Phosphatidylethanolamine made in the inner mitochondrial membrane is essential for yeast cytochrome bc₁ complex function

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Of the four separate PE biosynthetic pathways in eukaryotes, one occurs in the mitochondrial inner membrane (IM) and is executed by phosphatidylserine decarboxylase (Psd1). Deletion of Psd1 is lethal in mice and compromises mitochondrial function. We hypothesize that this reflects inefficient import of non-mitochondrial PE into the IM. Here, we test this by re-wiring PE metabolism in yeast by re-directing Psd1 to the outer mitochondrial membrane or the endomembrane system and show that PE can cross the IMS in both directions. Nonetheless, PE synthesis in the IM is critical for cytochrome bc₁ complex (III) function and mutations predicted to disrupt a conserved PE-binding site in the complex III subunit, Qcr7, impair complex III activity similar to PSD1 deletion. Collectively, these data challenge the current dogma of PE trafficking and demonstrate that PE made in the IM by Psd1 support the intrinsic functionality of complex III.

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The sequestration of enzymes and their substrates into different membrane compartments allows for the enrichment and regulation of metabolite synthesis in regions of the cell where they are essential. In eukaryotes, the essential phospholipid phosphatidylethanolamine (PE) is synthesized by four separate pathways, three of which localize to the endoplasmic reticulum (ER)\(^1\). A final pathway is dependent on phosphatidyserine decarboxylase (Psd1) which is embedded in the mitochondrial inner membrane (IM)\(^2\)\(^–\)\(^4\). The major PE production pathways are the Kennedy pathway, which synthesizes PE through the stepwise conjugation of CDP-ethanolamine to diacylglycerol, and the Psd pathway, which utilizes phosphatidyserine (PS) as substrate\(^1\). Deletion of either pathway is lethal during murine embryogenesis, highlighting the importance of PE generation in both the ER and mitochondria\(^5\)\(^–\)\(^7\).

Conservation of the Psd pathway from bacteria to humans suggests that mitochondrial PS/PE metabolism has been preserved to optimize mitochondrial performance\(^8\). Indeed, deletion of the nuclear-encoded Psd1 (Psd in mammals and PSD1 in yeast) in eukaryotic cells decreases cellular growth, impairs oxidative phosphorylation (OXPHOS), alters mitochondrial morphology, and diminishes PE levels in cells and mitochondria\(^8\)\(^–\)\(^10\). The Psd pathway is the predominant PE production pathway in Saccharomyces cerevisiae, producing up to 70% of PE in the cell\(^11\). Unlike mammals, yeast additionally contain Psd2 which localizes to either Golgi or endosomal compartments\(^4\)\(^,\)\(^12\). Deletion of PSD2 does not recapitulate the mitochondrial defects associated with loss of PSD1\(^13\). The combined absence of PSD1 and PSD2 produces a strain that is auxotrophic for ethanolamine that allows PE synthesis through the Kennedy pathway.

The substrate of Psd1, PS, is synthesized on the mitochondrial-associated membrane (MAM) of the ER by phosphatidylserine synthase (Cho1)\(^13\). Thus, the amphipathic PS must traverse two aqueous compartments, the cytosol and the mitochondrial intermembrane space (IMS), to reach the IM\(^14\). Whether a parallel pathway exists for PE import into the IM remains unclear. The lethal consequence of PISD deletion in mice and the failure of supplemental ethanolamine to rescue the respiratory defects of psd1Δ yeast suggest that PE made outside of the mitochondrial cannot compensate for the absence of Psd1\(^15\)\(^–\)\(^10\),\(^15\). However, it was recently reported that extramitochondrial PE can in fact improve OXPHOS in psd1Δ yeast\(^16\).

Previously, we generated a functional chimeric Psd1 protein in S. cerevisiae, ER-Psd1, targeted to the endosomal compartment\(^17\). In the current study, we further characterize ER-Psd1, together with an OM-targeted chimeric Psd1 (OM-Psd1), to test if the cytosol, IMS, or both, are barriers that prevent nonmitochondrially produced PE from functionally rescuing the absence of PE made in the IM. Alongside strains expressing these re-directed Psd1 constructs, we compare the mitochondrial function of psd1Δpsd2Δ yeast grown in the non-fermentable carbon source, lactate, with or without exogenous ethanolamine supplementation, to evaluate the ability of the ER-localized Kennedy pathway to support mitochondrial function. Our results establish that PE made outside the IM can indeed access this membrane, but at a significantly reduced abundance in comparison with PE made in the IM by Psd1. This altered PE biogenesis leads to general alterations in mitochondrial phospholipid content, which differentially impacts respiratory complexes III and IV. Our work supports a specific role of IM-synthesized PE in complex III activity, confirmed by site-directed mutations in a known lipid binding pocket of this enzyme. We conclude that in the context of bi-directional PE transport across the IMS, IM-localized Psd1 is required to support electron transport chain activity.

**Results**

**Ethanolamine does not fully rescue psd1Δ respiratory growth.** The ability of supplemental ethanolamine to rescue the respiratory growth defect of psd1Δ yeast has been reported by one group\(^16\) but not others\(^10\)\(^–\)\(^15\). If PE produced by the Kennedy pathway can replace IM-produced PE, this would imply robust trafficking mechanisms to move PE into mitochondria and, by extension, that IM-localized Psd1 is not required for mitochondrial function per se. Therefore, we tested the growth of wildtype (WT), psd1Δ, psd2Δ, and psd1Δpsd2Δ yeast in synthetic complete ethanol-glycerol (SCEG) or SC-lactate medium with or without ethanolamine supplementation (Fig. 1a). Consistent with previous findings\(^10\)\(^–\)\(^15\), we found that ethanolamine restored respiratory growth of psd1Δpsd2Δ yeast to psd1Δ levels but failed to fully restore the respiratory defect of psd1Δ yeast. Importantly, this basic result was confirmed in psd1Δ yeast from three additional strain backgrounds (Fig. 1b, c), regardless of the amount of ethanolamine provided, although subtle

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**Fig. 1** Ethanolamine only partially rescues the respiratory growth defect of psd1Δ yeast. The indicated strains were pre-cultured at 30 °C in YPD and spotted onto (a, b) synthetic complete dextrose (SCD), ethanol-glycerol (SCEG), or lactate medium with or without (+/−) 2 mM ethanolamine (+/−E) or (c) SCEG with or without (+/−) 10 mM ethanolamine and incubated at 30 °C for 2 days (SCD +/−E) or 3 days (all the rest)
differences between strains were noted. Overall, these findings indicate that the Kennedy pathway cannot fully compensate for the absence of Psd1.

Validation of Psd1 constructs expressed in the OM or ER. To interrogate whether the cytosol and/or the IMS is a barrier that prevents extra-mitochondrially produced PE from replacing PE made in the IM, we generated chimeric Psd1 constructs that localize to either the ER or OM membranes to redirect PS and PE metabolism (Fig. 2a). Both constructs, and the WT IM-localized Psd1 control (referred to as IM-Psd1 to distinguish it from strains expressing endogenous Psd1), contain a C-terminal 3XFLAG tag to track autocatalytic function of these chimeras by immunodetecting the released Psd1p α subunit. OM-Psd1 and ER-Psd1 each produced mature β and α subunits demonstrating that self-processing remained intact (Fig. 2b). The steady state levels of all three integrated constructs were similar to each other and over-expressed compared to endogenous Psd1. Importantly, both OM-Psd1 and ER-Psd1 rescued the ethanolamine auxotrophy of Δpsd1Δpsd2Δ yeast indicating that these constructs are capable of synthesizing levels of PE necessary for cellular growth (Fig. 2c).

Previously, ER-Psd1 localization was established by subcellular fractionation and its N-glycosylation status17. To confirm the OM localization of OM-Psd1, its protease accessibility was determined in intact mitochondria, OM-ruptured mitoplasts, and detergent-solubilized mitochondria and compared to IM-Psd1. Protease treatment of intact mitochondria expressing IM-Psd1 showed that IM-Psd1, like the IM control Tim54, was protected against degradation (Fig. 2d) while OM-Psd1 was completely degraded like the OM control Tom70, verifying that it was successfully re-localized to the OM facing the cytosol. Given the presence of inter-organelle contact sites, a proportion of ER-Psd1 co-fractionated with crude mitochondria (Supplementary Fig. 1) and demonstrated protease-sensitivity in intact mitochondria (Fig. 2d), a topology that is consistent with its N-glycosylation status (Fig. 2e). Thus, a portion of ER-Psd1 is retained in the ER and/or resides in an endosomal compartment that is co-purified with mitochondria.

OM-Psd1 and ER-Psd1 mitochondrial PE levels exceed WT. Next, the lipid content of sucrose gradient purified mitochondria, which none-the-less still contained some co-purified ER (Fig. 3a), was assessed in WT, psd1Δ, psd2Δ, psd1Δpsd2Δ, IM-Psd1, OM-Psd1 and ER-Psd1 yeast (Fig. 3b, c). PE was reduced in the absence of Psd1 and undetectable in the combined absence of Psd1 and Psd2 (Fig. 3d). In both psd1Δ and psd1Δpsd2Δ mitochondria, levels of phosphatidylcholine (PC) and phosphatidylinositol (PI) were increased (Fig. 3e, f). Notably, PSD2 deletion

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**Fig. 2** OM-Psd1 and ER-Psd1 constructs are functional and properly mis-localized. a Schematic of IM-Psd1, OM-Psd1, and ER-Psd1. All three constructs contain a 3XFLAG tag at the C-terminus (F, yellow). The Tom20 residues (1–100) that replace the mitochondrial targeting sequence (TM) and transmembrane (TM) domain of IM-Psd1 (green) are shown for OM-Psd1 (blue), and the carboxypeptidase Y signal sequence (residues 1–37) as well as an NXS motif are indicated for ER-Psd1 (purple). b The β and α subunits of Psd1 were detected in yeast whole cell extracts of the indicated strains by immunoblot. Tom70 served as a loading control. c The indicated strains were spotted onto synthetic complete dextrose (SCD) medium with or without (+/−) 2 mM ethanolamine (+E) and incubated at 30 °C for 4 days. d Protease protection assay in intact mitochondria (Mito), osmotically ruptured mitoplasts (MP), and detergent-solubilized mitochondria (Det.). Following incubation without or with (+/−) 100 μg proteinase K (Prot. K) for 30 min, samples were collected, resolved by SDS-PAGE and immunoblotted for Psd1p (β and α subunits), and the mitochondrial compartment-specific markers Tom70 (OM), Tim54 (IM), and Abf2p (matrix). e Illustration indicating the topology of (1) IM-Psd1, (2) OM-Psd1, and (3) ER-Psd1
modestly decreased mitochondrial PE (Fig. 3d) but did not result in a respiratory growth defect (Fig. 1a). Combined, these results indicate that Psd2 contributes to the mitochondrial-associated pool of PE but is unable to functionally replace PE made by Psd1.

Interestingly, OM-Psd1 and ER-Psd1 yeast contained significantly higher amounts of PE in mitochondrial-enriched membranes compared to IM-Psd1, which maintained levels similar to WT (Fig. 3d). As IM-Psd1, OM-Psd1, and ER-Psd1 are similarly over-expressed (Supplementary Fig. 2A–C), this suggests that OM-Psd1 and ER-Psd1 have increased access to their substrate and short-circuited normal mitochondrial PS/PE metabolism. OM-Psd1 and ER-Psd1 both normalized the absolute amount of Cho1 and its phosphorylated pool which were significantly increased in psd1Δpsd2Δ yeast grown in respiratory conditions (Supplementary Fig. 2A, and 2D–F). The relative abundance of PC was reduced in OM-Psd1 and ER-Psd1 (Fig. 3e). Intriguingly, the CL levels in OM-Psd1 mitochondria were significantly decreased compared to WT (Fig. 3g). The reduced levels of PC in OM-Psd1 and ER-Psd1 is notable as it might have been predicted that an increased production of PE would have resulted in augmented PC synthesis by ER-resident methyltransferases that convert PE to PC18. The steady state level of Kar2, the yeast equivalent of the Hsp70 chaperone Bip19, was not increased in OM-Psd1 or ER-Psd1 (Supplementary Fig. 2g), demonstrating that their altered membrane compositions did not induce ER stress. In contrast, Kar2 was significantly elevated in both psd1Δpsd2Δ and cho1Δ strains. Importantly, there were no dramatic differences in the mitochondrial architectures of strains with elevated or decreased PE levels (Supplementary Fig. 3), which is consistent with previous observations in psd1Δ yeast18. Overall, re-routing Psd1 to either the OM or ER results in a robust increase in mitochondrial-associated PE levels which may or may not reach the IM.

OM-Psd1 and ER-Psd1 phenocopy the psd1Δ respiratory defect. OXPHOS was initially evaluated in these strains by determining their growth on synthetic media containing dextrose with or without (+/−) ethanolamine, lactate, or ethanol-glycerol + /− ethanolamine (Fig. 4a). Compared to IM-Psd1, OM-Psd1 and ER-Psd1 only partially improved growth of psd1Δpsd2Δ yeast on respiratory carbon sources (lactate and ethanol-glycerol) and growth was not improved by ethanolamine (Fig. 4a). This suggests that PE made in either the OM or ER cannot functionally replace PE produced in the IM.

To directly assess OXPHOS capacity in these strains, oxygen consumption was monitored in mitochondria using an O2 electrode after the addition of ADP and ascorbate tetramethyl-p-phenyldiamine (TMPD) which promotes proton pumping by complex IV (Figs. 4b–c). The ADP-stimulated (State 3) respiratory rate is recorded after addition of ascorbate TMPD plus ADP and is calculated as the amount of O2 consumed over time. ADP facilitates proton pumping and the O2 consumption rate and upon its depletion by complex V, the respiratory rate returns to a resting state (State 4). Maximal respiratory capacity was evaluated by addition of carbonyl cyanide m-chlorophenyl hydrazine (CCCP), a proton ionophore that collapses the electrochemical gradient across the IM making it easier to pump protons.

Mitochondria lacking Psd1, but not Psd2, had reduced maximal respiratory rate compared to WT mitochondria. Even though psd2Δ mitochondria consumed O2 like WT, the combined
absence of Psd1 and Psd2 caused a more severe respiratory defect than when only Psd1 was missing. This indicates that in the absence of Psd1, PE made by Psd2 has some capacity to support respiratory activity. The uncoupled respiratory rate for OM-Psd1 and ER-Psd1 was significantly increased compared to psd1Δpsd2Δ mitochondria but still impaired relative to IM-Psd1 (Fig. 4b). The respiratory control ratio (RCR) is an indication of how well proton pumping by the electron transport machinery is coupled to ATP synthesis and is calculated by dividing the ADP-stimulated respiratory rate by the resting respiration rate (State 3/State 4). A decrease in the rate of State 3 over State 4 is coupled to ATP synthesis and is calculated by dividing the state 3 by state 4 respiratory rates. Statistical comparisons (ns, $P > 0.05$; 1 symbol $P \leq 0.05$; 2 symbols $P \leq 0.01$; 3 symbols $P \leq 0.001$; 4 symbols $P \leq 0.0001$) vs. WT (asterisk), psd1Δpsd2Δ (number sign), or IM-Psd1 (dollar sign) were performed by one-way ANOVA with Tukey’s multiple comparison test (mean ± S.E.M. for $n = 4$ biologically independent experiments).

**Reduced complex III activity when Psd1 is not in the IM.** The ability of ER-Psd1, but not OM-Psd1, to rescue complex IV activity to WT levels was surprising given that neither chimeric construct restored respiratory growth of the psd1Δpsd2Δ strain to this degree (Fig. 4a). Therefore, we postulated that the incomplete respiratory growth rescue of OM-Psd1 and ER-Psd1 could reflect a defect in complex III. Indeed, complex III activity was reduced in psd1Δ and psd1Δpsd2Δ mitochondria as previously reported, as well as in OM-Psd1 mitochondria but surprisingly, ER-Psd1 retained WT function (Fig. 5b). To determine if the different complex IV activities associated with OM-Psd1 and ER-Psd1 mitochondria reflected changes in its expression, we analyzed the steady state amounts of both nuclear and mtDNA-encoded subunits of complex IV (Fig. 5c). While the levels of the mtDNA-encoded subunit, Cox2, was significantly decreased in psd1Δpsd2Δ and OM-Psd1 strains, the steady state abundance of the two additional mtDNA-encoded subunits, Cox1 and Cox3, trended towards a decrease in psd1Δ, psd1Δpsd2Δ and OM-Psd1 that did not quite reach statistical significance (Supplementary Fig. 4). Additionally, the amount of a constituent encoded in the nucleus, Cox4, was decreased in OM-Psd1 but not psd1Δpsd2Δ. Blue native-PAGE analyses indicated that complex IV assembly into respiratory supercomplexes (RSCs) that consist of a complex III dimer affiliated with either one or two complex IV monomers, was normal regardless of the absence of PSD1 or PSD2, singly or in combination, as reported by others, or whether Psd1 was expressed in the IM, OM, or ER (Fig. 5d).
biologically independent experiments, except for mitochondrial extracts solubilized in 1.5% (w/v) digitonin. Cox4 and (as a positive control for RSC destabilization53.

Although there was proportionately more of the smaller supercomplex (Supplementary Fig. 4) or its assembly into supercomplexes (Fig. 5e), acceptor cytochrome c (cytochrome c) and markers of each mitochondrial compartment. Furthermore, the steady state levels of the complex III electron donor CoQ21, were equal with one exception (Fig. 5c and Supplementary Fig. 5). In B and F, statistical comparisons (ns, $P > 0.05$; 1 symbol $P \leq 0.05$; 2 symbols $P \leq 0.01$; 3 symbols $P \leq 0.001$; 4 symbols $P \leq 0.0001$) versus WT (asterisk), psd1Δpsd2Δ (number sign), or IM-Psd1 (dollar sign) were performed by one-way ANOVA with Tukey’s multiple comparison test. $P$ values for decreases that didn’t achieve significance are reported in red and were analyzed by student t-test versus WT.

ER-Psd1 did not reflect alterations in the steady state abundance of its subunits (Fig. 5c, Cor2, Rip1, Qcr6, and Qcr7, quantified in Supplementary Fig. 4) or its assembly into supercomplexes (Fig. 5e), although there was proportionately more of the smaller supercomplex (IIIIVV > IIIIV) detected in psd1Δpsd2Δ mitochondria. Furthermore, the steady state levels of the complex III electron acceptor cytochrome c were normal (Fig. 5c and Supplementary Fig. 4M). Similarly, subunits of the coenzyme Q (CoQ) synthome, a macromolecular complex that catalyzes the synthesis of the complex III electron donor CoQ21, were equal with one exception (Fig. 5C and Supplementary Fig. 5). In psd1Δpsd2Δ mitochondria, Coq1 was increased which could represent an attempt to diminish membrane stress22. Moreover, CoQ6 supplementation, which is capable of rescuing strains with reduced CoQ biosynthesis24, failed to improve respiratory growth of psd1Δ or psd1Δpsd2Δ yeast (Supplementary Fig. 6). Lastly, psd1Δ, psd1Δpsd2Δ, OM-Psd1, and ER-Psd1 respiratory growth was not further impaired in medium lacking para-amino benzoic acid (Supplementary Fig. 7), a molecule that can be used to produce CoQ by a secondary pathway23. In total, our results demonstrate that CoQ is not limiting for respiratory function in any of these strains. As such, they favor the hypothesis that the impaired complex III activity of psd1Δ, psd1Δpsd2Δ, OM-Psd1, and ER-Psd1 is intrinsic to the multisubunit holoenzyme itself.

Altered IM phospholipid profile with disrupted PE metabolism. Elevated mitochondrial PE levels have been suggested to be toxic for mitochondrial cristae morphology and respiratory function24. Therefore, given the increased levels of PE associated with OM-Psd1 and ER-Psd1 mitochondria (Fig. 3d), we sought to determine the IM lipid composition in our panel of yeast strains. As such, we adapted a detergent-free method that exploits
the ability of styrene maleic acid (SMA) copolymers to extract membrane protein complexes and their adjacent phospholipid microenvironment in nanodiscs. It was previously shown that complex IV affinity purified from SMA-extracted mitochondria contains the major IM phospholipids (Fig. 6a). Since complex IV is inarguably a resident of the IM, we reasoned that its local phospholipid environment will provide insight into the local lipid milieu surrounding the RSCs, which may reflect the overall IM phospholipid composition. To this end, we CRISPR-engineered a His tag onto the C-terminus of endogenous...
Cox8. Cox8-His was expressed, functional, and assembled normally into RSCs (Supplementary Fig. 8A–C). Affinity purification of SMA extracts with Ni-agarose was specific for Cox8-His, resulted in co-purification of additional complex IV subunits, and yielded a population of SMA-stabilized discoidal nanoparticles (Supplementary Fig. 8D–E).

We therefore appended a His tag at the C-terminus of Cox8 in our panel of re-wired Psd1 yeast and associated control strains (Supplementary Fig. 8F). A preparative scale Ni-agarose affinity purification was performed from SMA-extracted mitochondria and the resulting eluates were enriched in subunits of complex IV (Fig. 6b). While some complex III subunits were also present in the purified complex IV nanodiscs, OM proteins (Tom70, OM45, and Por1) were not detected (Fig. 6b). Thus, the purified complex IV nanodiscs are devoid of OM contaminants. Next, the phospholipid composition of the purified complex IV nanodiscs was determined by shotgun lipidomics (Fig. 6c–j). The amount of PE that co-purified with complex IV nanodiscs in the absence of Psd1 was even less than what was detected in gradient purified mitochondria (Figs. 6c and 3d, 6.7% vs 22% relative to WT PE levels, respectively). While PE was increased by 156% in mitochondria from OM-Psd1 and ER-Psd1 compared to WT, it was reduced by ~70% in their purified complex IV nanodiscs. This indicates that although PE made in the OM or ER can be transported across the IMS and incorporated into the IM, much of it remains in the OM and/or co-purified ER membranes. Surprisingly, the levels of PE, which were normal in mitochondria (Fig. 3d), was reduced by 59% in complex IV nanodiscs purified from IM-Psd1. While the basis for this observation is unclear, the fact that IM-Psd1 functionally rescued the absence of endogenous Psd1 indicates that the amount of PE in the IM is sufficient, at least for the IM functions tested. With the exception of phosphatidylglycerol (PG), the complex IV nanodisc-associated levels of other phospholipids were similar regardless of the absence of PSD1 or PSD2, singly or in combination, or whether Psd1 was expressed in the IM, OM, or ER. However, differences in the acyl chain profile of phospholipids associated with complex IV nanodiscs were identified that varied depending on if and where PE was made (Supplementary Fig. 9). Unexpectedly, the amount of complex IV nanodisc-associated PG, a precursor for CL that is of low abundance, was decreased when Psd1 was not in the IM (Fig. 6h). Another CL precursor, PA, was reduced in complex IV purified nanodiscs from OM-Psd1 (Fig. 6g), which also contained significantly less CL (Fig. 6i). Notably, when Psd1 is not present in the IM, the purified complex IV nanodiscs had a significantly lower phospholipid:protein ratio (Fig. 6j). In the absence of a clear compensatory increase in another lipid class, this is likely a direct consequence of their low PE levels. Collectively, our results demonstrate that while PE made in the OM or ER can access the IM, it fails to accumulate to the level needed to fully support complexes III and IV function.

Ethanolamine rescues complex IV but not complex III activity. As ER-Psd1 restores complex IV, but not complex III, function, we isolated psd1Δpsd2Δ mitochondria from cultures grown in rich lactate without or with (−/+) choline or ethanolamine, to evaluate the impact of PC and PE generation by the Kennedy pathways on respiratory activity (Fig. 7a, b). Similar to ER-Psd1, ethanolamine, but not choline, restored psd1Δpsd2Δ complex IV (Fig. 7a), but not complex III (Fig. 7b), function to WT levels. Combined, our results indicate that PE, but not PC, made in the ER by the Kennedy pathway (Fig. 7a) or in the endosomal system by either Psd2 or ER-Psd1 (Fig. 7b), can significantly rescue the severe complex IV dysfunction that occurs in psd1Δpsd2Δ yeast. Surprisingly, when we evaluated the phospholipid composition of psd1Δpsd2Δ yeast supplemented with choline or ethanolamine, we found that ethanolamine did not alter mitochondrial PE levels (Fig. 7c,d) and only modestly and yet significantly increased cellular PE abundance (Fig. 7f, g), consistent with the slight increase observed in1,28 but not16. Intriguingly, ethanolamine, but not choline, supplementation significantly increased CL in psd1Δpsd2Δ yeast to psd1Δ levels (Fig. 7e). Significant changes in the abundance of other phospholipid species in psd1Δpsd2Δ yeast supplemented with either choline or ethanolamine were not observed (Supplementary Fig. 10). As such, these results indicate that the Kennedy Pathway for PE production is metabolically linked to CL biosynthesis and/or stability. Moreover, they suggest that the ability of ethanolamine to improve complex IV activity in psd1Δpsd2Δ yeast coincides with its unanticipated capacity to increase CL levels, a phospholipid known to be important for complex IV function29. These findings demonstrate that both CL and PE are important for complex IV activity. Moreover, they further underscore that PE made within the IM is necessary for the full activity of complex III which is otherwise normally expressed, fully assembled, and not limited by the amount of either of its mobile electron carriers.

PE synthesis by Psd1 is required for complex III activity. Cho1 produces PS in the MAM of the ER through conjugation of free serine with DCP-diaclyglycerol13. In yeast, this feeds into both the Psd1 and Psd2 PS decarboxylation pathways (Fig. 8a). We generated CHO1 deletion strains in WT and psd1Δpsd2Δ (labeled cho1ΔΔ in Fig. 8k) yeast to deplete mitochondrial PS/PE levels while preserving Psd1 expression. Consistent with previous data17, deletion of Cho1 did not affect Psd1 accumulation or maturation (Fig. 8b) and resulted in an ethanolamine auxotrophy30 (Fig. 8c). As anticipated, PS and PE levels were drastically reduced in strains lacking Cho1 (Fig. 8d–f). In comparison to psd1Δpsd2Δ, cho1Δpsd1Δpsd2Δ yeast had a significant increase in CL and PI levels (Fig. 8g, h), the former of which may be associated with its enhanced respiratory growth (Fig. 8k). Deletion of CHO1 in the psd1Δpsd2Δ background restored PC to WT levels (Fig. 8i) but failed to increase the levels of the CL precursor PA (Fig. 8i). Growth of cho1Δ was decreased compared to WT in YPD and rich lactate media and similar to psd1Δpsd2Δ in SCEG containing ethanolamine (Fig. 8k). Notably, the activities of complexes III (Fig. 8i) and IV (Fig. 8m) were reduced in the absence of Cho1. Since Psd1 and essential subunits of complexes III and IV were expressed normally in the absence of Cho1, singly
Fig. 7 Ethanolamine rescues the activity of complex IV but not complex III. The indicated yeast strains were grown in rich lactate without (WT, psdΔ, and psd1Δpsd2Δ) or with choline (+C) or ethanolamine (+E). a Complex IV activity and (b) complex III activity in isolated mitochondria solubilized in 0.5% (w/v) DDM (mean ± S.E.M. for n = 6 biologically independent experiments, except for p<0.05, 1 symbol P ≤ 0.05; 2 symbols P ≤ 0.01; 3 symbols P ≤ 0.001; 4 symbols P ≤ 0.0001) vs. WT (asterisk) or psd1Δpsd2Δ (number sign) were performed by one-way ANOVA with Tukey’s multiple comparison test. P values for decreases that did not achieve significance are reported in red and were analyzed by student t test versus WT. c-e Mitochondrial phospholipids from the indicated strains were labeled overnight with 32Pi, and separated by TLC. c Representative TLC plate for mitochondrial 32Pi lipids. Quantitation of mitochondrial (d) PE and (e) CL levels (mean ± S.E.M. for n = 6 biological replicates). Significant differences compared to WT (asterisk) or psd1Δpsd2Δ (number sign) were calculated by one-way ANOVA with Holm-Sidak pairwise comparisons. f Representative TLC plate for cellular 32Pi lipids. g Quantitation of cellular PE levels (mean ± S.E.M. for n = 6 biological replicates). Significant differences compared to WT (asterisk) or psd1Δpsd2Δ (number sign) were calculated by student t-test.

or in combination with Psd1 and Psd2 (Fig. 8n), our combined results directly implicate mitochondrial PE depletion as the cause for the reduced respiratory function of psd1Δ, psd1Δpsd2Δ, and cho1Δ yeast.

Glu82 of Qcr7 may coordinate PE associated with complex III. PE and CL were identified in crystal structures of the yeast and mammalian cytochrome bc1 complex in association with the essential mtDNA-encoded catalytic subunit cytochrome b (Cob1) as well as the nuclear-encoded subunit Qcr731,32. Qcr7 is associated with the matrix-facing surface of Cob1 and it is postulated that hydrogen bonding interactions between the headgroup of PE and Glu82 of Qcr7 may help position the complex vertically within the bilayer (Fig. 9a). To test the importance of this residue in forming hydrogen bonds with the amine group of PE, we introduced a charge reversal by mutating Glu82 to Arg and also created a strain expressing Asp82 to Test the potential effect of shortening the distance of this interaction. Importantly, the amount of Qcr7E82R or Qcr7E82D in cell and mitochondrial lysates (Fig. 9b, c) was similar to WT (the Qcr7E82K variant was consistently upshifted compared to WT following SDS-PAGE). Further, Qcr7E82R and Qcr7E82D supported respiratory growth in rich or minimal medium in contrast to qcr7Δ whose respiratory growth was compromised (Fig. 9d). Despite being sufficiently functional to promote respiratory growth, complex III activity was significantly decreased for Qcr7E82K and Qcr7E82D to a similar degree as when Psd1 is missing (Fig. 9c). Surprisingly, complex IV activity was also decreased in Qcr7E82K but not Qcr7E82D (Fig. 9f). The impaired respiratory complex activity for Qcr7E82R and Qcr7E82D was independent of any changes in the steady state amount of their phospholipids, subunits, or subunit assembly into RSCs (Fig. 9c and g–i). These results provide the first molecular evidence of the functional importance of a conserved PE-binding site identified in the structures of yeast and human complex III. Collectively, these data demonstrate that PE made in the IM by Psd1 is critical to support the intrinsic functionality of complex III and suggest one likely mechanism.

Discussion
The results derived from this study support four important conclusions. First, PE flux across the IMS is bi-directional. Given the capacity of Psd1 to produce up to 70% of PE in the cell11, it has long been appreciated that PE made in the IM can be transported across the IMS to other membrane-bound compartments. Our biochemical determination that PE made in the OM or ER can access the IMS demonstrates that the mechanism(s) responsible for PE transport across the IMS, which has not yet been molecularly identified, can do so in both directions. This ability again raises the question as to why there is a PE-generating enzyme localized to the mitochondrial IM. In this regard, it is notable that the levels of PE in the IM, or at least in the immediate vicinity of complex IV, are significantly lower when Psd1 makes robust amounts of PE in either the OM or ER. Moreover, the resulting amount of PE in the IM is unable to fully support the activities of complex IV and especially complex III. These observations support a model whereby the final
**Fig. 8** Deletion of Cho1 impairs complex III and complex IV activities. a Metabolic pathways tied to PS synthesis by Cho1 in yeast. b Detection of the β subunit of Psd1 and Cho1 expression were verified in yeast whole cell extracts of the indicated strains by immunoblot. Kgd1 served as a loading control. 
*Cho1, phosphorylated Cho1. c The indicated strains were spotted onto synthetic complete dextrose (SCD) medium with or without (+/−) 2 mM ethanolamine (+E) and incubated at 30 °C for 2 days. d Mitochondrial phospholipids from the indicated strains were labeled overnight with 32Pi, separated by TLC, and quantitated by phosphoimaging (mean ± S.E.M. for n = 6 biological replicates). Significant differences compared to WT (asterisk) or psd1Δpsd2Δ (number sign) were calculated by one-way ANOVA with Holm–Sidak pairwise comparisons. k The indicated strains were spotted and incubated at 30 °C for 2 days on YPD and for 3 days on rich lactate (RL), and SCEG without or with 2 mM ethanolamine (+E). l Complex III activity and (m) complex IV activity in isolated mitochondria solubilized in 0.5% (w/v) DDM (mean ± S.E.M. for n = 6 biologically independent experiments, except for psd1Δpsd2Δ, n = 3). Analysis vs. WT by one-way ANOVA with Tukey’s multiple comparison test. n Steady state expression of mitochondrial proteins in mitochondria isolated from the indicated strains.

The distribution of PE between the IM and OM reflects a thermodynamic equilibrium between a high local gradient of PE in the IM established by Psd1 and PE transport across the IMS, which is in principle bi-directional. According to this model, the presence of Psd1 in the IM ensures that the levels of PE in this membrane are sufficient to fully support the activity of the respiratory complexes.

Second, the absence of PE made in the IM results in severe complex III and IV functional defects. Interestingly, PE made in the ER by ER-Psd1 or the Kennedy pathway upon ethanolamine supplementation can rescue complex IV but not complex III function in psd1Δpsd2Δ yeast. What is the basis for the capacity of extra-mitochondrially produced PE to rescue the activity of complex IV but not complex III? While the answer to this
question remains elusive, it is notable that CL, and not PE, was increased upon ethanolamine supplementation (Fig. 7e), a phenomenon that has been observed previously\textsuperscript{1,16}, suggesting that this rescue may be CL-dependent. Support for this possibility also stems from the failure of OM-Psd1, which has even lower levels of CL than psd1Δpsd2Δ (Figs. 3g and 6j), to increase the activity of complex IV (Fig. 5b). The importance of CL in the assembly and function of the RSCs is well documented\textsuperscript{29,33,34}. Although it is presently unclear how PE production in the ER promotes CL accumulation, it is known that the Ups1 and Ups2 lipid trafficking proteins have an inverse relationship with respect to CL/PE metabolism suggesting that this may be linked to PS/PA trafficking into the IM\textsuperscript{35,36}. Depletion of PS in the psd1Δpsd2Δ background restored CL levels to that of psd1Δ and cho1Δ which were still comparatively reduced vs WT (Fig. 8g). This increase in CL also coincided with improved growth for cho1Δpsd1Δpsd2Δ yeast (Fig. 8k). It is possible that in the absence of PS, metabolic pathways that either promote PA formation in the ER\textsuperscript{37} or promote PA import to the IM\textsuperscript{38,39} are stimulated. Recent evidence has also implicated ethanolamine in improving the respiratory function of CL-depleted strains\textsuperscript{40}. As respiration and complex assembly are improved in the absence of CL but coincides with
Fig. 9 A PE-coordinating residue in Qcr7 is important for complex III activity. a The crystal structure of yeast cytochrome bc/workspace. Using PyMOL, the region containing the catalytic subunit Cob1 (magenta) near the matrix-facing surface was enlarged to demonstrate hydrophobic interactions between this subunit and the acyl chains of PE. Arg51 of Qcr8 (orange) also shows hydrophobic interactions with a carbon atom from the ethanolamine headgroup. Glu82 of Qcr7 (blue) was predicted to form a hydrogen bonding interaction (3.4 Å distance) with the amine group of PE, whose atoms are depicted as spheres; gray: carbon, red: oxygen, and blue: nitrogen (hydrogen atoms are not represented). b WT and mutant Qcr7 was detected in yeast whole cell extracts of the indicated strains by immunoblot; Aac2 served as a loading control.

Table 1 Yeast strains used in this work. The names, genotypes, and sources of the yeast strains used in the present study

| Strain          | Genotype                                                                 | Source                  |
|-----------------|---------------------------------------------------------------------------|-------------------------|
| psd1Δ           | MATa, his3-11,15, leu2, ura3, trp1, ade8 [rho +, mit +]                    | Carla Koehler          |
| psd2Δ           | MATa, psd1Δ::His3MX6, leu2, ura3, trp1, ade8 [rho +, mit +]                 | ref. 17                |
| psd1Δpsd2Δ      | MATa, psd1Δ::His3MX6, leu2, ura3, trp1, ade8 [rho +, mit +]                 | ref. 17                |
| psd1Δpsd2ΔA      | MATa, psd1Δ::His3MX6, Psd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho +, mit +] | ref. 17                |
| psd1Δpsd2ΔA:OM-Psd1 | MATa, psd1Δ::His3MX6, Tom20-Psd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho +, mit +] | This study             |
| psd1Δpsd2ΔA:ER-Psd1 | MATa, psd1Δ::His3MX6, CPY*Psd3XFLAG::LEU2, ura3, psd1Δ::TRP1, ade8 [rho +, mit +] | This study             |
| choΔ            | MATa, choΔ::His3MX6, leu2, ura3, trp1, ade8 [rho +, mit +]                  | This study              |
| Qcr7p E82R      | MATa, his3-11, 15, leu2, ura3, trp1, ade8 [rho +, mit +] Qcr7p E82R Hi-CRISPR | This study              |
| Qcr7p E82D      | MATa, his3-11, 15, leu2, ura3, trp1, ade8 [rho +, mit +] Qcr7p E82D Hi-CRISPR | This study              |
| W303            | MATa, his3-11, ura3-1,15, trp-1, ade2-1, can-100 [rho +, mit +]             | Cathy Clarke           |
| psd1Δ W303      | MATa, psd1Δ::His3MX6, ura3-1,15, trp-1, ade2-1, can-100 [rho +, mit +]       | This study              |
| D273-10B        | MATa [rho +, mit +]                                                        | Carla Koehler          |
| BY4741          | MATa, his3-11, leu2-0, mit15-0, ura3-0 [rho +, mit +]                       | Euroscarf              |
| psd1Δ BY4741    | MATa, psd1Δ::KANMX4, his3-1, leu2-0, mit15-0, ura3-0 [rho +, mit +]         | Euroscarf              |
| abf2Δ          | MATa, his3-11,15, leu2, ura3, abf2Δ::TRP1, ade8 [rho +, mit +]              | This study              |

Table 1 (Continued)

To our knowledge, this is the first molecular evidence demonstrating the functional importance of a specific interaction of PE with complex III, which until now had only been postulated from crystal structures of yeast and human RSC31,32. A second PE is found adjacent to the dimer interface of this complex; as such, its acyl chains are thought to interact with both monomers. However, PE depletion did not disrupt dimer formation (Fig. 5d, e). PE at the dimer interface could instead potentially promote quinol-quinone exchange at the Q and Qo sites. More broadly, during quinol-quinone exchange, sidechain movement of Cob1 is thought to be necessary to transfer protons from His202 to ubiquinone42. If depletion of PE or mutagenesis of residues that interact with this lipid diminish the efficiency of electron transfer between complex III monomers or between complex III and its substrates, this could result in reduced complex III function, potentially as a means to prevent super-oxide production43. These structural observations will guide future efforts to determine the role(s) of PE as it relates to complex III activity. Future efforts will use a selective marker as indicated in Table 1.13 Psd1 containing a COOH-terminal 3XFLAG tag subcloned into pRS305 has been described15,44.

Methods

Yeast strains and growth conditions. All yeast strains used in this study are listed in Table 1 and were derived from GA74-1A unless otherwise noted. Deletion strains were generated by PCR-mediated gene replacement of the entire reading frame using a selectable marker as indicated in Table 1.13. Psd1 containing a COOH-terminal 3XFLAG tag subcloned into pRS305 has been described15,44.
To re-direct Psd1 to the mitochondrial OM, the first 100 amino acids of Psd1, encompassing its mitochondrial targeting sequence and transmembrane domain, were replaced by yeast mitochondria targeting sequence of the resident protein, Tom20. ER-Psd1, which is directed to the secretory pathway, was generated by replacing the first 57 amino acids of Psd1, encompassing its mitochondrial targeting signal (MTS), with the N-terminal signal sequence (amino acids 1–23) of carboxypeptidase Y, as previously described. Additionally, the ER-Psd1 construct contained a C-terminal NXS glycosylation signal immediately downstream of the CPY leader sequence to track its topology. Details of the primers used in this study are listed in Supplementary Table 1. The IM-Psd1, OM-Psd1, and ER-Psd1 constructs, which all contained the C-terminal 3XFLAG tag, were subcloned into the pKRP5 plasmid backbone, and integrated into the LEU2 loci of several N. crassa genomes. The LEU2 gene was disrupted in the parental strain and sterile filtered using a 0.20 µM filter (Corning, Inc). Individual colonies of yeasts were used to inoculate starter cultures in -pABA medium containing 2% (w/v) glucose and 2 mM ethanolamine. Solid growth of yeast on agar plates was evaluated in +/-pABA medium containing 1% (v/v) ethanol and 3% (v/v) glycerol (+/- 2 mM ethanolamine).

Preparation of yeast cell extracts. Overnight 2 mL cultures of yeast were grown in YPD, rich lactate, or SCD with or without 2 mM ethanolamine as indicated in figure legends. 1 OD_{600} of exponentially growing cells was collected in a 1.5 mL microcentrifuge tube by centrifugation for 10 min at 845 x g at room temperature. The supernatant was aspirated and the cell pellet resuspended in 1 mL of water. A freshly made NaOH-mercaptoethanol solution consisting of 1 mL 2 M NaOH and 80 µl of mercaptoethanol was prepared and added to the cell resuspension which was mixed by inversion. Tubes were incubated for 10 min on ice and mixed by inversion every 2 min. 75 µL of 100% (w/v) trichloroacetic acid (TCA) was added to precipitate proteins in each sample tube and incubated on ice for 10 min with mixing by inversion every 2 min. Samples were centrifuged for 2 min at 21,000 x g at 4 °C. The supernatant was aspirated and pellets washed using 500 µL of 100% cold acetone. Samples were centrifuged for 2 min at 21,000 x g at 4 °C and the acetone decanted. The pellet was briefly air-dried and then resuspended with 30 µL of 0.1 M NaOH. After dissolution of the pellets, an equal volume of 2X reducing sample buffer was added and samples boiled for 5 min at 95 °C. 5 µL of yeast extracts were evaluated by SDS-PAGE and immunoblotting.

Immunoblotting. Protein samples in reducing sample buffer were resolved on either 12% or custom-made 10–16% gradient SDS-PAGE gels at ~5.8 mAhpr. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc, 0.45 µM Catalog No. 1620115) at 30 Volts overnight (16 h) at room temperature. The quality of the transfer was checked by Ponceau S staining for 15 s, and membrane strips were used to probe for proteins of the appropriate molecular weight and size. As described, membranes were blocked with 5% (w/v) milk (Giant, Ellicott City, MD), 0.05% (v/v) Tween 20/XPBS for 1 h and then incubated with primary antibody to 1 h at room temperature. Following three successive 10 min washes with PBST (PBS with 0.2% Tween-20), HR- or IRDye 800CW-conjugated secondary antibodies were added for 45 min at room temperature. The membranes were washed three times for 10 min with PBST and twice for 10 min with PBS. When using HR-conjugated secondary antibodies, immunoreactive bands were visualized using the SuperSignal West Pico chemiluminescent substrate from Pierce and imaged using a Fluorchem Q (ProteinSimple). Immunoblots using IR 800 CW-conjugated secondary antibodies were imaged using an Odyssey CLX Imaging System. To reprobe a blot, membranes were incubated with stripping buffer (100 mM β-mercaptoethanol (β-ME), 2% (w/v) SDS, 62.5 mM Tris, pH 6.7) shaking at 37°C for at least 1 h and then washed 5 times, 10 min/wash, with PBST prior to blocking the membrane again.

Mitochondrial isolation and fractionation. Crude mitochondria were isolated as described. Yeast were maintained on rich lactate plates and grown in rich lactate media to prevent the loss of mitochondria. Yeast were transferred from plates and grown in YPD, rich lactate, psd1Δpsd2Δ yeast were grown in the presence of 2 mM choline prior to harvesting mitochondria (except where indicated in Fig. 7a, b and in Fig. 8). For mitochondrial isolation, starter cultures of 100 mL or (150 mL for psd1Δpsd2Δ) were grown at 30 °C for 36–48 h until saturation. 100 OD_{600} of the starter cultures were sterilely added to 2 L flasks containing 950 mL rich lactate and grown for ~18 h (or 24 h for psd1Δpsd2Δ) at which point the cultures had reached an OD_{600} of ~2.5–3.5 (two 2 L flasks were grown for each strain). Cells were harvested by centrifugation (5 min at 6,000 x g at room temperature) using 100 mL buckets and the cell pellets resuspended with ~100 mL of water and transferred to pre-weighted 250 mL flasks. Cells were collected at 2000 x g for 5 min, at room temperature by centrifugation. The supernatant was decanted and the cell pellets weighed (typically yield is ~6 g of yeast/L of culture). Cells were suspended in 50 mL of freshly prepared 0.1 M Tris-SO_{4} pH 9.4 (1 M Tris with pH adjusted with sulfuric acid) containing 15 mM dithiothreitol and incubated for 20 min at 30 °C shaking at 250 rpm. Cell pellets were collected by centrifugation at 2000 g for 5 min at room temperature and washed with 40 mL of 1.2 M sorbitol, 20 mM KPi, pH 7.4 buffer. Pellets were collected again at 2000 x g for 5 min at room temperature. To convert cells to spheroplasts, yeast pellets were resuspended in 1.2 M sorbitol, 20 mM KPi, pH 7.4, containing 3 mg of Zymolyase 20 T (nacalai tesque, INC.) per gram of yeast, using a final volume of 2 mL per gram of yeast, and the suspensions shaken at 220 rpm for 1 h at 30 °C. Spheroplast efficiency was monitored visually by checking for osmosilysis upon dilution of a small drop of yeast slurry in 10 µL H_{2}O. Spheroplasts were collected by centrifugation at 3500 x g for 5 min at 4 °C. From this point on, all operations were conducted at 4 °C and/or on ice. Cell pellets were resuspended with 1 mL with cold (4 °C) 12 M sorbitol, 0.1 M Tris, pH 7.4 buffer and collected by centrifugation at 3500 x g for 5 min at 4 °C. For homogenization, spheroplasts were resuspended in 50 mL of 0.6 M sorbitol, 20 mM K_{2}H_{2}O-MES,
pH 6 (BB6.0 buffer) containing 1 mM phenylmethysulfonyl fluoride (PMSF), transferred to a tight-fitting (type A) glass dounce homogenizer (pre-chilled on ice), was transferred to tubes containing 15 strokes. For centrifugation, the homogenate was placed and collected on ice to quantitate and analyze as the starting material (SM, Supplementary Fig. S1). The combined homogenate was centrifuged at 5 min at 1700 × g at 4 °C, and the supernatants collected. The residual pellets were re-homogenized in BB6.0 + 1 mM PMSF by 15 strokes in the same glass dounce vials. After this second centrifugation, the supernatant was aspirated, and the supernatants from both homogenizations were combined and then centrifuged for 10 min at 13,500 × g. The pellets following this centrifugation contain crude mitochondria. The resulting supernatants (S13) were either processed for additional isolation of crude mitochondria, or used directly to each sampling standard condition. A 10 μL sample of crude mitochondria, and 1 and 2 μL transferred to clean, unused glass tubes; the rest of the mitochondrial lipids were re-evaporated under a stream of liquid nitrogen. The 1 μL and 2 μL lipid samples were evaporated at 150 °C of 70% (w/v) perchoric acid and lipid extracted with 500 μL of a chloroform/methanol 2:1 ratio. For evaluation by thin layer chromatography, dried lipid samples were resuspended in 13 μL of chloroform and loaded on Silica Gel GHLF (Analtech) or SILGUR-25 (Machery-Nagel) TLC plates that were pretreated with 1.8% (w/v) boric acid in 100% ethanol and activated at 95 °C for at least 30 min. The loaded plates were resolved using a solvent system containing chloroform/ethanol/H₂O/ triethylamine (30:35:7:35). The plate was air-dried for 30 min and either sprayed manually using a TLC sprayer (CAMAG) and the molybdenum spray reagent (Sigma) or developed using a K-screen and FX-Imager (Bio-Rad Laboratories).

**Electron microscopy.** Cells were grown in rich lactate medium and harvested in mid-log phase by centrifugation. Cells were fixed in 3% glutaraldehyde contained in 0.1 M sodium cacodylate, pH 7.4, 5% sucrose, 5 mM MgCl₂, and 2.5% (w/v) sucrose for 1 h at room temperature with gentle agitation, spheroplasts, embedded in 2% ultra-low temperature agarose (prepared in water), cooled, and subsequently cut into small pieces (~1 mm³) as previously described. The cells were then post-fixed in 1% OsO₄, 1% potassium ferrocyanide contained in 0.1 M sodium cacodylate, pH 7.4, for 30 min at room temperature. The blocks were then washed thoroughly four times with double distilled H₂O, 10 min total, transferred to 1% thiocarbohydrazide at room temperature for 3 min, washed in double distilled H₂O (four times, 1 min each), and transferred to 1% OsO₄, 1% potassium ferrocyanide in 0.1 M sodium cacodylate, pH 7.4, for an additional 3 min at room temperature. These cells were washed in cold 32% (v/v) sucrose, 3 min, and stained en bloc in the presence of 2% (w/v) uranyl acetate for 2 h to overnight, dehydrated through a graded series of ethanol, and subsequently embedded in Spur resin. Sections were cut on a Reichert Ultracut T ultramicrotome, post-stained with uranyl acetate and lead citrate, and observed on a FEI Tecnai 12 transmission electron microscope at 100 kV. Images were recorded with a SOF Imaging System Megaview III digital camera, and figures were assembled in Adobe Photoshop with only linear adjustments in contrast and brightness.

**mtDNA quantitation.** Yeast cells were grown for 2 days in rich lactate and the collected cell pellets were vortexed at level 10 for 3 min with 200μl of 4 M ammonium acetate and 1 mL 10% (w/v) NaCl added to each tube which were vortexed for another 1 min. Tubes were centrifuged at 1000 rpm for 5 min at room temperature. The aqueous phase was aspirated and the residual phase was washed with 0.25 mL of 1:1 methanol:water. Tubes were vortexed for 30 s, centrifuged at 5 min at 1000 rpm and 5 min at room temperature and the lower organic phase transferred to new 5 mL borosilicate tubes using a clean glass Pasteur pipette. Lipid samples were dried down under a stream of liquid nitrogen. For DNA quantitation, equal amounts of each sample were dissolved in 100 μL of Tris-EDTA (TE) buffer pH 8.0 and phases were separated by centrifugation at 21,000 × g for 5 min. The aqueous phase was collected and DNA was precipitated by the addition of 100% ethanol and collected in the pellet after centrifugation at 21,000 × g for 5 min. The pellets were resuspended in 400 μL TE buffer pH 8.0 and RNA was digested with 10 μL of 30 μg/mL RNase A and incubated at 37°C for 5 min before addition of 10 μL of 4 M ammonium acetate and 1 mL 100% ethanol. DNA pellets were recovered by centrifugation at 21,000 × g for 3 min, dried, and resuspended in 30 μL TE buffer pH 8.0. DNA was stored at 80°C until ready for use, quantitated, and a portion diluted to 10 μg/μL to be used as a template for the qPCR reactions.

**Phospholipid analyses.** 1.2 mg of purified sucrose mitochondria were transferred to 5 mL borosilicate tubes containing 1.5 mL of 2:1 chloroform/methanol. After vigorous vortexing at room temperature for 30 min, 0.3 mL of 0.9% (w/v) NaCl was added to each tube which were vortexed for another 1 min. Tubes were centrifuged at 1000 rpm for 5 min at room temperature. The aqueous phase was aspirated and the residual phase was washed with 0.25 mL of 1:1 methanol:water. Tubes were vortexed for 30 s, centrifuged for 5 min at 1000 rpm at room temperature and the lower organic phase transferred to new 5 mL borosilicate tubes using a clean glass Pasteur pipette. Lipid samples were dried down under a stream of liquid nitrogen. For phospholipid quantitation, equal amounts of each sample were dissolved in 100 μL of chloroform/methanol (1:2 ratio), and 1 and 2 μL transferred to clean, unused glass tubes; the rest of the mitochondrial lipids were re-evaporated under a stream of liquid nitrogen. The 1 μL and 2 μL lipid samples were evaporated at 150 °C of 70% (w/v) perchoric acid and lipid extracted with 500 μL of a chloroform/methanol 2:1 ratio. The resulting supernatant was aspirated, and the supernatants from both homogenizations were combined and then centrifuged for 10 min at 13,500 × g. The pellets following this centrifugation contain crude mitochondria. The resulting supernatants (S13) were either processed for additional isolation of crude mitochondria, or used directly to each sampling standard condition. A 10 μL sample of crude mitochondria, and 1 and 2 μL transferred to clean, unused glass tubes; the rest of the mitochondrial lipids were re-evaporated under a stream of liquid nitrogen. The 1 μL and 2 μL lipid samples were evaporated at 150 °C of 70% (w/v) perchoric acid and lipid extracted with 500 μL of a chloroform/methanol 2:1 ratio. The resulting supernatant was aspirated, and the supernatants from both homogenizations were combined and then centrifuged for 10 min at 13,500 × g. The pellets following this centrifugation contain crude mitochondria.
were used at 100 nM concentration in a 20 µL reaction: COXI forward (5′-TCTAGATACGACATTTCCAAGA-3′), COXI reverse (5′-GTCCTGAAATAGTGAATACCCTGTCGTGTTTTG-3′), ACTI forward (5′-CATGATACCTTGGTGTCCGGTG-3′) and ACTI reverse (5′-CATGATACCTTGGTGTCCGGTG-3′). The reactions were performed in technical duplicate with three biological replicates. After completion of thermocycling in a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher), melting-curve data was collected to verify PCR specificity. The absence of primer dimers was ascertained by the nuclear (ACTI) and mitochondrial (COXI) target were computed as a measure of the mitochondrial DNA copy number relative to the nuclear genome.

Mitochondrial respiration measurements. As done previously, mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode in a magnetic stirrer, thermostatically controlled 1.5 mL chamber at 25 °C (Oxytherm-Hansatech). 100µg of mitochondria were suspended in 0.25 M sucrose, 0.25 mg/ml BSA, 20 mM KCl, 20 mM Tris-Cl, 0.5 mM EDTA, 4 mM KH2PO4, and 3 mM MgCl2, pH 7.2. After addition of 1 mM acetoxy-2,6-dimethoxyacetophenone and the Ct value difference between the nuclear (ACTI) and mitochondrial (COXI) was applied to glow-discharged, carbon coated, 400 mesh copper grids (Electron Microscopy Sciences). The grids were acquired on a Tecnai 12 G2 Spirit BioTWIN microscope (FEI, USA) and was cooled down and the SMA co-polymer was gradually precipitated by reducing the oxygen level reached zero. State 3 respiration was initiated by addition of 500 µM ADP. After state 4 rate was measured, 10M µM CCCP was added to induce uncoupled respiration, and the rate was followed for either 2 min or until oxygen level reached zero.

Complex III and IV activity measurements. Established methods were used to determine complex III and IV activities. To measure complex III activity, 25µg of mitochondria solubilized in 0.5% (w/v) n-dodecyl-β-D-maltoside were added to reactions containing 0.3 µM KMp, 2 µM EDTA, pH 7.4 with 0.008% (w/v) horse heart cytochrome c and 1 mM KCN. The reaction was started by adding 100µM deoxy-ubiquinol, and the reduction of cytochrome c followed at 550 nM. Complex IV activity was initiated by adding 5µg of solubilized mitochondria to reaction buffer with 0.008% (v/v) ferrocyanochrome c and measured by recording cytochrome c oxidation at 550 nm.

Blue native-PAGE. Sedimented mitochondria (150µg) were solubilized with 30 µL of 1.5% (w/v) digitonin (Biosynth International, Inc.) in 20 mM HEPEs-KOH, pH 7.4, 10% (v/v) glycerol, 50 mM NaCl, 5 mM MgCl2, and 25 mM MgCl2, pH 7.2. After addition of 1 mM ascorbate + 0.3 mM TMPD, state 2 rate was monitored for approximately 30 sec. State 3 respiration was initiated by addition of 500μM ADP. After state 4 rate was measured, 100µM CCcp was added to induce uncoupled respiration, and the rate was followed for either 2 min or until oxygen level reached zero.

Preparation of styrene maleic acid co-polymer (SMA). Styrene maleic acid co-polymer with ratio of 3:1 was prepared by alkaline hydrolysis of its anhydride polymer with ratio of 3:1 was prepared by alkaline hydrolysis of its anhydride precursor (SMA 3000, a gift from Gray Valley, USA) following a published protocol. Briefly, the styrene maleic anhydride co-polymer was dissolved in 1 M NaOH by heating and refluxing of the solution. Once dissolved, the solution was cooled down and the SMA co-polymer was gradually precipitated by reducing the pH to <5.0 with HCl. The precipitate was washed with water and re-dissolved in 0.6 M NaOH. The precipitation and washing cycle of SMA was repeated before the precipitate was finally dissolved in 0.6 M NaOH. The dissolved polymer was lyophilized using a freeze-dryer (Labconco) after adjusting the pH to 8.0 with HCl.

Negative staining and TEM imaging. A concentration of 5 µl of purified SMALPs was applied to glow-discharged, carbon coated, 400 mesh copper grids (Electron Microscopy Sciences, USA) and stained with 0.5 % uranyl acetate. TEM images of the grids were acquired on a Tecnai 12 G2 Spirit BioTWIN microscope (FEI, USA) operating at 120 kV.

Lipidomics. Individual samples (0.13–0.15 mg protein) were accurately transferred into disposable glass culture test tubes, and a mixture of lipid internal standards was added prior to conducting lipid extraction for quantification of all reported lipid species. Lipid extraction was performed by using a modified Bligh and Dyer procedure. Briefly, 4 mL chloroform/methanol (1:1, v/v) and an appropriate volume of 50 mM lithium chloride solution to bring the aqueous phase to a final volume of 2 mL was added to each sample. Following a brief 20 s vortex, the samples were centrifuged at 2700 r.p.m. for 10 min and the bottom organic layer transferred to a new borosilicate glass tube. The residual top layer was re-extracted with 2 mL chloroform. The combined organic layers were dried under a nitrogen stream with a nitrogen-evaporator. Individual lipid extracts were resuspended into a volume of 1 mL of chloroform/methanol (1:1, v/v) per mg of protein for mass spectrometric analysis. Individual suspensions were flushed with nitrogen, capped, and stored at –20 °C for future analysis.

For shotgun lipidomics, lipid extracts were further diluted to a final concentration of ~50 nmol total lipids/µl, and mass spectrometric analysis was performed on a triple quadrupole mass spectrometer (TSQ Allis, Thermo Fisher Scientific, San Jose, CA) and a Q exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) which were both equipped with an automated ESI nanospray device (TriVersa NanoMate, Advion Bioscience Ltd., Ithaca, NY) as previously described. Identification and quantification of lipid molecular species were performed using an automated software program. Data processing including ion peak selection, baseline correction, data transfer, peak intensity comparison, peak deisotoping, and quantitation were conducted using a custom programmed Microsoft Excel macro as previously described after considering the principles of lipidomics.

Antibodies. Antibodies used in the study are listed in Supplementary Table 3. Most of these antibodies were generated by our laboratory or in the laboratories of J. Schatz (University of Basel, Basel, Switzerland) or C. Koehler (UCLA) and have been described previously. Ab2 specific antibodies were raised in rabbits using His6-AmB2 as antigen. Mouse anti-FLAG (clone M2, catalog number F3165, Sigma), mouse anti-FLAG (clone M2, catalog number F3165, Sigma), mouse anti-Dmp1 (catalog number 113686, Abcam), rabbit anti-Qcr8, rabbit antisera reactive to Coq169, Coq470, Coq771, or Coq972, rabbit antisera raised against the C-terminus of Choi17, rabbit anti-Kar219 and horseradish peroxidase-conjugated (Thermo Fisher Scientific) or IRDye 800CW (LI-COR) secondary antibodies.

Miscellaneous. Uncropped and unprocessed scans of all immunoblots are presented in Supplementary Fig. 12. Immunoblots and TLC plates were quantitated by Quantity One Software (Bio-Rad Laboratories). Statistical comparisons (NS, P > 0.05; 1 symbol P ≤ 0.05; 2 symbols P ≤ 0.01; 3 symbols P ≤ 0.001; 4 symbols P ≤ 0.0001) were performed using SigmaPlot 11 software (Systat Software, San Jose, CA) or Prism 7 (GraphPad). In some cases, replicates of sample were loaded on the same SDS-PAGE gel, and thus borders of neighboring samples may be detected on the borders of some immunoblots. All graphs show the mean ± S.E.M.. At least three biological replicates represent each of the experiments performed in this study, unless otherwise indicated.
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