2*Δ mitochondria. The *S. cerevisiae* strains *crd1*Δ, *psd1*Δ, and *psd1*Δ *psd2*Δ in the BY4741 background[69] were grown in YPLac medium[39] at 30 °C to early logarithmic growth phase. Mitochondria were isolated by differential centrifugation, the protein concentrations were adjusted, and inner membrane vesicles were isolated by sucrose gradient centrifugation as previously described.[4,70] Phospholipids were extracted, separated by thin-layer chromatography, and analyzed as reported previously.[69,71,72] The amounts of individual phospholipid classes were determined using a phosphate solution with 1 mg/ml phosphor as standard.[73] Shown are the mean values of two determinations with range. DMPE, dimethylphosphatidylethanolamine; LP, lysophospholipids; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

absolute amounts of individual phospholipid classes in inner membrane vesicles from wild-type, *psd1*Δ, and *psd1*Δ *psd2*Δ mitochondria. Figure 1 shows that only the PE content was strongly reduced in the mutants, whereas the amounts of other phospholipids remained largely unaffected. In comparison, the levels of PE in total cell extracts and mitochondrial inner membrane vesicles from *crd1*Δ mutants were similar to the wild-type levels, whereas the levels of phosphatidylglycerol were strongly increased (Fig. 1 and Fig. S1b).[35,48,49]

For protein import experiments, mitochondrial precursor proteins were synthesized in reticulocyte lysates and labeled with [^{35}S]methionine. We used three presequence-containing preproteins and incubated them with mitochondria isolated from *psd1*Δ and *psd1*Δ *psd2*Δ strains: cytochrome c_1 is inserted into the inner membrane, whereas subunit β of the F_1F_o-ATP synthase ($F_1\beta$) and the model preprotein Su9-DHFR are translocated across the inner membrane into the matrix.[61,74–77] Each of these preproteins was imported in a $\Delta\psi$-dependent manner and the presequences were proteolytically removed (Fig. 2a). Import of the three preproteins was reduced in *psd1*Δ mitochondria and strongly reduced in *psd1*Δ *psd2*Δ mitochondria (Fig. 2a, lanes 5–7 and 9–11). The import of AAC via the carrier pathway was analyzed by monitoring assembly of AAC in the inner membrane.[80,81] We imported AAC into isolated mitochondria, lysed the mitochondria with the non-ionic detergent digitonin, and studied AAC assembly by blue native electrophoresis (Fig. 2b). The biogenesis of AAC was moderately reduced in *psd1*Δ and *psd1*Δ *psd2*Δ mitochondria (Fig. 2b, lanes 5–7 and 9–11).

It has been reported that in CL-deficient mitochondria, the stability of the TIM23 translocase is partially affected.[39,58–60] We thus tested whether depletion of PE also affected the stability of the TIM23 or TIM22 complexes. The protein levels of subunits of the TIM23 complex (Tim17, Tim23) and the TIM22 complex (Tim22, Tim54) were comparable in *psd* mutant and wild-type mitochondria (Fig. S2). The stability of the translocases was analyzed by blue native electrophoresis of digitonin-lysed mitochondria (Fig. 2c). The TIM23 translocase forms several blue native-stable complexes,[39,58,77] which were not dissociated but only slightly shifted to faster migrating forms in *psd1*Δ and *psd1*Δ *psd2*Δ mitochondria (Fig. 2c, lanes 2 and 3) (the slight mobility shifts may indicate that PE is bound to TIM23 in wild-type mitochondria but not critical for the stability of the translocase). The mobility of the TIM22 translocase was not affected in the mutant mitochondria (Fig. 2c, lanes 5 and 6). One hallmark of mitochondria lacking CL is the dissociation of AAC oligomers.[35,39] In contrast, depletion of PE did not block the oligomerization of AAC but only led to a slight mobility shift of AAC oligomers (Fig. 2c, lanes 8 and 9).

In summary, the import of presequence-carrying preproteins and carrier proteins is impaired in PE-depleted mitochondria. The main import machineries TIM23 and TIM22, as well as the AAC oligomers,

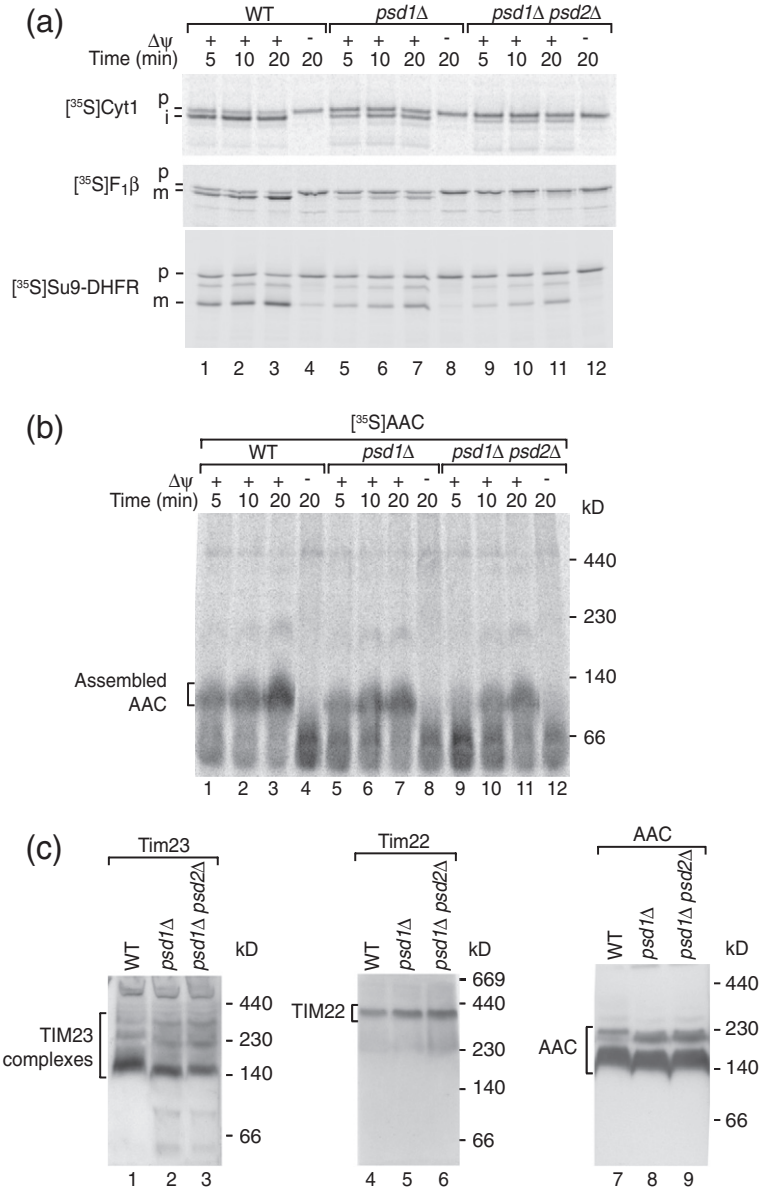

Fig. 2. PE-depleted mitochondria are impaired in import of preproteins into and across the inner membrane. (a) Isolated mitochondria from wild-type, *psd1*Δ, and *psd1*Δ *psd2*Δ yeast strains were incubated with the ^{35}S-labeled precursors of cytochrome c_1 (Cyt1), $F_1\beta$, and Su9-DHFR in import buffer [3% (w/v) bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 5 mM methionine, 2 mM KH_2PO_4, 10 mM Mops/KOH, pH7.2, 2 mM NADH, 5 mM creatine phosphate, 0.1 mg/ml creatine kinase, and 2 mM ATP] at 25 °C for the indicated periods. In control reactions, the membrane potential ($\Delta\psi$) was dissipated prior to import by addition of 8 μM antimycin A, 1 μM valinomycin, and 20 μM oligomycin. The import reactions were stopped by adding 8 μM antimycin A, 1 μM valinomycin, and 20 μM oligomycin. After washing with SEM buffer (250 mM sucrose, 1 mM ethylenediaminetetraacetic acid, and 10 mM Mops/KOH, pH7.2), the mitochondria were lysed under denaturing conditions and subjected to SDS-PAGE followed by digital autoradiography. p, precursor; i, intermediate; m, mature. (b) Isolated mitochondria from wild-type, *psd1*Δ, and *psd1*Δ *psd2*Δ yeast strains were incubated with ^{35}S-labeled AAC at 25 °C as indicated in the presence or absence of $\Delta\psi$. The mitochondria were lysed with 1% (w/v) digitonin in digitonin buffer [20 mM Tris/HCl, pH7.4, 50 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, and 10% (v/v) glycerol] and protein complexes were separated by blue native electrophoresis.[78,79] ^{35}S-labeled proteins were detected by digital autoradiography. (c) Wild-type, *psd1*Δ, and *psd1*Δ *psd2*Δ mitochondria were lysed with 1% (w/v) digitonin in digitonin buffer and subjected to blue native electrophoresis. Protein complexes were detected by Western blotting using the indicated antisera.

are not dissociated when PE is depleted, indicating that PE is not crucial for the stability of these complexes.

PE is required for the activity of the respiratory chain

We noticed that the mitochondrial import of the precursor of $F_1\beta$ was more severely affected by PE depletion than the import of Su9-DHFR (Fig. 2a). It was previously shown that the import of $F_1\beta$ requires a higher membrane potential and is thus more sensitive to a reduction of $\Delta\psi$ than the import of Su9-DHFR,[74] raising the possibility that the preprotein import defects observed in PE-depleted mitochondria may be related to a reduction of $\Delta\psi$ in the mutant mitochondria.

To assess the membrane potential of mitochondria, we used a $\Delta\psi$-sensitive fluorescent dye.[39,76,82] $\Delta\psi$ was partially reduced in *psd1*Δ mitochondria and strongly decreased in *psd1*Δ *psd2*Δ mitochondria (Fig. 3a). We determined the activity of the respiratory chain by oxygen consumption and observed that the rate of oxygen consumption was

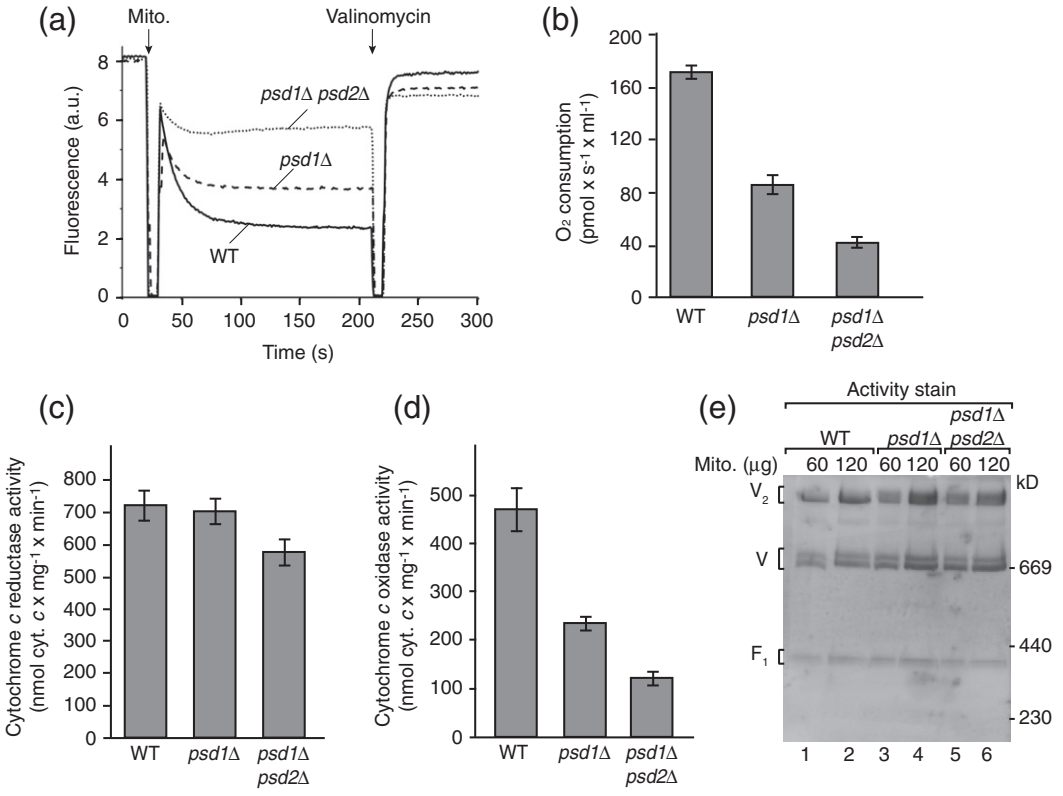

Fig. 3. PE is required for the activity of the respiratory chain. (a) The membrane potential ($\Delta\psi$) of wild-type, *psd1*Δ, and *psd1*Δ *psd2*Δ yeast mitochondria was assessed at 25 °C by fluorescence quenching using the $\Delta\psi$-sensitive dye $DiSC_3(5)$ (3,3′-dipropylthiadicarbocyanine iodide) in membrane potential buffer [0.6 M sorbitol, 0.1% (w/v) bovine serum albumin, 10 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid, and 20 mM KP_i, pH7.2] as described previously.[39,82] (b) The oxygen consumption of isolated wild-type, *psd1*Δ, and *psd1*Δ *psd2*Δ mitochondria was analyzed by oxygraph measurements at 25 °C. Isolated yeast mitochondria (100 μg protein) were added to 2 ml of buffer (10 mM Mops/KOH, pH7.2, 250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl, 5 mM KP_i, 1 mM ADP, and 1 mM NADH) and oxygen consumption was measured. The oxygen flux (negative time derivative of oxygen concentration) corrected for instrumental background flux was expressed in picomoles per second per milliliter. Shown are the mean values with standard error of the mean ($n=3$). (c and d) The activity of the cytochrome bc_1 complex (c) and the cytochrome *c* oxidase (d) was determined in submitochondrial particles prepared from wild-type, *psd1*Δ, and *psd1*Δ *psd2*Δ yeast cells as described earlier.[83,84] Ubiquinol-dependent cytochrome *c* reduction was measured as described by Palsdottir and Hunte[84] using 3 μg protein (submitochondrial particles), 50 μM horse heart cytochrome *c*, and 80 μM decylubiquinol for 1 ml assay volume (40 mM potassium phosphate buffer, pH7.4, 1 mM NaN_3, and 0.05 % β-D-undecylmaltoside). Reduction of cytochrome *c* was monitored at 550 nm and the activity was calculated with an extinction coefficient of 19.4 $mM^{-1}\,cm^{-1}$. The activity was fully sensitive to the specific inhibitor stigmatellin (1 μM). Cytochrome *c* oxidase activity was measured as described by Horvath *et al.*[85] with 50 μM reduced horse heart cytochrome *c* and 3–50 μg of protein (submitochondrial particles) in 1 ml assay volume (75 mM potassium phosphate buffer, pH7.4, 1 mM antimycin A, and 0.05% β-D-dodecylmaltoside). Oxidation of cytochrome *c* was monitored and quantified as for the cytochrome bc_1 complex. The activity was fully sensitive to the specific inhibitor sodium azide (1 μM). Specific enzyme activities are based on total protein determined by bicinchoninic acid assay (Pierce). Three preparations per strain were used and the activity measurements were repeated five times for each sample. Mean values with standard error of the mean are shown. (e) The activity of the mitochondrial ATPase was assessed by in-gel calcium phosphate precipitation upon ATP hydrolysis.[86,87] Mitochondria isolated from wild-type, *psd1*Δ, or *psd1*Δ *psd2*Δ strains were lysed with 1% (w/v) digitonin in digitonin buffer and protein complexes were separated by blue native electrophoresis. Subsequently, the gel was washed with water and incubated with ATP-containing buffer (50 mM glycine, pH8.4, 5 mM $MgCl_2$, and 20 mM ATP) for 20 min and transferred into 10% (w/v) $CaCl_2$ solution. Incubation was performed until calcium phosphate precipitation became visible and the reaction was stopped by transfer into water. V_2, ATP synthase dimer; V, ATP synthase monomer; F_1, F_1 part of the ATP synthase.

reduced in *psd1*Δ mitochondria and more severely decreased in *psd1*Δ *psd2*Δ mitochondria (Fig. 3b). Budding yeast does not contain complex I of the respiratory chain. The cytochrome bc_1 complex and cytochrome *c* oxidase are the two proton-pumping respiratory complexes and thus we analyzed their activities individually. The activity of the cytochrome bc_1 complex was only marginally affected

by the depletion of PE (Fig. 3c), whereas the activity of the cytochrome *c* oxidase was considerably reduced (Fig. 3d). The protein levels of several subunits of cytochrome *c* oxidase such as Cox1 were moderately reduced in the *psd* mutant mitochondria (Fig. S2), supporting the conclusion that cytochrome *c* oxidase was affected in the mutants. The F_1F_o-ATP synthase was visualized in native gels by ATPase activity staining,[86,87] revealing comparable activities in wild-type and *psd* mutant mitochondria (Fig. 3e).

Taken together, the stepwise decrease of PE levels in *psd1*Δ and *psd1*Δ *psd2*Δ mutants correlates with the stepwise decrease of cytochrome *c* oxidase activity, oxygen consumption, membrane potential, and preprotein import into and across the inner membrane. Thus, both PE and CL are important for the activity of the respiratory chain and the generation of a membrane potential. A decreased $\Delta\psi$ results in an impaired protein import into and across the inner membrane.[74,76]

Lack of PE stabilizes respiratory chain supercomplexes

The cytochrome bc_1 complex and cytochrome *c* oxidase form supercomplexes that can be resolved by blue native electrophoresis. The supercomplexes contain a dimer of the cytochrome bc_1 complex (III_2) and one (III_2/IV) or two copies (III_2/IV_2) of cytochrome *c* oxidase (Fig. 4a and b).[44,45] We lysed mitochondria with digitonin and analyzed the protein complexes by blue native electrophoresis and immunodecoration. Surprisingly, the supercomplexes were not dissociated in *psd1*Δ and *psd1*Δ *psd2*Δ mitochondria, but rather formed a larger oligomeric assembly, containing both cytochrome bc_1 complex and cytochrome *c* oxidase (Fig. 4a, lanes 2 and 3; Fig. 4b, lanes 2, 3, 5, and 6). The amount of free cytochrome *c* oxidase was decreased upon depletion of PE (Fig. 4b, lanes 2, 3, and 6), indicating that the lack of PE stabilizes the interaction of both complexes. For comparison, lack of CL in *crd1*Δ mitochondria has an opposing effect on the supercomplexes as the association of the cytochrome bc_1 complex with cytochrome *c* oxidase is destabilized (Fig. 4a, lane 5; Fig. 4b, lane 8).[38,39,47,48,82,88,89] Neither succinate dehydrogenase (complex II) nor the F_1F_o-ATP synthase (complex V) altered their blue native mobility upon depletion of PE (Fig. 4c), indicating that these complexes were not present in the large oligomeric assembly of the respiratory chain.

In conclusion, PE and CL are both important for mitochondrial function. Like CL, PE is required for maintaining the membrane potential, which is crucial for the import of preproteins into and across the inner membrane. The activity of the respiratory chain, in particular of cytochrome *c* oxidase, is decreased when PE (this study) or

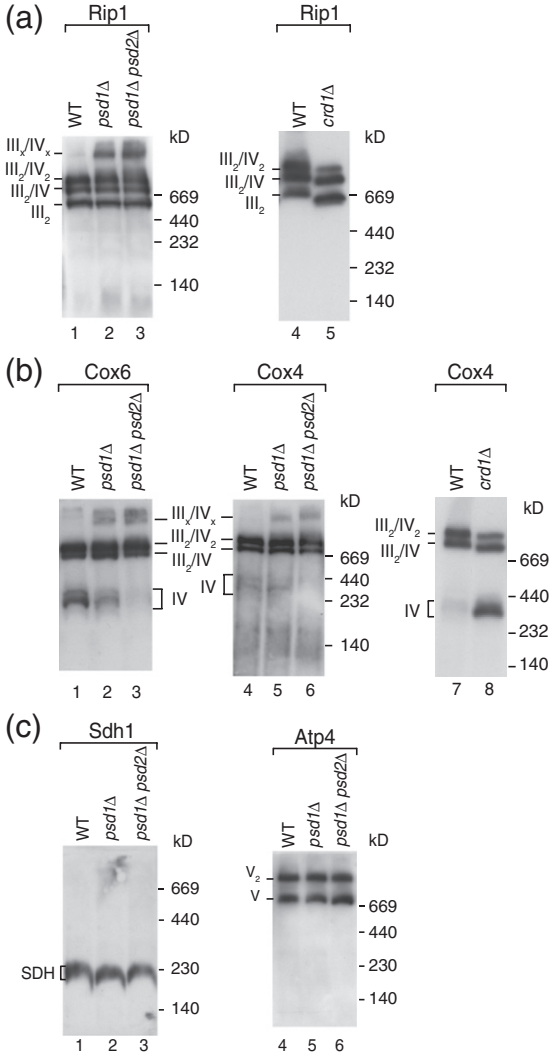

Fig. 4. Depletion of PE stabilizes respiratory chain supercomplexes. (a–c) Wild-type, *psd1*Δ, *psd1*Δ *psd2*Δ, and *crd1*Δ mitochondria were lysed with 1% (w/v) digitonin in digitonin buffer and subjected to blue native electrophoresis, followed by Western blotting using the indicated antisera. III, cytochrome bc_1 complex; IV, cytochrome *c* oxidase; V_2, ATP synthase dimer; V, ATP synthase monomer; Atp4, ATP synthase subunit 4 (subunit b); Cox4, cytochrome *c* oxidase subunit 4; Cox6, cytochrome *c* oxidase subunit 6; Rip1, Rieske iron–sulfur protein; SDH, succinate dehydrogenase (complex II).

CL[48,49] is depleted. However, PE and CL showed different effects on the molecular level when the stability of mitochondrial protein complexes was analyzed. Lack of CL results in dissociation of AAC oligomers and destabilization of respiratory chain supercomplexes.[35,38,39,48,49] In contrast, upon depletion of PE, the AAC oligomers remained stable and even higher forms of the respiratory chain supercomplexes were observed. CL and PE were shown to bind to the cytochrome bc_1 complex

and cytochrome *c* oxidase, likely including the interface of both complexes.[42,43,48,64,90,91] CL and PE are both non-bilayer-forming phospholipids. Their opposite effects on protein complex stability may provide an explanation why CL does not fully compensate for the loss of PE and *vice versa*.[6,27] CL has a negatively charged head group, whereas PE is a zwitterionic phospholipid of neutral charge, suggesting that the differently charged head groups may contribute to the differential effects on protein complex stability. Wenz *et al.* indeed showed that the negative charge of CL is important for maintaining the structural integrity of respiratory supercomplexes.[91] Wittig and Schägger[92] and Bultema *et al.*[93] proposed that the cytochrome bc_1 complex and the cytochrome *c* oxidase can be organized into higher oligomeric structures that are larger than the known supercomplexes and called them respiratory strings or megacomplexes. Our results suggest that such structures are stabilized when PE is depleted and thus megacomplexes of the respiratory chain can be detected on blue native gels.

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Supplementary Data

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