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Characterization of Phosphatidylserine Decarboxylase 1 from the Yeast

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Abstract

Phosphatidylethanolamine (PE) is one of the most abundant phospholipids found in yeast, plant and mammalian cells. In the yeast *Saccharomyces cerevisiae*, PE can be synthesized by (i) decarboxylation of the precursor phosphatidylserine (PS) through the action of the PS decarboxylases Psd1 and Psd2, (ii) re-acylation of lyso-PE catalyzed by Ale1 and Tgl3 or (iii) the CDP-ethanolamine (CDP-Etn) pathway using ethanolamine as a substrate. PE is not only an important membrane constituent but also serves as an acyl donor for the acyl-CoA independent synthesis of triacylglycerols (TAG) through Lro1. Our investigations on a possible link between TAG and PE metabolism elucidated that the CDP-Etn pathway contributed most to TAG synthesis. Defects in the CDP-Etn pathway lead to a decrease of TAG, microsomal and cellular PE levels, and reduced Lro1 activity whereas the transcription of *LRO1* was not affected. Our findings indicate that TAG and PE synthesis in the yeast are tightly linked and the local availability of PE in microsomes is crucial for TAG formation since enzymes of both biosynthetic pathways are located to the same compartment. Although the mitochondrial phosphatidylserine decarboxylase 1 (Psd1) is the major source of PE biosynthesis in yeast, its biogenesis is only poorly understood. Like most mitochondrial proteins, Psd1 is synthesized on free ribosomes and imported into mitochondria where processing/protein maturation occurs. In a detailed biochemical study, we showed that processing of Psd1 comprises three processing steps including the action of the mitochondrial processing peptidase (MPP), Oct1 and an autocatalytic cleavage at a highly conserved LGST motif yielding the mature form of the enzyme containing a β-subunit and an α-subunit. The β-subunit is localized to the inner mitochondrial membrane and anchors the soluble intermembrane exposed α-subunit. Proper maturation and correct localization to these mitochondrial subcompartments is crucial for enzymatic activity and maintenance of mitochondrial lipid homeostasis. Deletion of *PSD1* causes PE depletion in mitochondria which affects protein complexes of the outer and inner mitochondrial membrane. In a series of experiments, we observed an impaired function of the translocase of the outer membrane (TOM complex), decrease of the inner membrane potential affecting the import of preproteins into and across the inner membrane and reduced respiratory capacity whereas the formation of larger respiratory chain supercomplexes was initiated. Finally, we investigated whether alterations of the mitochondrial morphology in MINOS (inner membrane organizing system) mutants are related to the mitochondrial lipid status. Lack of MINOS core components did not affect the mitochondrial phospholipid composition. Thus, we excluded that defects observed in these mutants are secondary effects caused by changes in the mitochondrial lipid profile.

Zusammenfassung

Phosphatidylethanolamin (PE) ist ein Hauptbestandteil biologischer Membranen und zählt zu den am häufigsten vorkommenden Phospholipiden in Hefen, Pflanzen und tierischen Zellen. Decarboxylierung von Phosphatidylserin (PS) durch Psd1 und Psd2, Reacylierung von lyso-PE durch Ale1 und Tgl3, und der CDP-Ethanolamin (CDP-Etn) Biosyntheseweg führen in der Bäckerhefe *Saccharomyces cerevisiae* zur Bildung von PE. PE ist nicht nur ein wichtiger Membranbestandteil, sondern steht auch als Acyl-Donor für die acyl-CoA unabhängige Synthese von Triacylglyceriden (TAG) durch das Enzym Lro1 zur Verfügung. Unsere Untersuchungen der metabolischen Vernetzung von PE und TAG Metabolismus zeigten, dass der CDP-Etn Stoffwechselweg den größten Beitrag zur TAG Synthese liefert. Defekte in jenem PE Biosyntheseweg reduzierten die Lro1 Aktivität, den TAG sowie den zellulären und mikrosomalen PE Gehalt während die Transkriptionsrate von *LRO1* keine Veränderung aufwies. Diese Resultate zeigen eine enge Vernetzung von TAG und PE Biosynthese in der Hefe, die auf die lokale Verfügbarkeit von PE in den Mikrosomen und die Lokalisierung der Enzyme beider Stoffwechselwege in diesem Kompartiment zurückzuführen sind. Das mitochondriale Enzym Phosphatidylserine Decarboxylase 1 (Psd1) ist der Hauptproduzent von PE in *Saccharomyces cerevisiae*. In Analogie zu den meisten mitochondrialen Proteinen wird Psd1 an cytosolischen Ribosmen synthetisiert und in Mitochondrien importiert sowie prozessiert. Biochemische Analysen zeigten, dass die Psd1 Reifung in Mitochondrien drei Prozessierungsschritte durchläuft und von der mitochondrialen „processing peptidase“ (MPP), Oct1 und einer autokatalytischen Spaltung an dem konservierten LGST Motiv abhängig ist. Das reife Enzym besteht aus zwei Untereinheiten β und α, die in der inneren mitochondrialen Membran verankert bzw. im Intermembranraum lokalisiert sind. Eine korrekte Reifung und Lokalisierung beider Untereinheiten ist essentiell für die enzymatische Aktivität des Enzyms und die Aufrechterhaltung der mitochondrialen Lipidhomöostase. Deletion von *PSD1* führt zur Reduktion des mitochondrialen PE Gehalts und Veränderungen an Proteinkomplexen der äußeren und inneren mitochondrialen Membran. PE Depletion beeinträchtigt die Funktion des TOM Komplexes, das Potential der inneren mitochondrialen Membran, den Import von Proteinen in und durch die innere mitochondriale Membran sowie die respiratorsiche Kapazität. Zusätzlich wurde in *psd1Δ* Mutanten die Bildung von Megakomplexen der Atmungskette gefunden. Des Weiteren wurden mitochondriale Lipidprofile von MINOS (inner membrane organizing system) Mutanten untersucht. Wir konnten zeigen, dass eine veränderte mitochondriale Morphologie und die damit verbundenen Effekte nicht das Resultat eines veränderten Lipid-Status sind.

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General Introduction

The aim of this Thesis was to contribute to our understanding of the role of phosphatidylethanolamine (PE) to cellular structure and function. Synthesis of PE is closely linked to the function of Psd1, the mitochondrial phosphatidylserine (PS) decarboxylase. To study the biosynthesis of Psd1 and the role of PE in the lipid biosynthetic network, the yeast *Saccharomyces cerevisiae* was used as a model system.

Biological membranes are essential components of eukaryotic cells by serving as a diffusion barrier, restricting certain processes to specific subcellular compartments and protecting the cell from its environment. The major structural elements of membranes are phospholipids which provide the matrix for embedding sterols, sphingolipids and proteins. Membrane proteins catalyze the selective transport of molecules, act as enzymes in regulatory and metabolic processes or contribute as receptors to signalling processes [1]. Phospholipids are composed of a glycerol backbone esterified with fatty acids in the *sn-1* and *sn-2* position, and a phosphate group in the *sn-3* position which is further linked to a polar head group giving rise to specific lipid classes. In the yeast *Saccharomyces cerevisiae*, palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1) are regarded as the most abundant fatty acids found in phospholipids with saturated acyl chains preferentially incorporated in the *sn-1* position and unsaturated species connected to the *sn-2* position. The variation in the acyl chain composition and head group results in the occurrence of a huge spectrum of different phospholipid species. These specific compounds may influence membrane properties by assembling in different structures either favouring bilayers or inducing hexagonal (H_{II}) phase structures (Figure 1) [1-3].

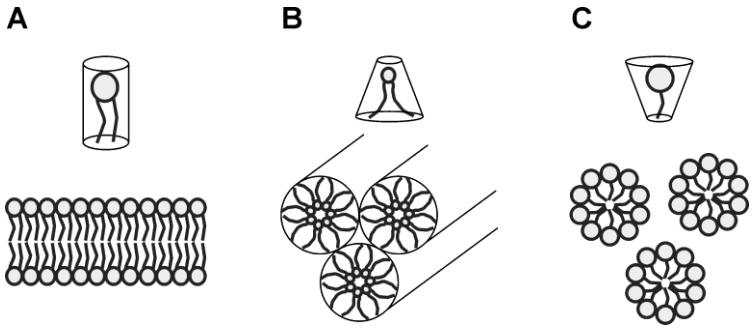

Figure 1. Shape-structure concept of lipid polymorphism. (A) Cylindrical-shaped lipid molecules form bilayer structures, (B) molecules with an conical shape such as CL and PE induce negative curvature and lead to hexagonal (H_{II}) phase structures whereas (C) positive membrane curvation favours micelle formation.

Cylindrical shaped phospholipids like phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylglycerol (PG) display a similar head group to acyl chain area and favour bilayer structures. Changes in the cross-section of head to acyl chain areas result in, positive (type I lipids) or negative (type II lipids) membrane curvation leading to micelle or inverted hexagonal phase structures (H_{II}), respectively. Type I lipids such as lyso-phospholipids with only one fatty acid moiety linked to the glycerol backbone contain a larger head to tail area. Phosphatidylethanolamine (PE) and cardiolipin (CL) belong to type II lipids forming an overall conical shape with a smaller head to tail area. They are regarded as non-bilayer forming lipids [4]. Non-bilayer forming phospholipids may directly affect the membrane function either by changing the flexibility and barrier properties or indirectly by influencing the activity of peripheral and integral membrane proteins [3].

The highest concentration of non-bilayer forming phospholipids is found in mitochondria where CL accounts for 10-15% of total phospholipids [5]. Mitochondria are specialized organelles whose functionality depends on a coordinated supply of proteins and lipids although some of these biomolecules are synthesized by the organelle itself. Chapter 1 of this Thesis will summarize the present knowledge about lipids of mitochondrial membranes with focus on the mitochondrial lipid composition, precise topography including distribution of lipids between the outer and inner mitochondrial membrane as well as transbilayer orientation within the bilayer. Furthermore, biosynthesis, regulation and transport of lipids to and within mitochondria are discussed. An important aspect is that lipids and mitochondrial proteins share a functional relationship to stabilize and assemble mitochondrial protein complexes involved in respiration, energy production and import processes.

PE is one of the most abundant phospholipids of cellular membranes and belongs to the group of non-bilayer forming lipids [3]. Unlike CL synthesis, which is restricted to mitochondria, PE biosynthesis occurs in different cellular compartments, including mitochondria (Psd1), the Golgi/vacuolar compartment (Psd2), the endoplasmic reticulum with its mitochondria associated membrane fraction (CDP-ethanolamine pathway, Ale1) and lipid droplets (Tgl3) (Figure 2A) [1;6-16]. PE plays not only a central role in aminoglycerophospholipid metabolism by serving as a precursor for PC formation but also in the acyl-CoA independent formation of triacylglycerols (TAG) [1;17-19]. TAG and

steryl esters (SE) are storage lipids of eukaryotic cells and accumulate in a specialized globular compartment of the cell termed lipid droplets (LD) (Figure 2B) [20]. LD are composed of a randomly packed TAG core which is surrounded by several more or less ordered SE shells. LD are sequestered from the cytosol by a phospholipid (PL) surface monolayer with specific proteins embedded [21;22]. The two major yeast TAG synthesizing enzymes are Dga1 and Lro1. Are1 and Are2 localized to the endoplasmic reticulum catalyze the synthesis of SE and contribute to the acyl-CoA dependent TAG formation with minor efficiency (Figure 2C) [17;23-26]. Dga1 catalyzes the acyl-CoA dependent TAG biosynthesis and is dually localized to the endoplasmic reticulum and LD [24;27]. The endoplasmic reticulum localized acyl-CoA independent phospholipid:diacylglycerol acyltransferase (PDAT) Lro1 preferentially uses PE as a co-substrate *in vitro* and transfers its *sn*-2 acyl group to diacylglycerol leading to TAG formation and lyso-PE [17-19]. In this Thesis, the link between TAG synthesis and PE metabolism was investigated in more detail and will be discussed in Chapter 2. The central question of this study was which PE biosynthetic pathway contributes most to TAG formation.

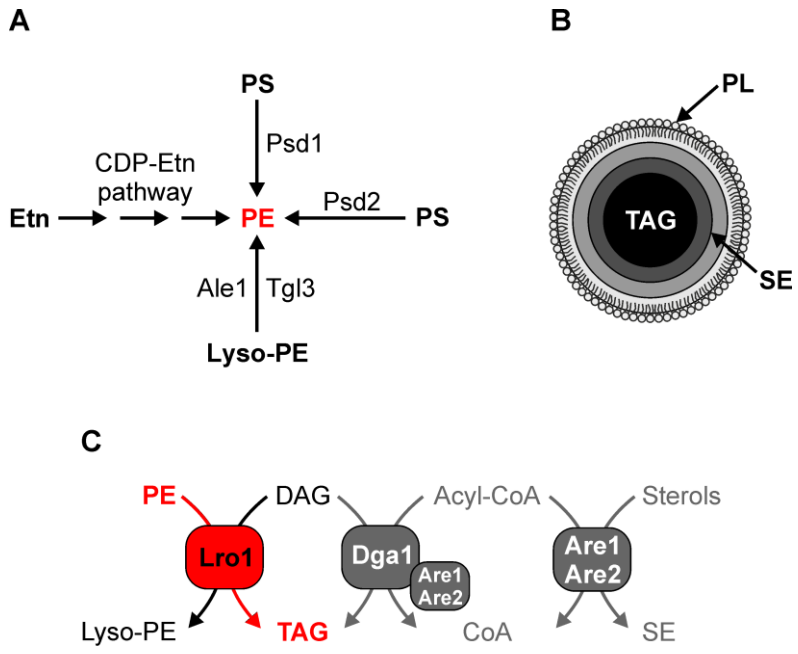

Figure 2. Phosphatidylethanolamine (PE) and triacylglycerol (TAG) metabolism in the yeast *Saccharomyces cerevisiae*. (A) PE is synthesized via decarboxylation, re-acylation of lyso-PE or the CDP-ethanolamine (CDP-Etn) pathway. (B) Model of a yeast lipid droplet. TAG form the inner core which is surrounded by several steryl ester (SE) shells and sequestered from the cytosol through a phospholipid (PL) monolayer. (C) TAG and SE biosynthesis in the yeast *Saccharomyces cerevisiae*.

In the yeast *Saccharomyces cerevisiae*, the mitochondrial phosphatidylserine decarboxylase 1 (Psd1) is the major site of PE biosynthesis. PE formed by Psd1 is not only incorporated into mitochondrial membranes but also supplied to other subcellular compartments [28]. Deletion of *PSD1* leads to ethanolamine auxotrophy on non-fermentable carbon sources, reduced growth on fermentable growth media and morphological alterations of mitochondria which cannot be completely compensated by the import of extramitochondrially synthesized PE. Thus, Psd1 is clearly a key player in yeast lipid metabolism and homeostasis [29;30]. Psd1 from the yeast *Saccharomyces cerevisiae* shares 35% sequence identity with Psd from *E. coli* and 44% sequence identity with PSD from Chinese hamster ovary cells [13;31-34]. Post-translational processing of the precursor occurs in three proteolytic steps leading to a mature α- and β-subunit with a size of 4 kDa and 46 kDa, respectively (Figure 3) [35]. It was suggested that a primary Psd1 translation product of 57 kDa is converted to a first intermediate of 52 kDa upon mitochondrial import by cleavage of the N-terminal targeting sequence. Removal of a 2 kDa fragment representing the intermembrane space sorting signal leads to a 50 kDa intermediate which is further processed through an autocatalytic step at the highly conserved LGST motif to the mature α- and β-subunit [35;36]. Autocatalytic processing at the LGST motif leading to a functional enzyme was investigated for the bacterial Psd starting with an ester bond formation between glycine and serine.

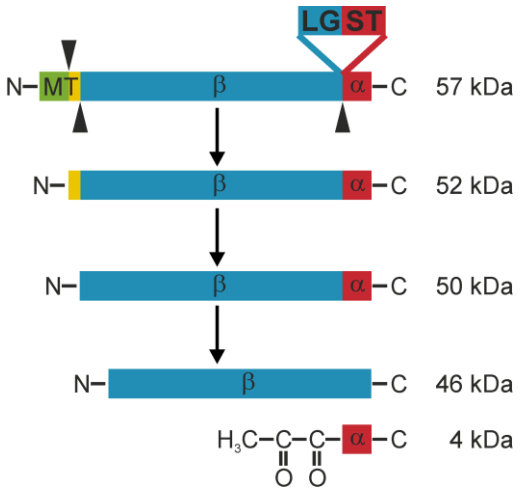

Figure 3. Post-translational processing of Psd1 from the yeast *Saccharomyces cerevisiae*. α, alpha-subunit; β, beta-subunit; MT, mitochondrial targeting sequences.

After an α,β-elimination, the mature β-subunit is released and the dehydroalanine at the N-terminus of the α-subunit undergoes further processing steps. These reactions include hydration and elimination of ammonia which subsequently forms the N-terminal pyruvoyl prosthetic group of the α-subunit and becomes the active site of the enzyme [34]. The exact import route of the Psd1 precursor into mitochondria, the nature of the three processing forms and the topology of the α- and β-subunits within mitochondrial compartments were not known. To address these questions, we performed a detailed biochemical study presented in Chapter 3.

Psd1 like the vast majority of mitochondrial proteins is encoded by nuclear genes, translated on cytosolic ribosomes and imported into mitochondria [35-37]. Coordinated protein trafficking and translocation are essential processes for mitochondrial biogenesis. The presence of four mitochondrial subcompartments, the outer membrane, intermembrane space, inner membrane and matrix require the assistance of versatile translocase machineries that operate in a collaborative manner to ensure correct protein import to its final destination. The environment for the assembly of translocation machineries is provided by phospholipids which are either synthesized within mitochondria like CL and PE or formed and transported into the organelle from other cellular compartments [1;5]. The non-bilayer forming phospholipid CL plays an essential role in the biogenesis of outer membrane proteins by affecting the mobility of the TOM (translocase of the outer membrane) and SAM (sorting and assembly machinery) complexes thereby regulating the assembly of β-barrel and some α-helical proteins [38]. Not only the outer but also the inner mitochondrial membrane is affected by CL depletion resulting in a reduced membrane potential, decrease of protein import through the presequence pathway TIM23 (translocase of the inner membrane) and alterations of various inner membrane complexes such as ADP/ATP carrier (AAC) and respiratory chain supercomplexes [39;40]. The evidence that the structural integrity of the outer and inner mitochondrial membrane as well as the functionality of proteins is influenced by the lipid surrounding suggests a close and dynamic interplay between lipids and proteins. In line with these observations, we investigated in collaboration with the group of N. Pfanner (Freiburg, Germany) the influence of mitochondrial PE depletion on protein complexes of the outer (Chapter 4) and inner mitochondrial membrane (Chapter 5).

The submitochondrial compartment with the highest protein to phospholipid ratio is the inner mitochondrial membrane which harbours protein complexes responsible for protein import and respiratory function as well as enzymes involved in the biosynthesis of CL and PE [5;41]. The architecture of the inner mitochondrial membrane reveals two distinct regions: the inner boundary membrane and a large tubular structure termed cristae membranes, which are connected by narrow structures with a defined diameter and length called cristae junctions [42]. Recently, a protein complex named MINOS (mitochondrial inner membrane organizing system) was identified which maintains cristae junctions connecting the inner boundary and cristae membrane. MINOS is composed of six inner membrane proteins exposed to the intermembrane space with Fcj1 and Mio10 as core components surrounded by Aim5, Aim13, Aim37 and Mio27. Loss of Fcj1 or Mio10 leads to an altered morphology of the inner mitochondrial membrane with large stacks of cristae membrane sheets due to disruption of cristae junctions. Besides the role of maintaining the inner membrane architecture, MINOS interacts with outer mitochondrial protein complexes. Fcj1 facilitates the connection of the TOM complex with the mitochondrial intermembrane space assembly machinery (MIA) guiding proteins into the intermembrane space [42]. In Chapter 6 of this Thesis, we describe the role of MINOS in protein biogenesis of the outer mitochondrial membrane. Lipid profiles of various MINOS mutants ruled out possible protein defects as secondary consequences of an altered lipid status.

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Chapter 1

Lipids of Mitochondria

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Abstract

A unique organelle for studying membrane biochemistry is the mitochondrion whose functionality depends on a coordinated supply of proteins and lipids. Mitochondria are capable to synthesize several lipids autonomously including phosphatidylglycerol, cardiolipin and partially phosphatidylethanolamine, phosphatidic acid and CDP-diacylglycerol, whereas phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sterols and sphingolipids have to be imported. The mitochondrial lipid composition, biosynthesis, import and regulation of these processes will be main issues discussed in this review article. Furthermore, interactions of lipids and mitochondrial proteins are highly important for various mitochondrial processes. Malfunction or loss of enzymes involved in mitochondrial phospholipid biosynthesis lead to dysfunction of cell respiration, affect the assembly and stability of the mitochondrial protein import machinery and cause abnormal mitochondrial morphology or even lethality. These aspects as well as diseases related to defects in the formation of mitochondrial membranes will be described.

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Abbreviations: CL, cardiolipin; CS, contact sites; DMPE, dimethylphosphatidylethanolamine; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; MAM, mitochondria associated membrane; PA, phosphatidic acid; PAM, presequence translocase associated motor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SAM, sorting and assembly machinery; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

Introduction

Biological membranes are multifunctional cellular constituents not only protecting the cell from external sources but also assigning specific processes to certain compartments. The main building blocks of membranes are phospholipids which provide a matrix for embedding proteins, sphingolipids and sterols. Lipids are not randomly distributed among biological membranes. Instead, they are highly specific and characteristic for each organelle influencing their shape, structure and function [1]. A specialized organelle for studying membrane biochemistry is the mitochondrion with its complex structure containing two membranes, the outer (OMM) and the inner mitochondrial membrane (IMM). The strongly folded cristae give rise to two aqueous compartments, the intermembrane space (IMS) and the matrix. Mitochondria are partially autonomous organelles. They harbour their own DNA, RNA and protein synthesizing machinery, but only 1% of proteins are formed on ribosomes in the mitochondrial matrix whereas the bulk of mitochondrial proteins is encoded by nuclear genes, translated on cytosolic ribosomes and imported into mitochondria [2;3]. Mitochondria are also capable of synthesizing some lipids on their own, but depend at the same time on the transfer and assembly of certain lipids mainly formed in the endoplasmic reticulum (ER) [4;5]. The continuous supply and exchange of lipids is required for maintaining mitochondrial membrane integrity and overall cellular function.

In this review, we will summarize the present knowledge about lipids of mitochondrial membranes. Characteristic features of the mitochondrial lipid composition compared to other cellular membranes will be addressed. Furthermore, the precise topology and distribution of lipids in the OMM and IMM as well as asymmetrical arrangement within the bilayer are presented. Central subjects of this article are biosynthesis, regulation and transport of lipids to and between mitochondrial membranes. Finally, possible interactions and functions of mitochondrial lipids with respect to stabilization and assembly of mitochondrial protein complexes involved in respiration, energy production and protein import are discussed.

Lipid composition of mitochondria

Each subcellular compartment of eukaryotes has a distinct set of proteins and lipids with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) being the most abundant phospholipids found in membranes from yeast to mammalian cells [6] (Table 1-3). In contrast to these bulk phospholipids, enrichment of phosphatidylserine (PS), sterols and sphingolipids are specific for the plasma membrane and the Golgi apparatus which also contains higher amounts of phosphatidylinositol (PI) [7-9]. The similarity of the Golgi apparatus and the plasma membrane may be caused by membrane flux through the secretory transport system [10]. Lysosomes from rat liver have increased levels of sphingolipids [11;12] whereas chloroplasts, e. g. from cauliflower, display an elevated phosphatidylglycerol (PG) content [6].

Besides these subcellular compartments, mitochondria of different organisms share the following specific characteristics: (I) Phospholipid to protein and sterol to protein ratios are low compared to other subcellular fractions. (II) PC and PE are the major phospholipids which account for about 80% of total phospholipids. (III) Mitochondria have a high cardiolipin (CL) content in the range of 10-15%. (IV) Sterols and sphingolipids are only found at low amounts (Table 1-3) [6-9;11-15]. In different mammalian cells and tissues, the mitochondrial lipid composition is more or less identical (Table 1). Exceptions are mitochondria from heart, brain, kidney, adrenal cortex and spleen which additionally contain PC and PE plasmalogens in the range of 5-30% of total phospholipids [16-22]. Plasmalogens are a class of phospholipids carrying a vinyl ether bond in the *sn-1* and an ester bond in the *sn-2* position of the glycerol backbone. This structure leads to strong lipophilic properties and allows plasmalogens to form inverse hexagonal phase structures, thereby favouring membrane fusion events [23;24]. Depending on the tissue, plasmalogens are important for the function of transmembrane proteins and membrane-related intracellular and extracellular cholesterol transport [24-27]. Phosphatidylglycerol (PG), the precursor of CL, is present in mitochondria of microorganisms only at small amounts but found in plants, mouse fibroblasts and mammalian cells/tissues, in particular, in mitochondria from bovine heart up to 11% of total phospholipids [13;18].

Table 1. Lipid composition of subcellular fractions of rat liver

	Mitochondria	Endoplasmic reticulum	Lysosomes	Golgi	Plasma membrane
Phospholipid (mg/mg protein)	0.175	0.374	0.156	0.825	0.672
Sterols (mg/mg protein)	0.003	0.014	0.038	0.038	0.128
			% of total phospholipids		
Phosphatidylcholine	44	60	48	51	40
Phosphatidylethanolamine	34	23	17	21	24
Phosphatidylinositol	5	10	6	12	8
Phosphatidylserine	1	2	3	6	9
Cardiolipin	14	1	1	1	1
Phosphatidic acid	<1	1	1	<1	1
Sphingomyelin	1	3	24	8	17

Data from Daum and Vance [6]

Table 2. Lipid composition of subcellular fractions of plant cells

	Mitochondria (Cauliflower)	Microsomes (Castor bean)	Chloroplasts (Cauliflower)	Peroxisomes (Castor bean)	Tonoplast (Mung bean)	Plasma membrane (Mung bean)
Phospholipid (mg/mg protein)	0.356	0.930	0.046	0.488	1.545	0.983
Sterols (mg/mg protein)	0.016	0.008	-	0.002	0.420	0.460
			% of total phospholipids			
Phosphatidylcholine	44	47	55	54	47	47
Phosphatidylethanolamine	34	31	6	29	32	35
Phosphatidylinositol	7	14	14	10	11	5
Phosphatidylserine	n.d.	2	n.d.	2	5	8
Phosphatidylglycerol	4	4	23	3	5	5
Cardiolipin	11	2	2	2	n.d.	n.d.

n.d.: not detected
Data from Daum and Vance [6]

Table 3. Lipid composition of subcellular fractions of the yeast *Saccharomyces cerevisiae*

	Mitochondria	Endoplasmic reticulum	Vacuoles	Plasma membrane
Phospholipid (mg/mg protein)	0.09	0.22	0.51	0.23
Sterols (mg/mg protein)	0.01	0.05	0.05	0.40
Sphingolipids (mg/mg protein)*	0.02	0.14	0.20	0.27
		% of total phospholipids		
Phosphatidylcholine	40	45	47	17
Phosphatidylethanolamine	27	22	19	20
Phosphatidylinositol	15	11	18	18
Phosphatidylserine	3	8	4	34
Cardiolipin	13	1	2	<1
Phosphatidic acid	2	4	2	4

Unless otherwise indicated, data are from Zinser and Daum [9]
* Daum and Vance [6]

Mammalian cells and cell lines like Morris hepatoma, Zajdela hepatoma, Hepatoma from rat and mouse and Fibroblasts (mouse, BHK) contain sphingolipids in the range of 1-12% of total phospholipids, whereas mitochondria of microorganisms and plants are devoid of this lipid class [13]. The highest level of lysophospholipids is found in mitochondria of sheep liver [28].

Phospholipids are, however, not only divided into classes defined by their head group but also contain acyl chains differing in length and degree of saturation giving rise to a huge variety of phospholipid species. Analysis of the fatty acid composition of mitochondria from mammalian cells as well as plants revealed a preference for palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and arachidonic (C20:4) acid [13]. Increased levels of palmitoleic (C16:1) and α-linolenic acid (C18:3) were detected in flight muscle of blowfly [29] whereas mouse brain [30], rat skeletal muscle [31] and rat heart [32] additionally contained C20:6 fatty acids. In contrast to plants, which contain high levels of linoleic (C18:2) and arachidonic (20:4) acid, *Saccharomyces cerevisiae* does not synthesize marked amounts of polyunsaturated fatty acids, and the mitochondrial composition of fatty acids is restricted to palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0) and oleic acid (C18:1) [13;33]. In *Pichia pastoris* the mitochondrial fatty acid pattern comprises oleic (C18:1), linoleic (C18:2), α-linolenic (C18:3), palmitic (C16:0), palmitoleic (C16:1) and stearic acid (C18:0) [34].

Lipid distribution within mitochondrial membranes

Mitochondria are one of the most specialized organelles with two membrane systems dividing the organelle in four compartments: the OMM, the IMS, the IMM and the matrix which are characterized by marker proteins and specific enzymes [9;35]. Standard protocols of mitochondrial subfraction isolation combine a variety of methods including differential and density gradient centrifugation steps, osmotic treatment (mild hypotonic shock, swelling, shrinking), sonication and in some cases controlled lysis with detergents [9;35;36]. Purified OMM and IMM vesicles differ in their shape, structure and lipid composition creating a specific environment for optimal membrane/enzyme function.

The OMM forms a smooth lipid rich surface of mitochondria envelope leading to an increase in membrane fluidity (Table 4) [13;37;38]. In contrast, the IMM is highly folded with an elevated protein level and lower lipid content compared to the OMM (Table 4). In all cell types, PC and PE are the major phospholipid classes with PE enriched in the IMM of mammalian cells and plants similar to the anionic phospholipid CL which is enriched in the IMM of all organisms. PI is present at a larger amount in the OMM of mammalian cells and plants and increased in the IMM of yeast [9;13;39] (Table 4).

Table 4. Lipid composition of the outer (OMM) and inner (IMM) mitochondrial membrane

	Mammalian cells (Rat liver)		Plant cells (Cauliflower)		Yeast* (*Saccharomyces cerevisiae*)	
	OMM	IMM	OMM	IMM	OMM	IMM
Phospholipid (mg/mg protein)	0.45	0.20	0.63	0.41	0.91	0.15
Sterols (mg/mg protein)	0.04	<0.01	0.08	0.03	<0.01	0.03
	% of total phospholipids					
Phosphatidylcholine	54	40	47	42	46	38
Phosphatidylethanolamine	29	34	27	38	33	24
Phosphatidylinositol	13	5	23	5	10	16
Phosphatidylserine	2	3	-	-	1	4
Cardiolipin	<1	18	3	15	6	16
Phosphatidic acid	1	-	-	-	4	2

Unless otherwise indicated, data are from Daum and Vance [6]
* Zinser *et al.* [91]

Sterol levels found in the OMM and IMM vary among different species. Mammals and plants display a significant higher sterol content in the OMM than in the IMM, whereas the yeast *Saccharomyces cerevisiae* contains higher sterol concentrations in the IMM [9;13;39] (Table 4). Studies on the lipid composition of mitochondrial membranes of the methylotrophic yeast *Pichia pastoris* grown on different carbon sources revealed an even higher sterol to protein ratio in the IMM (0.08 mg/mg) and OMM (0.04 mg/mg) compared to *Saccharomyces cerevisiae* [34]. However, mitochondria contain significantly lower portions of sterols than other subcellular compartments.

Asymmetric arrangement of lipids in mitochondrial membranes

Phospholipids are not evenly distributed across the lipid bilayers of mitochondrial membranes but rather arranged asymmetrically (Figure 1). The transbilayer orientation of phospholipids is measured by (I) selective and limited degradation of phospholipids catalysed by phospholipases; (II) spezific chemical modification of phospholipids from the outside of membrane vesicles by non-penetrating reagents such as trinitrobenzene sulfonate, fluorodinitrobenzene, fluorescamine and adriamycin; (III) reaction with phospholipid specific antibodies; and (IV) selective exchange of surface-exposed phospholipids with lipids of exogenous donor vesicles facilitated by lipid transfer proteins [40-46]. The transversal distribution of phospholipids across the bilayer may also be influenced by the isolation method of the respective membrane.

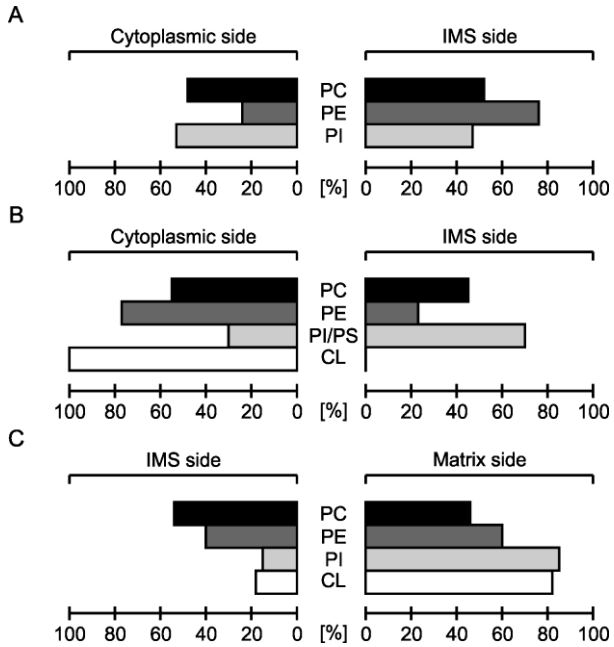

Figure 1. Asymmetric distribution of phospholipids in the outer and inner mitochondrial membrane. Transbilayer orientation of phospholipids in the outer mitochondrial membrane (OMM) of the yeast *Saccharomyces cerevisiae* (A) and rat liver (B) is indicated as % of individual phospholipid facing the cytosolic side or the intermembrane space (IMS), respectively. (C) Asymmetric distribution of phospholipids in the inner mitochondrial membrane from mammalian cells is expressed as % of individual phospholipids distributed between intermembrane space (IMS) and the matrix side. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin. Data from Sperka-Gottlieb *et al.* [38]; Hovius *et al.* [47]; Daum and Vance [6].

In the yeast *Saccharomyces cerevisiae*, the OMM shows an equal distribution of PC and PI at both sides of the bilayer, whereas 76% of PE are found on the inner aspect of the OMM facing the IMS [38]. In rat liver mitochondria, PC is almost equally distributed between both sides of the OMM bilayer. CL is exclusively exposed to the cytosolic surface whereas PI and PS (70%) are preferentially oriented towards the IMS. In contrast to yeast, mammalian OMM display an enrichment of PE up to 77% at the outer aspect of the bilayer facing the cytoplasm [47]. The presence and accumulation of anionic lipids at the surface of the OMM suggests tight interaction with positively charged mitochondrial protein presequences and protein import at initial steps of translocation. According to model systems using liposomes, mitochondrial precursor proteins are efficiently bound to membranes containing a high content of CL which is assumed to sequester proteins in a translocation-competent conformation [48-52]. The binding efficiency and affinity of

preproteins for negatively charged phospholipids was found to be in the order CL>PG>PI>PS inducing a partial unfolding of the precursor protein. Investigations on the asymmetric arrangement of phospholipids within the IMM from mammalian cells are divers and tissue specific [41;43-46]. In summary, CL and PI are predominantly exposed to the matrix side of the IMM whereas PC and PE are nearly equally distributed between the two leaflets of the membrane [41;43;44;46].

Contact sites between outer and inner mitochondrial membranes

In mammals and yeast, the OMM and IMM are in close contact to each other through junctions which were detected by electron microscopy [53-55]. These junctions between OMM and IMM were considered as possible sites of protein import as well as phospholipid translocation [56-58]. In yeast, mitochondrial membrane contact sites (CS) combine characteristics of OMM and IMM regarding phospholipid to protein and ergosterol to protein ratios (Table 5). In contrast to the OMM and IMM, CS display an increased level of non-bilayer forming lipids such as PE and CL promoting hexagonal phase structures and therefore stabilizing the local arrangement of membrane junctions. Further features of this isolated membrane fraction are decreased PC, PI, PS and PA levels compared to both mitochondrial membranes (Table 5) [57]. In mouse liver mitochondria, two populations of CS with different physicochemical properties were found. These properties depend on the isolation protocol which leads to enrichment of either OMM or IMM constituents [59].

Table 5. Lipid composition of mitochondrial membrane contact sites (CS)

	Yeast* (*Saccharomyces cerevisiae*)	Mammalian cells‡ (Mouse liver)	
	CS	CS/OMM	CS/IMM
Phospholipid (mg/mg protein)	0.37	0.37	0.36
Sterols (mg/mg protein)	0.02	0.019	0.001
	% of total phospholipids		
Phosphatidylcholine	31	26	26
Phosphatidylethanolamine	35	22	22
Phosphatidylinositol	5	8	8
Phosphatidylserine	<1	<1	<1
Cardiolipin	17	20	24
Phosphatidic acid	1	n.d.	n.d.

CS, contact sites; CS/OMM, outer mitochondrial membrane contact sites; CS/IMM, inner mitochondrial membrane contact sites; n.d., not detected. Data are from * Simbeni *et al.* [57], ‡ Ardail *et al.* [59].

The OMM-CS are characterized by a higher sterol to protein ratio and more saturated fatty acyl chain moieties compared to the IMM-CS. Similar to yeast, both populations of mammalian CS show an increase of CL up to 24% whereas PC with ~26% of total lipids is decreased in comparison to the OMM and IMM. Contrary to yeast, PE with a level of ~22% was found to be decreased in both fractions of mammalian CS. Anisotropy measurements of mitochondrial CS from mouse liver revealed higher fluidity of CS derived from the IMM which contains a decreased level of sterols compared to CS-OMM (Table 5) [59].

Recently, a mitochondrial complex termed MINOS, MitOS, or MICOS was identified [60-62]. This IMM associated complex is responsible for maintaining mitochondrial morphology by connecting the inner boundary membrane to the cristae membrane *via* cristae junctions. Mitofilin/Fcj1 is not only a central component of MINOS but also a functional interaction partner of the OMM protein import machineries [63]. Therefore, it was also suggested that MINOS is required for the formation of CS between OMM and IMM facilitating the transfer of proteins. Moreover, the mitochondrial phospholipid distribution was not altered in MINOS mutants displaying aberrant mitochondrial morphology [60;63].

Effects influencing the lipid composition of mitochondria

Cell development influences lipid species

The mitochondrial lipid composition is dynamic and responds to aging, growth state, nutritional supplements, cultivation conditions, temperature and mutations in lipid biosynthetic pathways [13]. During development, rat liver mitochondria display changes in the fatty acid pattern of PE and PC to a high degree of saturation, and the cholesterol to phospholipid ratio increases whereas the level of PE decreases [64;65]. In rat heart mitochondria, a significant age-dependent decrease of negatively charged phospholipids like CL, PA and PS with an increase of PC was observed [66]. Furthermore, higher amounts of palmitic (C16:0) and stearic (C18:0) acid were incorporated while polyunsaturated fatty acids decreased during ageing with the exception of arachidonic acid (C20:4) which was elevated under these growth conditions [66]. In contrast, mitochondria

of rabbit liver did not display any changes in the phospholipid pattern during development [67;68]. Transition from exponential growth to stationary phase causes an elevation of PC levels of yeast mitochondria at the expense of PE, whereas no significant changes in the concentration of other phospholipids were detected [69].

Nutrients affect mitochondrial lipid species

Not only ageing but also carbon sources and nutritional supplementation by choline, inositol and unsaturated fatty acids affect growth characteristics and mitochondrial lipid composition. In general, yeast cells grown on non-fermentable carbon sources have fully developed mitochondria. Such cells have a gradual increase of the phospholipid to protein ratio from exponential to stationary phase and display elevation of cellular PI and CL levels [69]. Mitochondria of cells grown on lactate or ethanol contain higher levels of CL, PC and PE whereas PI, DMPE and lyso-phospholipids are decreased compared to cells on glucose [70]. The ergosterol level in mitochondria is not significantly changed in the presence of different carbon sources. The ratio of unsaturated to saturated fatty acids, however, is higher in cells grown under non-fermentable growth conditions especially by increased incorporation of oleic acid (C18:1) at the expense of palmitic acid (C16:0) [70]. Similar effects were observed with the methylotrophic yeast *Pichia pastoris* [34]. Higher CL levels were found in the IMM of *Pichia pastoris* grown on oleic acid or methanol accompanied by a strong proliferation of mitochondria. Under these conditions proliferation of peroxisomes is also induced. Since both subcellular compartments contain enzymes of the glyoxylate cycle and rely on the exchange of metabolites, a functional connection of peroxisomal and mitochondrial machineries was suggested [34]. In *Pichia pastoris*, the incorporation of unsaturated fatty acids into mitochondrial phospholipids is elevated using either sorbitol or oleic acid (C18:1) as carbon sources. Mitochondria of cells grown on methanol display high levels of linoleic acid (C18:2) at the expense of oleic acid (C18:1) [34].

Rats fed with a choline deficient diet exhibit structural and compositional changes of hepatocytes by accumulation of triacylglycerols and altered phospholipid distribution. In the OMM, an increased PE level at the expense of PC is observed while the overall phospholipid content is not changed [71]. Furthermore, mammalian lipids can be

controlled and modified in a reversible manner by dietary fatty acid balance. Such manipulations influence the fatty acid incorporation in mitochondrial lipids as well as the lipid distribution. Compared to soya-bean oil diet, high-erucic acid rapeseed oil causes an increase of mitochondrial PC and TAG, whereas PE levels are decreased. Low-erucic acid rapeseed oil results in elevation of CL whereas the total phospholipid content is not affected in rat heart [72;73]. Phospholipid species are also subject to changes in dietary fatty acid balance. Soya-bean or rapeseed based diet, respectively affect the fatty acid composition of the major mitochondrial lipids in a reversible way by incorporating of either polyunsaturated or monounsaturated fatty acids [74]. The fatty acid turnover of CL appears to be slower than that of PC and PE, which might be due to a longer half-life of fatty acids or the asymmetric distribution of CL within the IMM [74]. Furthermore, adaption of the fatty acid composition of intracellular membranes in rats depends on the tissue. As an example, rat brain mitochondria are most resistant to dietary alterations and maintain the fatty acid composition more efficiently than mitochondria from liver or heart [75].

Temperature as a simple parameter to change lipid species composition – cold adaptation

Besides growth state, culture conditions and nutritional supplementation, mitochondrial membranes strongly respond to temperature changes by altering the degree of fatty acid saturation and distribution of phospholipids. Most investigations were carried out by shifting organisms from temperatures between 25°C and 37°C to lower temperatures ranging from 4°C to 10°C. In rat liver mitochondria, cold adaptation causes an increase of PE levels and unsaturated fatty acids such as arachidonic acid (C20:4), whereas PC and linoleic acids (C18:2) decrease [76]. This alteration of the membrane composition leads to an increase of mitochondrial respiratory activities by modulating the adenine nucleotide translocase. Decreased cholesterol to protein ratios and content of sphingomyelin were observed in mitochondrial fractions of rat epididymal adipocytes of cold-acclimated rats compared to controls [77].

However, differences in the degree of fatty acid saturation are not only due to temperature adaption but also species dependent [78]. Large amounts of arachidonic acid (C20:4) and

docosahexaenoic acid (C22:6) are found in mitochondrial lipids from rat brown adipose tissue whereas in mitochondrial lipids from hamster oleic acid (C18:1) is increased and more saturated fatty acids are incorporated in a reversible manner [78;79]. Lipid modulation through cold adaption also affects mitochondrial morphology by increasing the number of cristae and expending the IMM, which is attributed to a decrease of the PC to PE ratio and an overall increase of the mitochondrial phospholipid content found in rat brown adipose tissue [80;81].

Similar effects of temperature acclimation on sterol and phospholipid distribution in mitochondrial membranes were observed with *Neurospora crassa* [82]. This microorganism incorporates fatty acids with a higher degree of unsaturation upon lowering the growth temperature from 37°C to 15°C. Mitochondrial membranes display a higher free sterol to phospholipid ratio at low temperature (15°C), whereas the cellular content of free sterols is decreased under this condition. At high growth temperature (37°C), free sterols comprise about 67% of total cellular sterols. Adaption from high to low growth temperatures (15°C) increases the conversion of free sterols to sterol esters leading to an equilibrium of both lipid species. Cells grown at 37°C results in elevated mitochondrial level of PC and CL at the expense of PE compared to cells grown at 15°C [82]. Also in *Saccharomyces cerevisiae* the degree of membrane lipid unsaturation increases when cells are shifted from higher to lower temperature thus permitting adaptation of membrane fluidity [83-85]. Studies with psychrophilic (-2 to 20°C), mesophilic (5 to 35°C), thermotolerant (8 to 42°C) and thermophilic (25 to 45°C) yeast strains revealed a direct correlation between growth temperature and degree of lipid saturation [83]. In general, higher temperature leads to a larger degree of fatty acid saturation. Thermophilic yeast strains contain 30-40% saturated fatty acids compared to the psychrophilic and mesophilic yeast with less than 10% and 10-30% saturated fatty acids, respectively. Highly unsaturated fatty acids found in psychrophilic yeast allow membranes to remain in a sufficient fluid state which is important for various functional metabolic processes. Psychrophilic yeast strains contain increased amounts of PE whereas thermophilic strains were found to be enriched in CL with the exception of *C. slooffii*. This respiratory deficient thermophilic strain is characterized by the absence of CL which is compensated by elevated levels of PI and PS [83].

Lipid biosynthesis and the mitochondrial contribution to lipid homeostasis

Mitochondria harbour a certain set of enzymes involved in lipid biosynthesis and degradation [86-88]. The capacity of mitochondria to synthesize their own lipids is limited to PE, PA, PG and CL. Therefore, the import of extra-mitochondrially formed lipids such as PI, PS and PC is very important. The major sites of lipid biosynthesis are the ER and its specific subfraction, the mitochondria associated membrane (MAM) [89-91]. Several mechanisms describe the import of lipids from their site of synthesis into mitochondria. These mechanisms include vesicular transport, protein-mediated translocation, direct transfer *via* membrane contact sites and membrane tethering complexes [6]. Localization of lipid synthesis to specific organelles has been studied by using highly purified subcellular fractions [9;89;91]. In the following chapter lipid biosynthesis in mammalian, plant and yeast cells with emphasis on the contribution of mitochondria will be discussed.

Biosynthesis of phosphatidylethanolamine

In the yeast *Saccharomyces cerevisiae*, several subcellular compartments including mitochondria, the Golgi/vacuolar compartment, the ER with its specialized subfraction, the MAM, and lipid droplets contribute to PE biosynthesis. The major route of PE formation in the yeast is accomplished by the mitochondrial phosphatidylserine decarboxylase 1 (Psd1) (Figure 2) [92-94] . The *PSD1* gene encodes a 57 kDa precursor protein which is synthesized on cytosolic ribosomes and imported into mitochondria [92;95]. Post-translation processing of the Psd1 precursor occurs in three proteolytic steps comprising the action of the mitochondrial proteases MPP and Oct1, and autocatalysis at the highly conserved LGST motif. These processing steps lead to maturation of the enzyme consisting of a beta- and an alpha subunit localized to the IMM and the IMS, respectively [96;97]. Autocatalytic cleavage of the enzyme in alpha- and beta-subunits has been described for several Psd1 homologues from various species [94;98-102]. Correct localization of Psd1 to the IMM and IMS interface is crucial for developing full enzymatic activity [96].

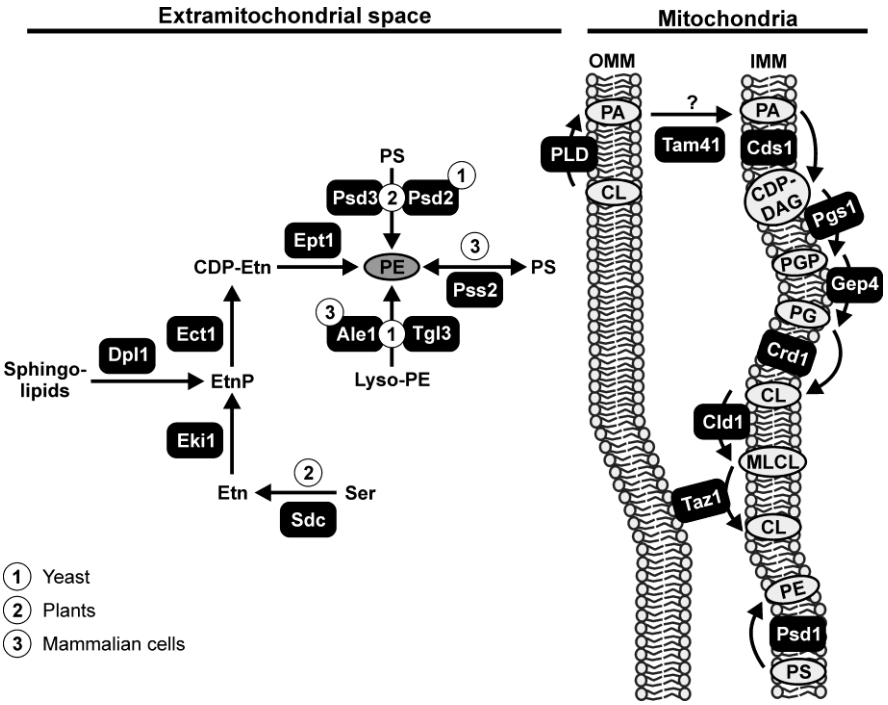

Figure 2. Phosphatidylethanolamine (PE) and cardiolipin (CL) biosynthesis in yeast, mammalian cells and plants. Biosynthetic pathway restricted to yeast (1), plants (2) and mammalian cells (3). Pathways without indications are found in yeast, mammalian cells and plants. Ale1, acyltransferase for lyso-PE; CDP-DAG, CDP-diacylglycerol; Cds1, CDP-diacylglycerol synthase; Cld1, cardiolipin-specific deacylase; Crd1, cardiolipin synthase; Dpl1, dihydrosphingosine phosphate lyase; Ect1, ethanolaminephosphate cytidylyltransferase; Eki1, ethanolamine kinase; Ept1, *sn*-1,2-diacylglycerol ethanolaminephosphotransferase; Etn, ethanolamine; EtnP, ethanolaminephosphate; Gep4, genetic interactors of prohibitins; IMM, inner mitochondrial membrane; MLCL, monolysocardiolipin; OMM, outer mitochondrial membrane; PA, phosphatidic acid; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; Pgs1, phosphatidylglycerolphosphate synthase; PLD, phospholipase D; PS, phosphatidylserine; Psd, phosphatidylserine decarboxylase; Pss2, phosphatidylserine synthase-2; Sdc, serine decarboxylase; Ser, serine; Tam41, translocator assembly and maintenance protein 41; Taz1, taffazin; Tgl3, triacylglycerol lipase.

In the yeast, Psd1 shares 19% sequence identity with Psd2 from the Golgi/vacuolar compartment [103;104]. Similar to Psd1, Psd2 contains a GGST motif and is predicted to undergo post-translational processing leading to the formation of a pyruvoyl prosthetic group at the amino terminus of the alpha subunit [103]. Besides the putative Golgi localization/retention sequence, Psd2 contains a C2 domain which was first described for the protein kinase C family as a sequence responsible for Ca^{2+} binding and regulation of enzyme activity. In Psd2, the role of the C2 domain remains ambiguous. Processing as well as targeting of the enzyme to the correct organelle might be possible functions of this sequence [104]. Since only 4-12% of the total cellular PS decarboxylase activity found in yeast wild type cells were derived from Psd2, we assigned Psd1 as the major player in PE biosynthesis [104]. Deletion of both decarboxylases causes auxotrophy for ethanolamine

which is incorporated and converted to PE *via* the CDP-ethanolamine pathway described by Kennedy and Weiss [105;106]. The initial step of the CDP-ethanolamine pathway is catalyzed by ethanolamine kinase (Eki1) using ATP to generate ethanolaminephosphate [107]. Eki1 and the choline kinase Cki1 have overlapping substrate specificities for choline and ethanolamine [107;108]. Ethanolaminephosphate cytidylyltransferase Ect1 activates ethanolaminephosphate with CTP leading to CDP-ethanolamine [109]. The activated ethanolaminephosphate moiety is transferred to DAG by ethanolaminephosphotransferase (Ept1) forming PE [110-112]. Ept1 has a broad substrate specificity utilizing CDP-ethanolamine, CDP-monomethylethanolamine, CDP-dimethylethanolamine and CDP-choline [111;112]. Furthermore, the CDP-ethanolamine pathway is linked to sphingolipid catabolism through the enzyme dihydrosphingosine phosphate lyase Dpl1 which catalyzes the formation of long chain aldehyde and ethanolaminephosphate from phosphorylated sphingoid bases (Figure 2) [113;114]. Finally, re-acylation of lyso-PE by Ale1 and Tgl3 contributes to PE biosynthesis in yeast [115-117]. Ale1 is an acyl-CoA dependent enzyme and localized to the MAM of the ER whereas Tgl3 is mainly localized to lipid droplets [115-117]. Tgl3 is one of the three TAG lipases found in yeast so far and catalyzes triacylglycerol mobilization. Besides a GXSXG lipase motif, Tgl3 like Tgl5 and Tgl4 contain an additional acyltransferase HXXXXD motif. It was shown that Tgl3 and Tgl5 can also act as acyltransferases converting lyso-PE or lyso-PA to PE and PA, respectively. The involvement of Tgl3 in sporulation of yeast cells has been attributed to the acyltransferase activity of the enzyme [115].

The two main biosynthetic routes of PE formation in mammalian cells are the PS decarboxylation and the CDP-ethanolamine pathways (Figure 2) [106;118]. Similar to yeast, mammalian phosphatidylserine decarboxylase (PSD) is exposed to the outer aspect of the IMM and undergoes posttranslational processing during recognition and translocation into mitochondria [92;94;96;119;120]. In contrast to yeast, only a single PSD activity is present in mammalian cells and responsible for *de novo* PE biosynthesis [88]. The mature PSD enzyme, sharing 44% identity with Psd1 from yeast, is derived from a 46 kDa precursor protein which gives rise to the two-non identical alpha- and beta- subunits with a size of 4 kDa and 34 kDa, respectively [92;119]. The preference for either decarboxylation or CDP-ethanolamine pathway depends on the tissue and mammalian species. In Chinese hamster ovary cells, more than 80% of PE is derived from *de novo*

synthesis by PSD [121-123], whereas in rat liver the CDP-ethanolamine pathway is predominant [124-126]. Enzymatic steps of the CDP-ethanolamine branch of the Kennedy pathway are similar to yeast (see above) (Figure 2) [127]. In mammals two genes were identified which encode ethanolamine kinase 1 and 2. Both kinases phosphorylate ethanolamine, but ethanolamine kinase 1 can also use choline as a substrate [127;128]. After phosphorylation, ethanolamine phosphate which is also synthesized *via* sphingosine-1-phosphate lyase [129;130] is activated by CTP:phosphoethanolamine cytidylyltransferase (Pcyt2) [124;125;131-133]. Under most metabolic conditions this enzyme catalyzes the rate-limiting step of PE synthesis. Overexpression of the mammalian ethanolamine specific kinases accelerates the CDP-ethanolamine pathway [127]. The final step of PE biosynthesis is catalysed by the CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) present in the ER [134-137]. A dual substrate specificity of EPT for CDP-ethanolamine and CDP-choline has been described [135], whereas the human EPT (hEPT1) is specific for CDP-ethanolamine [137]. The CDP-ethanolamine pathway and decarboxylation of PS contribute differently to PE species formation. PSD preferentially synthesizes PE with polyunsaturated fatty acids, whereas the CDP-ethanolamine pathway mainly forms PE with mono- or di-unsaturated fatty acids in the *sn-2* position [138]. Furthermore, PE species newly synthesized through the Kennedy pathway rapidly equilibrate with PE pools of the ER and mitochondria. In contrast, PE formed by PSD seems to be largely retained in mitochondria at its site of synthesis [138]. Finally, mammalian cells are able to form PE through a Ca^{2+} dependent base-exchange reaction catalyzed by PS synthase-2 in the ER or the lyso-PE re-acylation pathway. Both pathways are considered to be of minor importance compared to other PE biosynthetic pathways in mammalian cells (Figure 2) [139;140].

In plants, the ethanolamine moiety of PE directly derives from free serine through decarboxylation. This reaction is catalyzed by a pyridoxal 5'-phosphate-dependent l-serine decarboxylase (Sdc) found in soluble extracts from spinach leaves, *Arabidopsis* and rapeseed [141]. Ethanolamine is further incorporated into the CDP-ethanolamine pathway which is the major route for PE biosynthesis in plants [142;143]. The CDP-ethanolamine pathway from plants is similar to yeast and mammalian systems comprising the sequential action of ethanolamine kinase (EAK) [144], CTP-ethanolaminephosphate cytidylyltransferase (ECT) localized to the external leaflet of the OMM and ER [145], and

amino-alcohol phosphotransferase (AAPT1) which has similar substrate binding affinities to CDP-ethanolamine and CDP-choline in soybean [146]. Unlike yeast, *Arabidopsis* harbours three PS decarboxylases, with one enzyme being located to mitochondria (PSD1) and two in the endomembrane system (PSD2, PSD3) [142]. A triple mutant *psd1Δpsd2Δpsd3Δ* does not exhibit major changes in the phospholipid composition of whole leaves, although the mitochondrial PE content was decreased. Thus, the alternative pathways of PE synthesis seem to be predominant [142]. The mitochondrial Psd1 from tomato (LePSD1) is homologous to the mitochondrial PSDs from yeast and Chinese hamster ovary cells with approximately 37% identity to both enzymes [102]. The 50 kDa precursor protein LePSD1 from tomato contains the typical features of PS decarboxylases comprising a mitochondrial targeting peptide followed by an IMM sorting sequence and the characteristic GST motif close to the carboxyl-terminus. Posttranslational processing leads to formation of alpha and beta-subunits with a size of 5 kDa and 35 kDa, respectively, confirming similarities to the mammalian and yeast enzyme counterparts [102].

Biosynthesis of phosphatidylglycerol and cardiolipin

CL is a unique phospholipid with a dimeric structure containing four acyl chains and two phosphatidyl moieties linked to glycerol [147]. The fatty acid pattern in CL from most mammalian tissues and higher plants is restricted to C18 chains, whereas in yeast CL with equal amounts of oleoyl (C18:1) and palmitoleoyl (C16:1) residues are found [148]. In the presence of divalent cations, at low pH and high ionic strength CL favours to from inverted hexagonal instead of lamellar structures. The local formation of these non-bilayer structures within biological membranes facilitates membrane processes including assembly, transbilayer movement of proteins and formation of membrane contact sites [149-152]. CL is synthesized on the matrix side of the IMM and acts as a specific marker for the development of mitochondrial membranes [153-155]. The cellular amounts of CL differ under various growth conditions.

In the yeast, the highest level of CL is reached by growth on non-fermentable carbon sources in the stationary phase when mitochondria are fully developed [155]. The biosynthesis of CL starts with the formation of CDP-diacylglycerol from PA by Cds1

(CDP-diacylglycerol synthase) which is localized to the ER and has also been detected in yeast mitochondria (Figure 2) [87;156]. Studies by Kutik *et al.* [157] showed that the translocator assembly and maintenance protein 41 (Tam41) is required at an early stage of CL biosynthesis. Deletion of *TAM41* causes accumulation of PA but displays normal levels of CL synthesizing enzymes Pgs1 and Crd1. Phosphatidylglycerolphosphate synthase (Pgs1) catalyses the rate-limiting step of CL biosynthesis by forming phosphatidylglycerolphosphate (PGP) from CDP-diacylglycerol and *sn*-glycerol 3-phosphate (G3P) [158]. Deletion of the *PGS1* gene and consequent depletion of PG and CL are not lethal for the cell but seriously compromise mitochondrial function by causing temperature sensitivity, growth dependence on fermentable carbon sources and petite lethal phenotype [158]. Gep4, a phosphatase localized to the mitochondrial matrix and peripherally attached to the IMM, dephosphorylates PGP to phosphatidylglycerol (PG) [159]. Loss of *GEP4* leads to PGP accumulation, destabilization of the respiratory chain supercomplexes, impaired cell growth at elevated temperature and an inability to grow in the presence of ethidium bromide which is likely caused by cell wall defects [159]. The product of Gep4, PG, is either converted to CL by reacting with CDP-diacylglycerol under the control of cardiolipin synthase (Crd1) localized to the IMM or degraded to diacylglycerol and glycerophosphate by Pgc1, a C-type phospholipase dually localized to lipid droplets and mitochondria with the *in vitro* activity restricted to the latter compartment [160-163]. Lack of CL in a *crd1Δ* mutant caused an increase of the PG level up to 13% when yeast cells were grown on non-fermentable carbon sources, whereas cells grown on glucose did not contain detectable amounts of PG. Lack of CL is compensated by elevated amounts of PE and PA, whereas PC levels are decreased in mitochondria [161]. CL is not essential for growth of yeast cells on fermentable or non-fermentable carbon sources [162]. Similar to *crd1Δ*, deletion of *PGC1* does not affect cell growth on various carbon sources and at different temperatures, but leads to PG accumulation which is most pronounced in the absence of inositol. Thus, Pgc1 was regarded as a novel regulator of CL biosynthesis by removing excess of PG [163].

CL synthesis is completed by successive remodelling of the acyl chain arrangement catalyzed by the phospholipase Cld1 and the transacylase Taz1 leading to CL species with four unsaturated acyl chains [164;165]. The cardiolipin-specific deacylase 1 (Cld1) is exclusively localized to mitochondria and shows distant homology to the mammalian

protein CGI-58, an activator of the mammalian triacylglycerol lipase ATGL [166]. With its phospholipase A activity, Cld1 preferentially catalyses the removal of palmitic acid (C16:0) from premature CL leading to formation of monolysocardiolipin (MLCL) [164]. Deletion of *CLD1* in the yeast does not impair mitochondrial function and cellular growth, but specifically affects the fatty acid composition of CL leading to an increase of palmitic (C16:0) acid residues and a decrease of palmitoleic (C16:1) as well as oleic (C18:1) acid moieties [164]. The last step of CL maturation is accomplished by tafazzin 1 (Taz1), an acyltransferase localized to the OMM facing the IMS and responsible for re-acylation of MLCL and lyso-PC with unsaturated fatty acids [167;168]. Transport of Taz1 into mitochondria requires the receptor Tom5 of the translocase of the outer membrane (TOM complex), the small Tim proteins of the IMS, but does not depend on the sorting and assembly complex (SAM) [165;168;169]. In yeast, deletion of *TAZ1* leads to growth defects on non-fermentable carbon sources, increase of MLCL, PE and PS whereas unsaturated fatty acyl CL species are decreased [165]. Additionally, in the presence of lactate a *taz1Δ* mutant synthesizes 2-fold more triacylglycerols compared to wild-type [167]. In humans, a severe genetic disorder, the Barth syndrome, is associated with impaired CL acyl-chain remodelling through mutations in the *TAZ* gene encoding Tafazzin homologues to the yeast counterpart Taz1 [170;171]. The Barth syndrome is an X-linked disease causing an acute decrease in tetralinoleoyl (C18:2) cardiolipin which is regarded as the major molecular CL species found in the human heart [172]. Barth syndrome patients suffer from cyclic neutropenia, skeletal and cardiac myopathies. Main causes of death are heart failure or opportunistic infections.

Similar to yeast, biosynthesis of CL in mammalian cells is entirely confined to the mitochondria (Figure 2) [173]. It starts with the acylation of glycerol 3-phosphate in the OMM and formation of CDP-diacylglycerol by CDP-diacylglycerol synthase which is dually localized to the IMM and microsomal fractions with 90%-95% of the activity restricted to the latter compartment [88;153;174;175]. The subsequent steps are localized to the IMM and comprise formation of the intermediates PGP and PG leading to CL which undergoes a re-acylation cycle to result in specific acyl composition [173]. The initial step of PG synthesis depends on the fatty acid composition of CDP-diacylglycerol [176]. In general, CDP-diacylglycerols with unsaturated or short-chain fatty acids such as dioleoyl and dilinoleoyl or dilauroyl and dimyristoyl, respectively, are preferred substrates and

result in more efficient PG synthesis [176]. Mammalian cardiolipin synthase is an integral protein of the IMM exposed to the matrix site. The activity of the enzyme depends on the presence of divalent cations and the lipid environment with PE and CL as stimulating components, whereas lysophospholipids display inhibitor effects [153;176]. Formation of the specific tetralinoleoyl-CL in rat liver mitochondria requires the aid of a specific phospholipid acyltransferase which directly transfers linoleoyl groups from PE and PC to premature CL [177].

In plants, CL biosynthesis is similar to mammalian cells and yeast [13;178;179]. The two specific enzymatic reactions for PG formation in plants comprising PGP synthase and PGP phosphatase are associated with the IMM, the ER and the chloroplast, whereas CL synthase (CLS) is localized to mitochondria (Figure 2) [179;180]. CL synthases from various plant mitochondria have an absolute dependence on divalent cations [181]. CL synthase from mung bean mitochondria exhibits an alkaline pH optimum of 9 like the yeast and mammalian counterpart and maximal enzymatic activity in the presence of Mn^{2+} cations [178]. Unlike the enzyme of mung beans, other eukaryotes depend on the presence of either Co^{2+} or Mg^{2+}.

Biosynthesis of phosphatidylcholine

Phosphatidylcholine (PC) is the most abundant phospholipid of mitochondrial membranes comprising ~40% of total phospholipids [13]. Mitochondria are not able to synthesize PC and depend on its import. The three pathways of PC synthesis are: (i) sequential methylation of PE, (ii) phosphorylation of choline and transfer to a diacylglycerol acceptor (CDP-choline pathway), and (iii) base exchange. The S-adenosylmethionine dependent methylation pathway of PC synthesis in yeast and mammals comprises three steps of conversion using PE as a substrate (Figure 3). In yeast, two methyltransferases Pem1 and Pem2 were identified and localized to the ER [87;182]. Pem1 catalyzes the first step of PC synthesis leading to the formation of phosphatidylmonomethylethanolamine (PMME) whereas Pem2 exhibits a broader specificity catalyzing all steps with preference for the second and third methylation [182]. Furthermore, both enzymes especially Pem1 have a preference for converting di-C16:1 PE to PC. The specific methylation of PE species shows that methyltransferases play a crucial role in shaping the steady state profile of PC

molecular species in yeast [183]. PE, the substrate for the methylation pathway is mainly derived from Psd2, whereas PE formed by Psd1 and the CDP-ethanolamine pathway is preferentially incorporated into membranes to maintain PE homeostasis. Deletion of *PSD2* causes limitations in the substrate availability for de novo PC synthesis thereby enhancing the CDP-choline pathway [184]. Disruption of the methylation pathway leads to choline auxotrophy of yeast cells, whereas deletion of either *PEM1* or *PEM2* decreases the PC level although not affecting cell viability [182].

In mammalian cells, one *PEMT* gene encodes two forms of phosphatidylethanolamine N-methyltransferases. Pemt from rat liver is able to catalyse all three methylation steps [185;186]. The highest activity is assigned to the ER harbouring Pemt1, whereas Pemt2 is localized to the MAM. The activity of the methyltransferases is regulated through availability of the substrates, PE and S-adenosylmethionine, as well as through the product level of S-adenosylhomocysteine. Gene expression is influenced during development and by the supply of choline in the diet [187].

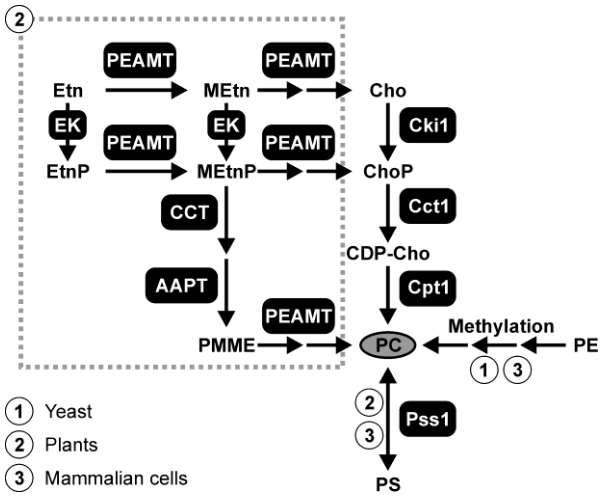

Figure 3. Phosphatidylcholine (PC) biosynthesis in yeast, mammalian cells and plants. Biosynthetic pathway restricted to yeast (1), plants (2) and mammalian cells (3). Pathways without indications are found in yeast, mammalian cells and plants. AAPT, amino-alcohol phosphotransferase; Cct1 (CCT), cholinephosphate cytidylyltransferase; Cho, choline; ChoP, cholinephosphate; Cki1, choline kinase; Cpt1, choline phosphotransferase; Etn, ethanolamine; EK, ethanolamine kinase; EtnP, ethanolaminephosphate; MEtn, monomethylethanolamine; MEtnP, monomethylethanolaminephosphate; PE, phosphatidylethanolamine; PEAMT, methyltransferase; PMME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine; Pss1, phosphatidylserine serine synthase-1.

Plants do not directly methylate PE. Instead, a branched biosynthetic route combining the methylation and CDP-choline pathway is used for PC formation (Figure 3) [188]. Plants are able to convert ethanolamine to choline at the phosphobase level which is missing in mammalians and yeast. The initial step is catalyzed by ethanolamine kinase (EK) leading to ethanolaminephosphate (EtnP) which is subsequently methylated to monomethylethanolaminephosphate (MEtnP) by the methyltransferase (PEAMT) [189-192]. Monomethylethanolaminephosphate can either be converted to cholinephosphate (ChoP) by further methylation steps or directly channelled into the Kennedy pathway leading to phosphatidylmonomethylethanolamine (PMME) through the action of CCT (choline phosphate cytidylyltransferase) and AAPT (amino-alcohol phosphotransferase) [146;193;194]. In a final step, PMME is methylated at the phosphatidylbase level to PC [189;190]. The classical Kennedy pathway in plants depends on the availability of choline and the action of choline kinase (CK), choline phosphate cytidylyltransferase (CCT) and amino-alcohol phosphotransferase (AAPT). In plants, choline kinase is specific and does not utilize ethanolamine as a substrate, whereas CCT displays a broader specificity accepting also ethanolaminephosphate derivatives as substrates [189;190;195]. CCT is considered as the rate-limiting step of the Kenney pathway [194;196;197]. CDP-choline and CDP-ethanolamine are substrates for AAPT with similar binding affinity for both substrates [146].

In yeast, choline kinase (Cki1) is responsible for the initial step to synthesize PC via the CDP-choline pathway (Figure 3). Unlike in plants, Cki1 has overlapping substrate specificity thereby also displaying ethanolamine kinase activity [108]. Cholinephosphate cytidylyltransferase (Cct1) [198] activates cholinephosphate to CDP-choline which reacts with diacylglycerol to form PC catalysed by Cpt1 (choline phosphotransferase). Cpt1 converts only CDP-choline, whereas Ept1 can utilize both CDP-choline and CDP-ethanolamine [110-112;199]. In yeast, PC synthesized either *via* the sequential methylation of PE or the CDP-choline pathway differs with respect to the acyl chain composition. To this end, the CDP-choline pathway contributes most to the molecular diversity of PC species [183;200]. In mammalian cells, PC is predominantly derived from the CDP-choline pathway and only in hepatocytes/liver the PE methylation pathway contributes with 30-40% to PC synthesis [125;201;202]. Similar to yeast, choline kinase of mammals is the initial enzyme of the pathway, exhibits also ethanolamine kinase activity and is encoded by

two distinct genes [203-206]. Like in plants and yeast, the rate of PC synthesis in mammals *via* the Kennedy pathway is regulated by the CTP:phosphocholine cytidylyltransferase [124;125;207]. The final step of PC synthesis comprises the action of the cholinephosphotransferase which is an integral membrane protein present in the ER [88].

Biosynthesis of phosphatidylserine

In yeast, phosphatidylserine synthase (Pss1) is highly abundant in MAM, a subfraction of the ER (Figure 4) [89]. The enzyme converts CDP-diacylglycerol and serine to PS [89;208]. Deletion of *PSS1* causes auxotrophy for choline and ethanolamine indicating that Pss1 is the only PS synthase and essential for yeast [209]. This pathway is absent in mammalian cells. Instead, PS is synthesized through a Ca^{2+} dependent base-exchange reaction using pre-existing phospholipids as substrates and replacing the head-group by L-serine [88]. Two PS synthases with different substrate specificities were found in mammalian cells, namely PS synthase-1 and PS synthase-2 [210]. PS synthase-1 exchanges serine with the choline head-group of PC, whereas PS synthase-2 uses preferentially PE as a substrate [211-215]. The overexpression of either PS synthase-1 or PS synthase-2 appears to affect the phospholipid metabolism differentially. Cells overexpressing PS synthase-1 show an inhibition of the CDP-ethanolamine pathway and an increased rate of PS synthesis, whereas overexpression of PS synthase-2 has no effects [216;217]. Similar to yeast, both mammalian PS synthases are enriched in the MAM and barely detectable in the bulk ER [90;218;219]. Plants display features of PS synthesis from yeast and mammalian cells by synthesizing PE either from CDP-diacylglycerol and serine, or by head-group exchange of an existing phospholipid with L-serine [220;221].

Biosynthesis of phosphatidylinositol

The importance of PI is documented by its role in cellular signalling, as precursor for the synthesis of GPI-anchored proteins and inositol-containing sphingolipids, and as a substrate for phosphoinositide synthesis [222;223]. The highest activity of PI synthase (Pis1) is assigned to the MAM fraction [89;224] (Figure 4). CDP-diacylglycerol and inositol are used as substrates for the formation of PI. The disruption of *PIS1* is lethal in

the yeast *Saccharomyces cerevisiae* [225]. In *Arabidopsis*, PI is synthesized through the action of AtPIS which requires myo-inositol and CDP-diacylglycerol as well as Mg^{2+} or Mn^{2+} ions for activity [226]. Furthermore, AtPIS is capable of performing head group exchange with either endogenous or exogenous PI molecular species [227]. Similar to plants and yeast, mammalian cells display PI synthase activity which is predominantly localized to the ER [228]. In non-photosynthetic plant tissues PI is the third most abundant phospholipid comprising 21% of total phospholipids after PC and PE [229].

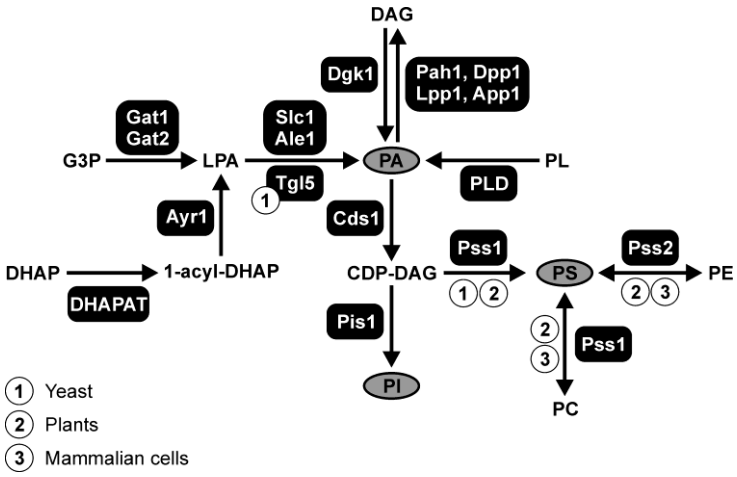

Figure 4. Phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) biosynthesis in yeast, mammalian cells and plants. Biosynthetic pathway restricted to yeast (1), plants (2) and mammalian cells (3). Pathways without indications are found in yeast, mammalian cells and plants. Ale1, acyltransferase for lyso-PE; App1, actin patch protein (phosphatidate phosphatase); Ayr1, 1-acyldihydroxyacetone-phosphate reductase; Cds1, CDP-diacylglycerol synthase; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; DHAPAT, dihydroxyacetone phosphate acyltransferase; Dgk1, diacylglycerol kinase; Dpp1, diacylglycerol pyrophosphate phosphatase; G3P, glycerol-3-phosphate; Gat1/2, glycerol-3-phosphate acyltransferase; LPA, lyso-phosphatidic acid; Lpp1, lipid phosphate phosphatase; Pah1, phosphatidic acid phosphohydrolase; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Pis1, phosphatidylinositol synthase; PL, phospholipids; PLD, phospholipase D; Pss, phosphatidylserine synthase; Slc1, 1-acylglycerol-3-phosphate acyltransferase; Tgl5, triacylglycerol lipase.

Biosynthesis of the intermediates phosphatidic acid and CDP-diacylglycerol

Phosphatidic acid (PA) is an important substrate for many enzymes involved in phospholipid and triacylglycerol metabolism [230]. In mammalian cells and yeast, two different acylation pathways lead to PA biosynthesis, namely the glycerol 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) pathway (Figure 4) [231-234]. In yeast, glycerol 3-phophate is acylated by Gat1 and Gat2 to yield lyso-PA which is further converted to PA with the aid of the 1-acylglycerol 3-phosphate acyltransferases Slc1 and Ale1. Ale1 was identified as a lyso-PE specific acyltransferase by studies of Riekhof *et al.* [116;117] whereas Jain *et al.* [235] found that Ale1 (or Lpt1) can utilize a variety of lyso-phospholipid species as substrate. Gat1 and Gat2 display overlapping substrate specificities for G3P and DHAP. The latter component is also a substrate of the dihydroxyacetone phosphate pathway comprising DHAP acyltransferase, 1-acyl DHAP reductase (Ayr1) and Slc1 as well as Ale1 [234-237]. Gat1, Gat2, Ayr1 and Slc1 were found in lipid droplets and microsomes whereas DHAP acyltransferase activity was located to mitochondria [238]. Deletion of *GAT1* and *GAT2* is lethal for the cell [239].

In plants, PA is synthesized from the precursor glycerol 3-phosphate which is acylated in three different compartments [240-242]. A soluble acylation system is present in chloroplasts and two membrane associated systems in mitochondria and the ER. In mammalian cells, two isoforms of glycerol 3-phosphate acyltransferases were found in the OMM and associated with the ER [243;244]. The mitochondrial enzyme preferentially utilizes saturated acyl-CoAs whereas the microsomal enzyme equally incorporates saturated and unsaturated species [243;244]. The second acylation step is catalysed by an enzyme primarily localized to the ER and using preferentially unsaturated fatty acyl-CoAs as substrates [88].

Besides *de novo* biosynthetic pathways, PA can also be derived from alternative routes including hydrolysis of phospholipids by phospholipase D and phosphorylation of diacylglycerol by DAG kinase [238]. Phospholipase D activities were characterized from yeast to mammals and play an important role in the regulation of cell functions [245]. PLDs share a conserved motif ($HXKX_4DX_6G^G/_S$) and belong to a gene superfamily which includes bacterial PLDs, phosphatidyltransferases/phospholipid synthases, endonucleases and viral envelope proteins [246-249]. Two PLDs were characterized in the yeast *Saccharomyces cerevisiae* displaying either Ca^{2+} dependence or independence [250-253].

The membrane-bound Pld2 found in mitochondria and plasma membrane is strictly dependent on Ca^{2+}, activated in the presence of non-fermentable carbon sources and preferentially hydrolyzing PS and PE. The Ca^{2+} independent Pld1/Spo14 is specific for PC and catalyses both phospholipid hydrolysis and transphosphatidylation. Pld1 is activated by phosphatidylinositol 4,5-biphosphate similar to PLDs from plants and mammalian cells. Deletion of *SPO14* results in a severe sporulation defect [246;251-255]. Besides hydrolysis of phospholipids, PA can also be derived from phosphorylation of diacylglycerol *via* diacylglycerol kinase 1 (Dgk1). Unlike bacteria, plants and animals, the yeast enzyme utilizes CTP instead of ATP as a co-substrate. It shows dependence on Ca^{2+} and Mg^{2+} and is strongly inhibited by *N*-ethylmaleimide [256]. Dgk1 counteracts with the *PAH1*-encoded PA phosphatase [257]. Overexpression of *DGK1* or deletion of *PAH1* results in PA-enriched membranes around the nucleus and causes an expansion without proliferation of the cortical ER membrane. Thus, PA metabolism is a critical factor to maintain nuclear membrane biogenesis and structure [256;257]. PA phosphatases (PAP) were recognized as important enzymes for balancing the synthesis of triacylglycerols and phospholipids at the branch point of PA dephosphorylation yielding diacylglycerol and inorganic phosphate [258]. PAP enzymes are classified with respect to their cofactor requirement for catalytic activity. Pah1 depends on Mg^{2+} whereas PAP2 enzymes like Lpp1 and Dpp1 are Mg^{2+} independent. In contrast to Pah1 specificity towards PA, PAP2 enzymes display a broad substrate tolerance including PA, diacylglycerol pyrophosphate, lyso-PA, sphingoid base phosphates and isoprenoid phosphates [259]. Most recently, another Mg^{2+} dependent PA phosphatase, the cytosolic protein App1, was identified which is sensitive to *N*-ethylmaleimide and associates with membranes [260]. In contrast to the function of Pah1 in the regulation of triacylglycerol and phospholipid synthesis, or PAP2 enzymes in signalling functions of PA, DAG pyrophosphate and lyso-PA in vacuole and Golgi membranes, the role of App1 in cell physiology has not been identified. It was speculated that App1 is involved in vesicle formation through its recruitment from the cytosol to cortical actin patches mediated by endocytic proteins [260].

In mammalian cells, lipin proteins play a dual function in lipid metabolism by acting as PAP enzymes and as transcriptional regulators [261]. Mutations in the *LIPIN 1* gene cause deficiency in adipogenesis, neonatal hypertriglyceridemia and hepatosteatosis, insulin resistance and increased susceptibility to artherosclerosis. The PAP activity of lipins

depends on Mg^{2+} or Mn^{2+}. Similar to yeast Pah1, lipin-1 possesses a highly conserved DXDXT catalytic motif at the C-terminal end of the protein which is termed C-LIP domain, present in most lipin homologs and responsible for PAP activity [261].

Another important branch point in phospholipid biosynthesis is the formation of CDP-diacylglycerol by CDP-diacylglycerol synthase (CDS) (Figure 4) [262-265]. CDS are integral membrane proteins located to mitochondria and microsomes of all eukaryotes [88;127;221]. In mitochondria, the enzyme appears to provide the substrate for the mitochondrial biosynthesis of PG and CL, whereas the microsomal activity serves to form the precursor for the synthesis of PI and its phosphorylated derivatives. In yeast, CDP-diacylglycerol is also utilized for the *de novo* synthesis of PS [88;156]. Besides microsomal and mitochondrial CDS proteins, plants harbour a plastidial CDS activity in the inner envelope membrane providing the substrate for the synthesis of PG in plastids [266]. Yeast possesses a single *CDS1* gene that encodes the total cellular CDS activity which is essential for cell viability and spore germination. In mammals two *CDS* genes have been identified. The microsomal activity predominates, whereas the mitochondrial enzyme accounts for only 5-10% of cellular CDS activity [127;156;174].

Minor lipid components of mitochondrial membranes – sterols and sphingolipids

Sterols and sphingolipids are minor constituents of mitochondrial membranes. Sterols synthesized *via* the cytoplasmic mevalonate pathway are important structural components of the plasma membrane and precursors of steroid hormones in vertebrates and plants [267;268]. Mammalian sterol biosynthesis is restricted to the ER and peroxisomes with cholesterol as final product, whereas in yeast sterol biosynthesis occurs in the ER and lipid droplets with ergosterol as the major sterol [269-274]. Plants produce a variety of phytosterols which display various function in plant development [267].

Sphingolipid synthesis occurs in several organelles including the ER, the Golgi apparatus and the plasma membrane [275]. Sphingolipids are key players in various cellular processes, serve as structural components of membranes and act as signalling molecules. Furthermore, sphingolipid metabolism is closely coordinated with other processes along the secretory pathway as well as sterol and phospholipid metabolism [276]. Ceramide, an intermediate in sphingolipid biosynthesis, is an important signalling molecule and

synthesized by a family of six ceramide synthases (CerS) which give rise to several ceramide species with distinct acyl chain lengths in mammalian cells [277]. These CerS are involved in other biological processes such as cancer and tumour suppression, responding to chemotherapeutic drugs, in apoptosis and in neurodegenerative diseases.

Import and assembly of lipids into mitochondria

Interorganelle transport of lipids

Mitochondria have the capacity to synthesize some of their own lipids, namely PE, PA, PG, CL and CDP-DAG [86-88]. Moreover, the permanent supply of other lipid classes to mitochondria is essential to maintain mitochondrial function and cell survival [6;13]. Several mechanisms have been suggested for lipid transport into mitochondria including: (i) membrane contact site formation between the ER and mitochondria with the aid of membrane complexes or through the MAM fraction; (ii) vesicular transport; or (iii) the occurrence of specific lipid binding and transfer proteins.

Phospholipids are able to migrate between membranes *via* contact sites between donor and acceptor membranes. This transfer across bridges between ER and mitochondria, the MAM-fraction, appears to be independent of vesicular traffic (Figure 5A) [278]. Contact sites between both organelles were identified by co-sedimentation of ER (microsomal) derived components with mitochondria and by electron microscopic observations with rat liver cells and DT40 cells [5;279-281]. Tomographic analysis of isolated rat-liver mitochondria revealed connections of OMM and smooth ER at a length of 9-16 nm. This space between OMM and rough ER in situ is ~20 nm, and the measured lengths of tethers ~19-30 nm. Association and tethering between the two organelles is important for cell function and survival [281]. In yeast, 80-110 of these contact sites between ER and mitochondria per cell were calculated [4]. It was demonstrated that these contact domains contain enzymes involved in phospholipid biosynthesis supporting the collaborative action in lipid production. It was assumed that membrane contact sites mediate lipid channelling through formation of a hydrophobic tunnel allowing translocation of lipids between organelles [89;90;282].

In yeast, entrance of PS from the site of synthesis to the site of conversion to PE in the IMM does not require energy [4;283], whereas in mammalian cells PS transport to the site of Psd1 is ATP dependent [284]. However, experiments with isolated donor (microsomes) and acceptor membranes (mitochondria) strongly suggested that only the final step of lipid transfer does not require ATP both in mammalian cells and yeast [285]. The major difference between the two experimental systems is that steps preceding the final lipid transfer to mitochondria require ATP [286]. In mammalian cells, PS is formed through a base exchange reaction where ethanolamine (Etn) and choline (Cho) are replaced by serine (Ser) [286]. Entrance of newly synthesized PS to MAM and further to mitochondria requires the aid of ATP [218].

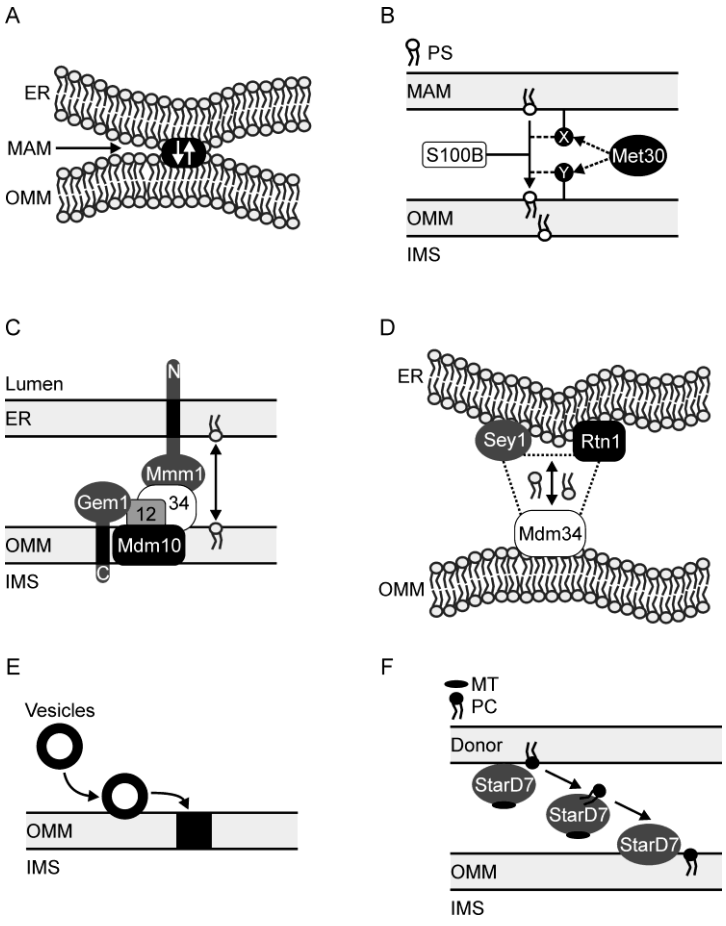

Figure 5. Interorganelle lipid transport. (A) Lipids migrate between membrane contact sites. (B) Phosphatidylserine transport is facilitated by Met30 and S100B. (C) ERMES tethers mitochondria to the ER and affects the transport of lipids. (D) Exchange of lipids between the ER and mitochondria is mediated by ER shaping proteins. (E) Vesicular transport of lipids *via* fusion of endosomes and lysosomes with mitochondria. (F) StarD7 specifically catalyzes PC transfer between the ER and mitochondria. ER, endoplasmic reticulum; ERMES, ER mitochondria encounter structure; IMS, intermembrane space; OMM, outer mitochondrial membrane; MAM, mitochondria associated membrane; PC, phosphatidylcholine.

Investigations of PS transport in permeabilized mammalian cells led to the identification of S100B (NP_006236) (Figure 5B) [287]. This protein affects lipid transport between ER/MAM and mitochondria. It was speculated that S100B is involved in stabilizing junctions between ER and mitochondria. In yeast, a screening of mutants compromised either in transport of PS to mitochondria or in export of PE from mitochondria to the ER identified a strain named *pstA1*. This mutant exhibits ethanolamine auxotrophy, a reduced mitochondrial PE level and much denser mitochondria than wild type cells caused by a reduced phospholipid:protein ratio [288]. Complementation experiments identified *MET30* encoding a protein subunit (Met30) of a multi-component E3 ubiquitin ligase to cause the above mentioned effects [289]. A substrate for this multi-component E3 ubiquitin ligase system is the transcription factor Met4 which was speculated to disturb PS transport from the ER to mitochondria [290-292]. Upon ubiquitination Met4 is inactivated and PS transfer between both organelles becomes functional again [288]. However, the mechanism how Met30 regulates PS transport between mitochondria and the ER is not known [278;290-292]. Investigations of PS transport in mammalian cells identified specific proteins, e. g. R41, which affect the lipid distribution between the OMM and IMM independently of the mitochondrial protein import/transport machinery (Figure 6E) [293].

Mammalian cells contain a number of proteins tethering ER and mitochondria, e. g. mitofusins (Mfn1, Mfn2), glucose-regulated protein 75 and PACS-2 (phosphofurin acidic cluster sorting protein 2) [294]. The OMM localized MFN2 belongs to the family of dynamin-like GTPases and mediates mitochondrial fusion and interaction with Miro (mitochondrial rho GTPase)/Milton complex [295;296]. Recent studies with the yeast identified a five-protein complex tethering the ER to the OMM. This complex which was termed ER-mitochondria encounter structure (ERMES) is composed of Mmm1, Mdm34, Mdm10, Mdm12 and Gem1 (Figure 5C) [297-299]. Mmm1 is an integral ER membrane protein with a glycosylated N-terminal domain exposed to the luminal side of the ER and a cytosolic C-terminal domain interacting with further ERMES subunits associated and integrated to the OMM [297;299;300]. The β-barrel protein Mdm10 is integrated with major parts into the OMM, whereas Gem1 has a C-terminal single transmembrane segment and is exposed with a large domain towards the cytosol [299]. The cytosolic protein Mdm12 and the putative OMM protein Mdm34 form a bridge between Mmm1 and Mdm10 in the complex. Since Mdm10 is present in both the sorting and assembly

machinery (SAM) and the ERMES complex, interaction of mitochondria and ER seems to provide a platform coordinating mitochondrial biogenesis and membrane dynamics [297;299;300]. Gem1 is found in substoichiometric amounts in the ERMES complex and displays rather a regulatory than a structural function for its assembly [298]. In addition, Mmm1, Mdm12 and Mdm34 contain an SMP (synaptotagmin-like, mitochondrial and lipid-binding proteins) domain involved in binding of hydrophobic ligands or lipids. This feature suggests a possible function of the ERMES complex as mediator of lipid transport/exchange [301]. In ERMES mutants mitochondria display an abnormal morphology with large spherical shapes or aggregation of small spheres accompanied by a decrease of mitochondrial CL [302-304]. Besides Gem1 which is a regulator of ERMES function in phospholipid exchange, overexpression of the IMM protein Mdm31 rescues growth defects of ERMES mutants, restores CL levels and the tubular mitochondrial morphology. Mdm31 genetically interacts with ERMES components but is not required for the formation of ER-mitochondria contact sites [298;304].

Mechanisms of tethering mitochondria to ER and thereby affecting the transport of lipids across this complex are still a matter of dispute. Studies of Nguyen *et al.* [305] argued that ERMES and its substoichiometric component Gem1 [298;299] are not involved in the transport of phospholipids from the ER to mitochondria. These authors demonstrated that neither PS transport from the ER to mitochondria nor conversion of PS to PE are affected by the absence of ERMES components. Incorporation of PC in ERMES mutants was not altered which is in contrast to results obtained by Kornmann *et al.* [297] who reported a moderate decrease in the aminoglycerophospholipid biosynthetic route from PS to PC. Nguyen *et al.* [305] further demonstrated that Gem1 does not regulate the assembly or maintenance of the ERMES complex. Moreover, it was suggested that the ERMES complex primarily fulfils a structural role maintaining mitochondrial morphology. Due to the lack of these components the mitochondrial phospholipid transport may be affected.

Studies of Voss *et al.* [306] showed that exchange of lipids between the ER and mitochondria is facilitated by ER-shaping proteins (Figure 5D). The ER structure is a network of sheets and tubules which is maintained by the reticulons Rtn1 and Rtn2, the reticulon-like protein Yop1, dynamin-like GTPases atlastins in mammals and Sey1 in *Saccharomyces cerevisiae*. Yeast lacking ER-shaping proteins and the ERMES complex have an altered steady-state level of phospholipids and an impaired transfer of

phospholipids [306]. PS transfer decreases in cells lacking Mdm34 in combination with either Rtn1 and Yop1 or Rtn1 and Sey1, respectively. ER-shaping proteins similar to ERMES play a role in facilitating functional contact between the ER and mitochondria. It was suggested that the shape of ER and contact sites affect the lipid exchange between both organelles.

Besides phospholipid biosynthesis and translocation, the MAM fraction appears to be involved in steroid biosynthesis of vertebrates by shuttling cholesterol to the mitochondrial IMS (Figure 5A) [307]. Imported cholesterol is converted to the steroid precursor pregnenolone which is then transported back to the ER for further processing. The uptake of ergosterol in yeast is independent of ATP and does not require the membrane potential of the IMM similar to the import of PS, PI and PC which is facilitated by membrane contact [308;309]. In mammals, additional pathways of cholesterol transport into mitochondria were suggested including transfer through cholesterol binding proteins or vesicular transport via fusion of endosomes and lysosomes with mitochondria (Figure 5E) [273]. The sterol carrier protein-2 (SCP-2) was identified to participate in cholesterol transfer from lysosomal to mitochondrial membranes [310]. As additional factors of sterol transport into mitochondria the translocator protein TSPO and steroidogenic acute regulatory proteins (StAR) were detected. TSPO, a peripheral-type benzodiazepine receptor, is located to mitochondria and exhibits high-affinity to cholesterol. In contrast, StAR is a hormone induced protein which is targeted to mitochondria and initiates cholesterol transfer into the organelle [311;312]. Steroidogenic acute regulatory protein-related lipid transfer (START) domains present in 15 mammalian proteins are suggested to participate in the intracellular lipid transport system. StarD7 comprises an N-terminal mitochondrial targeting sequence and is localized to the OMM. *In vitro* purified recombinant StarD7 specifically catalyzes PC transfer between vesicles (Figure 5F). Overexpression of StarD7 accelerated the incorporation of PC into mitochondria indicating that PC transport occurs in a non-vesicular manner [313]. Horibata and Sugimoto [313] proposed that StarD7 is translated in the cytoplasm where PC is either extracted from cytoplasmic surfaces of the ER, Golgi apparatus or plasma membrane or accepted from other PC-transporting proteins. After PC binding, the precursor StarD7 with its N-terminal mitochondrial targeting sequence is recognized by the translocase of the outer mitochondrial membrane, and PC is inserted into the OMM.

Intramitochondrial transport of lipids

Intramitochondrial transport of lipids is important to supply the lipid biosynthetic enzymes of the IMM with the substrates CDP-DAG, PA and PS for efficient CL and PE synthesis. Moreover, the IMM strongly depends on the continuous import of PI and PC which cannot be produced by mitochondria themselves.

PA transport between OMM and IMM is mediated by Ups1, a protein localized to the intermembrane space (Figure 6A) [314-316]. The transport occurs in distinct steps and starts with PA binding to Ups1 at the surface of the OMM. After extraction of PA from the membrane, Ups1 assembles with Mdm35 which stabilizes and protects the client protein against the intermembrane space proteases Yme1 and the metallopeptidase Atp23 [317]. Association of both proteins as heterodimer leads to a transfer competent conformation and enables a bidirectional PA transport occurring irrespective of the acyl chain composition. Interaction of negatively charged phospholipids such as CL, PG or CDP-DAG with the heterodimer facilitates the dissociation of Mdm35 from the complex, thereby releasing PA to the acceptor membrane. High concentration of CL impairs detachment of Ups1 from the acceptor membrane and inhibits the PA flux between both membranes, thereby providing a tool to limit and regulate CL accumulation in the IMM [314]. The function of Ups1 to regulate mitochondrial shape and topogenesis of Mgm1, a dynamin-related GTPase required for fusion, morphology, inheritance and genome maintenance of mitochondria is conserved in eukaryotes [318]. In human cells, four proteins are related to the yeast Ups proteins with PRELI (protein of relevant evolutionary lymphoid interest) displaying the highest homology to Ups1 and functionally replacing the yeast counterpart [319].

Several models of PE biogenesis and intramitochondrial transport have been discussed. It was suggested that (i) PS decarboxylation occurs at the inner leaflet of the OMM prior to its transport to the IMM; (ii) trafficking and decarboxylation of PS is coupled and mediated by Psd1; or (iii) PE biosynthesis is coordinated by two separate steps including transfer of PS from the OMM to the IMM and decarboxylation at the latter compartment by Psd1 [283;285;320;321]. Studies by Tamura *et al.* [322] support the latter model and describe PS trafficking and decarboxylation as two separate processes with a Psd1 independent PS transfer between OMM and IMM. Under physiological conditions, PE biosynthesis occurs in a time dependent manner controlled by the availability of Psd1 in mitochondria (Figure 6B). In the yeast, deletion of *UPS1*, *MDM31* or *GEP5* causes a decrease in Psd1 levels and

consequently a reduction of PE formation under fermentable growth conditions [302;303;316;317;322;323]. Ups1 facilitates import of Psd1 into mitochondria, mediates the export of PE from the IMM to the OMM, accelerates the conversion of PE to PC, but appears to be dispensable for PS transfer [304;322]. In addition, Mdm31 and Gep5 are possible factors affecting Psd1 import and stabilization through maintaining mitochondrial morphology and normal respiratory growth [302;303;323]. Moreover, fragmentation of mitochondria due to the impaired mitochondrial fusion machinery containing Fzo1, Ugo1 and Mgm1 leads to decreased Psd1 levels and compromised PE biosynthesis [322;324].

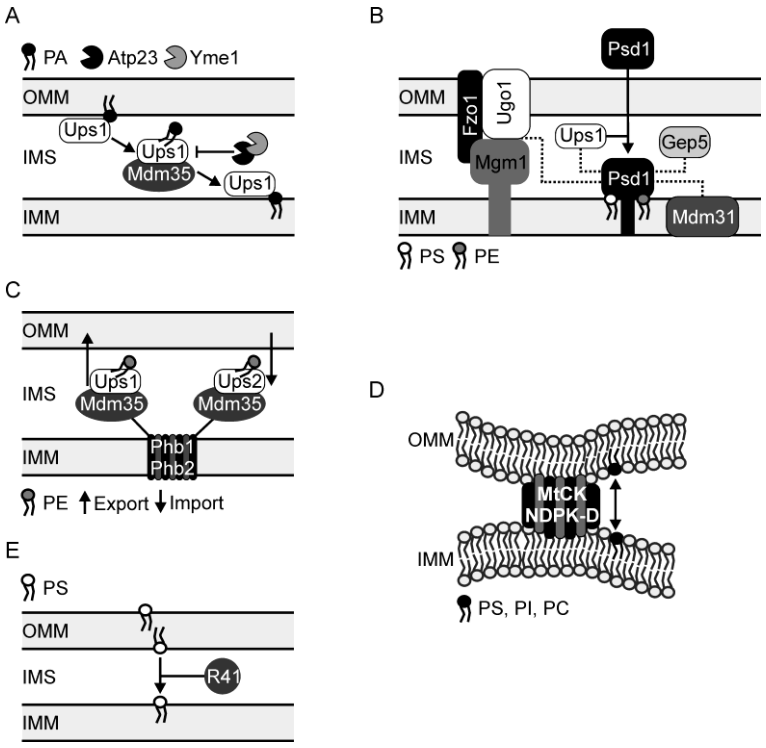

Figure 6. Intramitochondrial lipid transfer. (A) PA transport between the OMM and IMM is mediated by the IMS protein Ups1. (B) PE homeostasis in mitochondria depends on the availability of Psd1 which is affected by Ups1, Mdm31, Gep5, Fzo1, Ugo1 and Mgm1. (C) Mitochondrial PE levels are controlled by Ups1 and Ups2. (D) Briding the OMM and IMM with protein complexes facilitates the intramitochondrial lipid transfer. (E) Specific proteins affect lipid distribution between the OMM and the IMM independently of the mitochondrial protein import/transport machinery. IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Like *UPS1*, *UPS2* (also termed *GEP1*) is a genetic interactor of prohibitin. The multimeric high-molecular weight prohibitin complex is composed of two homologous subunits Phb1 and Phb2 localized to the IMM [315;325-327]. It was suggested that Ups2 might control mitochondrial PE levels by inhibiting of either PE specific lipases or the export of PE to other subcellular compartments (Figure 6C). Psd1 activity and uptake of PS was not affected by Ups2 [315]. Thus, Ups2 and Ups1 appear to coordinately regulate the levels of PE and CL in mitochondria. Deletion of *UPS2* leads to an increase of CL, whereas overexpression significantly reduces CL and enhances PE levels [315].

Import into mitochondria, mitochondrial stability and accumulation of Ups proteins are regulated by Mdm35, a protein of the IMS [302;316;317;328;329]. Mdm35 prevents Ups proteins from arresting at the TOM complex and facilitates unidirectional movement across the OMM [316]. Complex formation of Mdm35 with each Ups protein causes a protection against IMS proteases and leads to accumulation of the protein [316;317]. Additionally, cells lacking Mdm35 display a decrease of the PE level in mitochondria and morphological defects similar to *ups2Δ*. Therefore, Mdm35 can be regarded as another factor in the highly complex process of phospholipid distribution and regulation in mitochondria [317].

Intramitochondrial PS, PI, and PC translocation is suggested to occur at CS between the OMM and IMM independently of an electrochemical potential across the IMM or ATP supply [56;57;59;309]. Bridging OMM and IMM and thereby facilitating lipid transfer is a novel function assigned to the mitochondrial creatine kinase (MtCK) and nucleoside diphosphate kinase (NDPK-D) (Figure 6D). Both proteins associate in highly symmetrical oligomeric entities which ensure cross-linking of membranes. The structural arrangement of these complexes promotes the transfer of lipids. It is controlled by the oligomerization state of MtCK between dimer and octameric form with the latter form comprising the active state [330]. Furthermore, PS transport in mammalian cells is specifically affected by the protein R41 (Figure 6E) [293].

The functional liaison of mitochondrial lipids and proteins

Lipids and proteins of the outer mitochondrial membrane

The OMM provides a lipid rich envelope for proteins and several protein complexes required for protein translocation across and into the bilayer [6;91;95]. In general, two major classes of proteins are found in the OMM. Proteins are either inserted into the bilayer by multiple transmembrane β-strands or by hydrophobic α-helical segments. The translocation processes of β-barrel and α-helical proteins into the OMM are different and strongly respond to alterations of the lipid surrounding [95;331-340]. A brief summary of protein insertion into the OMM is shown in Figure 7.

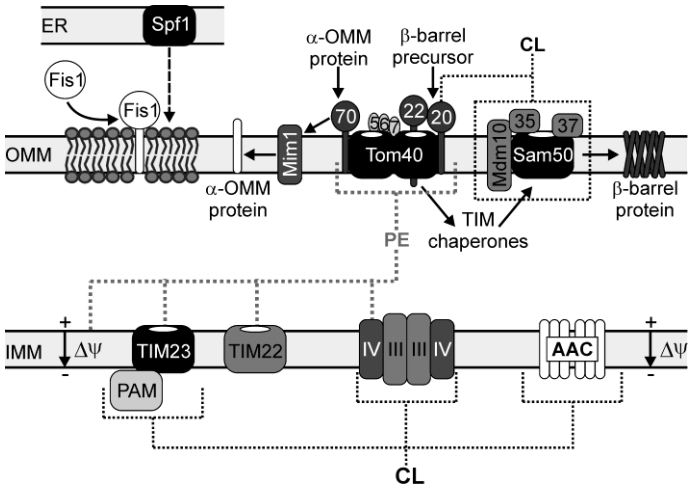

Figure 7. Lipid-protein interaction in mitochondria. CL and PE differentially affect the protein complexes of the OMM and the IMM. Import and assembly of tail-anchored α-helical proteins are facilitated by the specific lipid composition of the OMM. AAC, ADP/ATP carrier; CL, cardiolipin; IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; PE, phosphatidylethanolamine; SAM, sorting and assembly machinery; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane; III, cytochrome bc_1; IV, cytrochrome c oxidase.

The general entry gate for proteins into mitochondria is the translocase of the OMM (TOM complex) consisting of the general import pore Tom40, the three receptors Tom20, Tom70 and Tom22, and the small Tom proteins (Tom5, Tom6, Tom7) which are crucial to maintain the translocase complex in a dynamic conformation [341-348]. β-barrel proteins

are targeted and imported *via* the TOM complex, bind to small Tim chaperone complexes in the IMS and are guided to the sorting and assembly machinery (SAM complex) which is also located to the OMM. The SAM complex is composed of a β-barrel core protein Sam50, the receptor Sam35 and Sam37 which is involved in releasing the β-barrel precursor into the OMM. Furthermore, the SAM complex associates with Mdm10, a subunit of the ERMES complex which facilitates the transport of phospholipids from the ER to mitochondria by tethering both organelles [297;346;347;349-357].

Both complexes, TOM and SAM, respond to alterations of the OMM lipid composition, in particular to the levels of the non-bilayer forming phospholipids PE and CL [333] (our own unpublished data). CL is present in the OMM at a concentration of 6% of total phospholipids [91]. Mutations in the biosynthetic route of CL lead to growth defects and a decrease of the IMM potential ($\Delta\psi$) at elevated temperatures. The CL induced decrease of $\Delta\psi$ partially impairs protein translocation across the IMM but can be reversed by lowering the growth temperature to 21°C. Under these conditions steady-state protein levels of the IMM and the OMM import machinery are restored close to that of wild type [333]. CL also affects the association of the Tom20 receptor (α-helical protein) with the TOM complex, whereas other core components such as Tom40, Tom22 and small Tom subunits are not influenced. In addition, CL impairs the organization of the SAM complex which is involved in the assembly of β-barrel proteins into the OMM. These observations demonstrate that alterations of CL formation in *crd1Δ*, *tam41Δ* and *taz1Δ* mutants specifically block the import machineries of the OMM by affecting insertion of certain α-helical proteins and the assembly of β-barrel proteins at the level of the SAM complex [333]. In contrast, PE is required to maintain the function of the TOM complex and the import of β-barrel proteins, whereas biogenesis of α-helical OMM proteins or the stability of TOM and SAM complexes are not affected (our own unpublished data). It was suggested that PE is required for β-barrel protein (Tom40) assembly at early stages before binding to the SAM complex. Thus, loss of non-bilayer forming phospholipids CL or PE differentially manipulate the import machineries of the OMM by either destabilizing TOM and SAM complexes or impairing the function of the TOM complex, respectively.

In contrast to β-barrel proteins, import and assembly of tail-anchored α-helical proteins into the OMM were described to be facilitated by the lipid composition [331;332;334;335;338-340]. As an example, Fis1, a protein which is inserted into the

OMM by a single transmembrane segment at the C-terminus, depends on the specific lipid composition of the OMM, in particular on the low ergosterol content [335]. *In vitro* import of Fis1 into liposomes mimicking the lipid composition of yeast OMM had similar insertion efficiency as observed with isolated yeast mitochondria. Additionally, liposomes with an increased level of ergosterol inhibited Fis1 import, suggesting that elevated fluidity due to the low ergosterol content of the OMM supports specific protein targeting to the membrane [91;335]. Studies of Merklinger *et al.* [337] demonstrated that membrane integration of OM45, a mitochondrial protein with an N-terminal signal, does not require cytosolic factors or the TOM complex. Similar to Fis1, the low ergosterol content of the OMM seems to be crucial for OM45 import [337]. Recently, the ER ATPase Spf1 was found to affect the insertion of tail-anchored proteins into the OMM [336]. Spf1 is required to maintain the lipid composition of intracellular compartments. In *spf1Δ* mutants a higher ergosterol to phospholipid ratio in the OMM similar to the ER was found. The similar sterol content of both compartments causes a non-specific targeting of tail-anchored OMM proteins to the ER. Thus, Spf1 directly or indirectly regulates the ergosterol level and as a consequence protein insertion into the OMM [336].

Lipids and proteins of the inner mitochondrial membrane

In the IMM two pathways contribute to protein translocation, namely the presequence and carrier pathway [95]. Preproteins with a cleavable presequence use the TIM23 complex whereas carrier proteins containing internal targeting signals are integrated in the IMM by the TIM22 complex. Import across and insertion into the IMM *via* both routes depends on an electrochemical gradient ($\Delta\psi$) across the lipid bilayer. Yeast mitochondria with decreased levels of PE through *PSD1* and *PSD2* deletions display a reduced activity of cytochrome c oxidase leading to an impaired membrane potential ($\Delta\psi$) which affects the import efficiency of proteins *via* the TIM22 and TIM23 complexes (Figure 7) [358]. Despite the impaired function of the IMM translocases due to PE depletion, the stability of both IMM import machineries as well as the ADP/ATP carrier (AAC) oligomerization were not affected. Furthermore, lowering the mitochondrial PE level favours the stabilization of respiratory chain supercomplexes to larger oligomeric structures containing both the cytochrome bc_1 complex and cytochrome c oxidase [358]. CL has the opposite

effect by destabilizing the association of the cytochrome bc_1 complex with cytochrome c oxidase (Figure 7) [358;359]. At elevated temperature, *CRD1* mutants lose viability and their mitochondrial genome. Lack of CL in mitochondria affects the respiratory rate, ATPase and cytochrome oxidase activities as well as protein import through a reduced membrane potential. Impaired mitochondrial function cannot be completely bypassed by increased amounts of PG which accumulate in the presence of non-fermentable carbon sources. Nevertheless, PG restores ATPase and cytochrome c oxidase activities suggesting substitution of CL to some extent with the exception for AAC function and oligomerization [359]. Deletion of *TAM41* and *TAZ1* resembles the observed defects in mitochondria lacking CL [157;168]. Tamura *et al.* [319] described that CL levels controlled through Ups1 and Ups2 affect the assembly state of the TIM23 complex with the presequence translocase-associated motor (PAM). Decrease of CL alters the conformation of the TIM23 complex leading to PAM dissociation and resulting in impaired mitochondrial protein import [319]. Studies of Harada *et al.* [360] questioned the influence of CL on the TIM23 complex. These authors showed that overexpression of Art5 suppressed growth defects of *tam41Δ* and partially restored the presequence pathway for protein import (TIM23 complex) without changing the CL level [360].

Specific physical interactions of non-bilayer forming phospholipids with IMM protein complexes were suggested by increased mobility of AAC and TIM23 complexes in the absence of CL and PE, respectively. Both complexes display an increased mobility with a small size shift towards a lower molecular weight depending on the presence/absence of bound phospholipids [358;359]. Further lipid-protein interactions were identified by using X-ray structures of cytochrome bc_1 complex identifiying several tightly bound phospholipids including CL [361]. The mitochondrial cytochrome bc_1 complex (ubiquinol:cytochrome c oxidoreductase or complex III) is a multi-subunit protein complex embedded in the IMM which couples proton translocation across the membrane with electron transfer. Site-directed mutagenesis of lipid binding sites in the yeast complex III revealed a structural and functional lipid-protein interaction [361]. The function of CL for stabilizing respiratory chain supercomplexes has also been observed by Böttinger *et al.* [358] and Zhang *et al*. [362]. Depletion of CL causes dissociation of respiratory chain supercomplexes by releasing cytochrome c oxidase (complex IV) [363]. This defect cannot be rescued by elevated levels of PG. Studies of Wenz *et al.* [361] suggested that CL

stabilizes respiratory supercomplex formation through neutralization of charged lysine residues between cytochrome bc_1 and cytochrome c oxidase complexes.

The ADP/ATP carrier (AAC) of the IMM is another interaction partner of CL. In yeast, Aac2 is the major isoform of three ADP/ATP carriers and equivalent to the heart and muscle-specific human AAC1 [364]. Experiments with reconstituted ADP/ATP transporter suggested the binding of six molecules of CL per AAC dimer [365]. This effect is caused rather by the headgroup structure of CL than the acyl chain composition. Crystal structure of bovine AAC from heart muscle mitochondria displayed the presence of three CL molecules [366]. Homotetramer formation of AAC2 homodimers is facilitated by the presence of CL [359]. AAC2 is also part of other protein complexes such as respiratory chain supercomplexes and smaller AAC2-containing complexes with phosphate carriers Pic1 and Pic2, the dicarboxylate transporter Dic1 and the GTP/GDP transporter Ggc1. Interaction of AAC2 with respiratory supercomplexes and other mitochondrial carrier complexes leads to formation of 160-670 kDa complexes [367]. In the presence of CL, ATP production is increased through facilitating the physical interaction of AAC2 with respiratory chain supercomplexes and stimulating oxidative phosphorylation [367].

Phospholipids and morphological aspects in mitochondria

Prohibitins form large multimeric ring complexes in the IMM by assembly of Phb1 and Phb2 which is mediated by the TIM23 complex and facilitated through the IMS located Tim8-Tim13 complex [327;368-371]. The function of prohibitins has been linked to mitochondrial DNA stabilization, maintenance of mitochondrial morphology and respiration [372]. Prohibitin deficient yeast cells are viable as long as several genes essential for cell survival termed genetic interactors of prohibitins (GEP) are intact [315;372]. These genes predominantly encode mitochondrial proteins which regulate mitochondrial phospholipid biosynthesis and metabolism, respiratory chain assembly and other membrane processes such as membrane fusion and fission events. In the absence of prohibitins the enzymes Psd1 and Crd1 become crucial to maintain cell viability [315;373]. Furthermore, the two GEP genes *UPS1* and *UPS2* were identified as regulators of mitochondrial CL and PE levels which suggested a functional link of prohibitins to mitochondrial phospholipid metabolism [315]. Downregulation of prohibitins and a low

amount of PE lead to a severe disturbance of the IMM, dissipation of $\Delta\psi$ and cell death. These results suggested that prohibitins and the mitochondrial lipids PE and CL contribute to similar processes in the IMM. Prohibitins were also suggested to support the formation of mitochondrial membrane domains that are specifically enriched in CL and PE which is critical for mitochondrial structure and integrity [315;372].

Conclusions

Mitochondria are unique organelles for several reasons. First, they consist of a complex structural network based on the existence of two membranes, the OMM and the IMM. These membranes although in close vicinity or even attached to each other have distinct features regarding their protein and lipid composition and their function. Secondly, mitochondria and their subcompartments fulfil distinct and important biochemical roles. An appropriate environment of enzymes in mitochondrial membranes and soluble compartments is prerequisite for cellular fitness. Finally, mitochondria are derived from two independent genetic and biosynthetic systems. The majority of mitochondrial components is synthesized outside the organelle and has to be imported. Only a small portion of mitochondrial constituents is formed by the organelle autonomously. The dual origin of mitochondrial components applies to proteins and lipids as well.

The distinct role of mitochondrial lipids has been restricted for a long time to the mitochondria specific phospholipid cardiolipin. It has been realized that occurrence of this lipid in the IMM is essential for one of the major functions of mitochondria, the respiration. More recently, however, our understanding of the role of mitochondrial lipids was extended to more or less all lipid classes. Although not essential in every case, lipids of mitochondrial membranes support structural and functional features of the organelle in a rather specific way. Especially the organization of mitochondrial supercomplexes has been realized as target for fine tuning by lipids.

During the last decade, more and more protein components were identified which contribute to lipid homeostasis in mitochondria. Gaps in the mitochondrial lipid biosynthetic pathways were closed and regulatory aspects of these pathways were recognized. Components governing import of lipids from their extramitochondrial sites of

synthesis to their appropriate intramitochondrial locations on one hand and regulating the intramitochondrial lipid traffic and assembly on the other hand were identified. Larger complexes involved in communication of mitochondria with other organelles, especially with the ER, were detected. These subcellular structures seem to contribute to lipid transport between organelles, although the mechanism(s) involved are still a matter of dispute.

As outlined above, mitochondria appear to be a major acceptor of cellular components. However, major contributions to the total cellular metabolic network made by mitochondria have to be mentioned. These components are linked to the energy metabolism, the flux of central metabolites and also to lipids. Export of phosphatidylethanolamine (PE) as a precursor for the major cellular phospholipid, phosphatidylcholine (PC), and as an essential building block of all organelle membranes, fatty acid beta-oxidation in higher eukaryotes and traffic of steroids are the most prominent examples for such contributions. Thus, a well-balanced lipid metabolism in mitochondria is an important aspect in the cellular puzzle of lipid and biomembrane homeostasis.

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Chapter 2

Metabolic link between phosphatidylethanolamine and triacylglycerol metabolism in the yeast Saccharomyces cerevisiae

Metabolic link between phosphatidylethanolamine and triacylglycerol metabolism in the yeast *Saccharomyces cerevisiae*

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Abstract

In the yeast *Saccharomyces cerevisiae* triacylglycerols (TAG) are synthesized by the acyl-CoA dependent acyltransferases Dga1p, Are1p, Are2p and the acyl-CoA independent phospholipid:diacylglycerol acyltransferase (PDAT) Lro1p which uses phosphatidylethanolamine (PE) as a preferred acyl donor. In the present study we investigated a possible link between TAG and PE metabolism by analyzing the contribution of the four different PE biosynthetic pathways to TAG formation, namely *de novo* PE synthesis *via* Psd1p and Psd2p, the CDP-ethanolamine (CDP-Etn) pathway and lyso-PE acylation by Ale1p. In cells grown on the non-fermentable carbon source lactate supplemented with 5 mM ethanolamine (Etn) the CDP-Etn pathway contributed most to the cellular TAG level, whereas mutations in the other pathways displayed only minor effects. In *cki1*Δ*dpl1*Δ*eki1*Δ mutants bearing defects in the CDP-Etn pathway both the cellular and the microsomal levels of PE were markedly decreased, whereas in other mutants of PE biosynthetic routes depletion of this aminoglycerophospholipid was less pronounced in microsomes. This observation is important because Lro1p similar to the enzymes of the CDP-Etn pathway is a component of the ER. We conclude from these results that in *cki1*Δ*dpl1*Δ*eki1*Δ insufficient supply of PE to the PDAT Lro1p was a major reason for the strongly reduced TAG level. Moreover, we found that Lro1p activity was markedly decreased in *cki1*Δ*dpl1*Δ*eki1*Δ, although transcription of *LRO1* was not affected. Our findings imply that (i) TAG and PE synthesis in the yeast are tightly linked; and (ii) TAG formation by the PDAT Lro1p strongly depends on PE synthesis through the CDP-Etn pathway. Moreover, it is very likely that local availability of PE in microsomes is crucial for TAG synthesis through the Lro1p reaction.

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Keywords: phosphatidylethanolamine, triacylglycerol, acyltransferase, CDP-ethanolamine, yeast, *Saccharomyces cerevisiae*

Abbreviations: CF, cellular fraction; CL, cardiolipin; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DMPE, dimethylphosphatidylethanolamine; ER, endoplasmic reticulum; Etn, ethanolamine; LP, lipid particle; LPL, lysophospholipid(s); MMGlu, minimal glucose media; MMLac, minimal lactate media; MAM, mitochondria associated membrane; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PDAT, phospholipid:diacylglycerol acyltransferase; RT-PCR, reverse transcription polymerase chain reaction; SE, steryl ester; TAG, triacylglycerol; TLC, thin-layer chromatography; YPD, complex glucose media; YPLac, complex lactate media

Introduction

Storage lipids in all types of eukaryotic cells accumulate mostly as triacylglycerols (TAG) and steryl esters (SE) which are the major components of a specialized globular compartment of the cell named lipid particle (LP), lipid droplet, lipid body or oil body [1]. LP consist of a randomly packed TAG core surrounded by several more or less ordered SE shells and a phospholipid surface monolayer with a small set of specific proteins embedded [1-3]. Mechanism(s) of LP biogenesis are still a matter of dispute [4], but the most convincing hypothesis for this process is budding of LP from the endoplasmic reticulum (ER) [2, 5-7]. According to this model, TAG and SE formed in the ER accumulate in certain microdomains of the ER membrane. Upon further synthesis of neutral lipids, LP precursors are formed, which at a critical size bud off the ER and are released into the cytosol [2, 4].

In *Saccharomyces cerevisiae* two proteins localized to the ER, Are1p and Are2p, catalyze the synthesis of SE with slightly different substrate specificity. Are2p is the major SE synthase of the yeast and prefers ergosterol as a substrate, whereas Are1p uses ergosterol and its precursors at nearly equal efficiency with a slight preference for lanosterol. Mutants lacking *ARE1* and *ARE2* are completely devoid of SE and accumulate free sterols [5, 8]. In addition, Are1p and Are2p can also catalyze TAG synthesis although with minor efficiency compared to the two major yeast TAG synthesizing enzymes, Dga1p and Lro1p [8-12]. In *Saccharomyces cerevisiae* two primary mechanisms of TAG formation were identified, namely an acyl-CoA dependent reaction catalyzed by the diacylglycerol acyltransferase (DGAT) Dga1p, and an acyl-CoA independent pathway involving the phospholipid:diacylglycerol acyltransferase (PDAT) Lro1p [9-14]. Dga1p is dually localized to the ER and LP and appears to be more efficient than Lro1p under standard growth conditions when cells are in the stationary phase [3, 9-11]. In contrast, Lro1p which requires phospholipids as acyl donor is exclusively localized to the ER [12-15]. Lro1p preferentially uses phosphatidylethanolamine (PE) as co-substrate *in vitro* and transfers its *sn-2* acyl group to diacylglycerol (DAG), resulting in the formation of TAG and lyso-PE [13-15]. This reaction has no counterpart in mammalian cells.

The link between TAG synthesis and PE metabolism through Lro1p as described above led us to investigate metabolic interactions between biosynthesis/degradation of these two

lipids in some more detail. In *Saccharomyces cerevisiae*, PE synthesis occurs by four different pathways. In brief, two of these pathways are accomplished by phosphatidylserine (PS) decarboxylases Psd1p and Psd2p which use PS as a substrate. While Psd1p is localized to mitochondria, Psd2p is a component of a Golgi/vacuolar compartment [16-21]. Yeast PE can also be synthesized through the cytidyldiphosphate ethanolamine (CDP-Etn) branch of the so-called Kennedy pathway using Etn and DAG as substrates [16, 22, 23]. The CDP-Etn pathway is also linked to sphingolipid metabolism through the action of the dihydrosphingosine phosphate lyase Dpl1p which sets Etn-P free [24, 25]. Moreover, the lyso-PE acyltransferase Ale1p which is present in the mitochondria associated ER (MAM) catalyzes an alternative pathway to form PE [26, 27]. Surprisingly, Tgl3p, the major yeast TAG lipase, can also act as a lyso-PE acyltransferase [28].

In a series of studies from our laboratory [29-33] we observed preferences in the incorporation of newly formed PE through the different pathways into different cellular compartments. These findings led us to speculate that also distinctions regarding the relative contributions of PE biosynthesis to TAG formation by Lro1p may exist between the different PE synthesizing routes. We used haploid single and multiple deletion strains bearing defects in PE biosynthesis, namely *psd1*Δ, *psd2*Δ, *psd1*Δ*psd2*Δ, *cki1*Δ*dpl1*Δ*eki1*Δ (CDP-Etn mutant) and *ale1*Δ to address this question. Yeast cells used for these experiments were grown on the non-fermentable carbon source lactate because under these conditions the level of PE in the cell becomes more critical for cell growth and viability than in glucose grown cells [31]. Here we report a clear metabolic link between TAG formation in the yeast and PE synthesis *via* the CDP-Etn branch of the Kennedy pathway, but not by other PE biosynthetic routes. In the light of these findings, the lipid metabolic network of PE and TAG metabolism in the yeast is discussed.

Materials and Methods

Strains and culture conditions

Strains used throughout this study are listed in Table 1. Cells were cultivated aerobically in 2 l Erlenmeyer flasks to the stationary growth phase (A_{600} ~ 4) at 30°C in minimal lactate medium consisting of 2.66% lactate (Roth), 0.67% yeast nitrogen base without amino acids (USBiological), 0.073% amino acid mix (Roth, Fluka) supplemented with 5 mM Etn (Merck) and adjusted to pH 5.5 with KOH. Main cultures were inoculated to an A_{600} of 0.1 from precultures grown aerobically for 48 h in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid) and 2% glucose (Merck) at 30°C.

For synthesis of radioactively labeled [^{14}C]phospholipids, a *dga1*Δ*lro1*Δ double deletion strain was grown on minimal glucose media containing 2% glucose (Merck), 0.67% yeast nitrogen base without amino acids (USBiological) and 0.073% amino acid mix (Roth, Fluka). Details of the labeling procedure will be described below.

Table 1. Yeast strains used in this study.

Strain	Genotype	Source/Reference
wild type	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
psd1Δ	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::His3MX6*	This study
psd2Δ	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd2Δ::KanMX4*	Euroscarf
ale1Δ	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ale1Δ::KanMX4*	Euroscarf
lro1Δ	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 lro1Δ::KanMX4*	Euroscarf
dga1Δ	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dga1Δ::KanMX4*	Euroscarf
cki1Δdpl1Δeki1Δ	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cki1Δ::His3MX6 dpl1Δ::LEU2 eki1Δ::KanMX4*	This study
psd1Δpsd2Δ	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::His3MX6 psd2Δ::KanMX4*	This study
dga1Δlro1Δ	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dga1Δ::His3MX6 lro1Δ::KanMX4*	This study

Strain construction

Single-step deletion of chromosomal genes was carried out using the PCR-mediated technique described by Longtine *et al.* [34]. The marker module His3MX6 including the *Schizosaccharomyces pombe his5*$^{+}$ gene on the vector pFA6a was used to replace *YNL169c* encoding Psd1p and *YOR245c* encoding Dga1p in the single deletion strains *psd2*Δ*::KanMX4* and *lro1*Δ*::KanMX4*, respectively [34-36]. Deletion cassettes containing homologous regions to start and stop regions of either *PSD1* or *DGA1* and the entire *HIS5*$^{+}$ gene were constructed with primers listed in Table 2. A 1.4 kbp PCR fragment was

generated with *ExTaq* DNA polymerase (Takara, Otsu, Japan) by using 150 ng of plasmid as a template in a standard PCR mixture containing PCR buffer (20 mM Mg^{2+}), 0.2 mM deoxynucleoside triphosphates, each, and a 1 µM solution of primers in a total volume of 100 µl. After a denaturation step of 2 min at 94°C, fragments were amplified for 10 cycles of 15 s at 94°C, 60 s at 55°C, and 100 s at 72°C; and for 25 cycles of 15 s at 94°C, 90 s at 68°C, and 60 s at 72°C, followed by a final elongation step for 10 min at 72°C.

Overnight cultures (A_{600} ~0.8) of single mutants *psd2*Δ*::KanMX4* and *lro1*Δ*::KanMX4* were used for transformation with the high-efficiency lithium acetate transformation method [37]. Transformants were grown on plates lacking histidine for 3 days at 30°C. Plates used for cultivation of *psd1*Δ*psd2*Δ transformants contained in addition 5 mM Etn to permit growth. Large colonies were transferred to fresh plates for further selection. Clones yielding colonies were considered as positive transformants and further checked for correct integration of the respective deletion cassette. Verification of the correct replacement of *PSD1* and *DGA1* by the His3MX6 module was done by colony PCR. In brief, oligonucleotides were designed to bind outside the target locus and within the marker module [36, 38]. Correct integration of the marker resulted in the appearance of the respective PCR fragment.

Table 2. Primers used for the construction of deletion strains and for RT-PCR. The underlined sequences are homologous to the *His3MX6* disruption cassette (PSD1-H1, PSD1-H2, DGA1 Del_for, DGA1 Del_rev). Amplified plasmid DNA with the primers PSD1-H1 and PSD1-H2 led to *PSD1* deletion, whereas DGA1 Del_for and Del_rev to *DGA1* deletion after homologues recombination to the target loci YNL169c and YOR245c, respectively. Lro1 FP, Lro1 RP, Dga1 FP, Dga1 RP, Act1 FP and Act1 RP were used to determine expression levels of *LRO1*, *DGA1* and *ACT1*.

Primer	Primer sequence (5´-3´)
PSD1-H1	GCCAGTTAAGAACGCCTTGGCGCAAGGGAGGACGCTCCTCCGGATCCCCGGGTTAATTAA
PSD1-H2	CAGGTATGTGGTTCCAAGTGTTTGTCGCTCTTTGAATTTGGAATTCGAGCTCGTTTAAAC
DGA1 Del_for	ATAAGGAAACGCAGAGGCATACAGTTTGAACAGTCACATAACGGATCCCCGGGTTAATTAA
DGA1 Del_rev	TTCCTGTAAGTTAATACTCTTACTTAAGATATACAGCCCGAATTCGAGCTCGTTTAAAC
Lro1 FP	ATGGGCACACTGTTTCGAAGAAATG
Lro1 RP	AACAGGATGTTTGGCCTCGATATTA
Dga1 FP	AAGAAGGAAGAAGGAAGCCCTACAG
Dga1 RP	CCTGGTTGCGATAGTCAATAGTAGA
Act1 FP	GGTCCCAATTGCTCGAGAGAT
Act1 RP	GAAGTCCAAGGCGACGTAACA

Isolation and characterization of subcellular fractions

Total cell-free homogenate (3,000 x g supernatant) and 100,000 x g microsomes were prepared from cells grown to the stationary growth phase as described previously [21, 39]. Proteins from isolated fractions were precipitated with trichloroacetic acid at a final concentration of 10%, the obtained protein pellet was solubilized in 0.1% SDS, 0.1 M NaOH, and proteins were quantified by the method of Lowry *et al.* [40] with bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [41], and Western blot analysis by the method of Haid and Suissa [42]. Proteins were detected by enzyme-linked immunosorbent assay using rabbit antisera as the first antibody and peroxidase-conjugated goat anti-rabbit IgG as the second antibody. Antibodies used in this study were directed against the mitochondrial and microsomal marker proteins, Por1p and 40-kDa protein, respectively [21].

Lipid extraction and analysis

Lipids were extracted from total cell-free homogenate and microsomes of yeast cells grown to the stationary growth phase by the procedure of Folch *et al.* [43] using chloroform/methanol (2:1; v/v). After washing the organic phase with 0.034% $MgCl_2$ solution (w/v), 2 N KCl/methanol (4:1; v/v), and methanol/water/chloroform (48:47:3; per vol), extracts were taken to dryness, dissolved in 50 µl chloroform/methanol (2:1; v/v) and applied to thin-layer chromatography (TLC) plates (Silica gel 60; Merck, Darmstadt, Germany) with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4, Muttenz, Switzerland). For analyzing neutral lipids, TAG and SE, chromatograms were developed in an ascending manner to the half-distance of the plate by using light petroleum/diethyl ether/acetic acid (70:30:2; per vol). After brief drying, TLC were further developed to the top of the plate using light petroleum/diethyl ether (49:1; v/v) as the second solvent system. For separating DAG from ergosterol light petroleum/diethyl ether/acetic acid (70:10:2; per vol) were used as a solvent system and chromatograms were developed to the top of the plate. Quantification of ergosteryl esters was accomplished by densitometric scanning at 275 nm using a Shimadzu CS-930 dual-wavelength chromatoscanner and ergosterol (Sigma) as a standard. TAG were visualized by post-chromatographic staining after dipping TLC plates into a solution containing 0.8 g $MnCl_2 \times 4H_2O$, 120 ml water, 120 ml methanol and 9 ml concentrated sulfuric acid, and charring at

105°C for 30 min. Quantification of TAG was carried out by densitometric scanning at 400 nm with triolein (NuCheck, Inc., Elysian, Maine) as a standard. For analyzing total phospholipids, the same solvent systems as described above were used to separate phospholipids from neutral lipids. Lipids were visualized by iodine vapor; phospholipids were scraped off the plate and quantified by the method of Broekhuyse [44].

Individual phospholipids were separated by two-dimensional thin-layer chromatography on silica gel 60 plates (Merck) using chloroform/methanol/25% NH_3 (68:35:5; per vol) as the first and chloroform/acetone/methanol/acetic acid/water (53:20:10:10:5; per vol) as the second developing solvent system. Phospholipids were visualized by staining with iodine vapor, scraped off the plate and quantified by the method of Broekhuyse [44].

Preparation of radiolabeled lipid substrates and measurement of TAG synthase activity in vitro

Radioactively labeled [^{14}C]phospholipids were synthesized by incubating *dga1*Δ*lro1*Δ yeast mutants with 10 µCi [^{14}C]oleic acid (PerkinElmer Life Sciences) for 24 h at 30°C in 100 ml minimal glucose media (see above). After harvesting and disrupting cells with glass beads (Sartorius, 0.25-0.30 diameter), lipids were extracted and total phospholipids were separated from neutral lipids as described above. Phospholipids were scraped off the plate and extracted from the silica gel with chloroform/methanol (1:4; v/v) for 3 h. The organic phase was collected and the remaining silica gel was again extracted twice for 1 h, each. After combining the organic phases and drying under a steam of nitrogen, phospholipids were dissolved in 1 ml chloroform/methanol (2:1; v/v), and radioactivity was measured by liquid scintillation counting using LSC Safety (Baker, Deventer, The Netherlands) with 5% water as a scintillation cocktail [11]. Additionally, phospholipid concentration was quantified by the method of Broekhuyse [44].

The phospholipid:diacylglycerol acyltransferase (PDAT) assay was performed in a final volume of 200 µl containing phospholipids labeled with [^{14}C]oleic acid (27 nmol; 70,000 dpm), 200 µg protein from 100,000 x g microsomes, 150 mM TrisCl (pH 7.0), 15 mM KCl, 15 mM $MgCl_2$, 0.5 mM CHAPS, and 0.05 mM dioleoylglycerol [3, 13]. The acyl-CoA:diacylglycerol acyltransferase (DGAT) assay was performed in a final volume of 200 µl containing 16 nmol unlabeled oleoyl-CoA and 0.68 nmol [^{14}C]oleoyl-CoA (0.02 µCi), 100 µg protein from cell-free homogenate, 150 mM TrisCl (pH 7.0), 15 mM KCl, 15 mM

$MgCl_2$, 0.5 mM CHAPS, and 0.025 mM dioleoylglycerol [3, 11]. Incubations were carried out for 30 min at 30°C and terminated by addition of 3 ml chloroform/methanol (2:1; v/v). Lipids were extracted and neutral lipids were separated as described above. After visualization with iodine vapor, TAG were scraped off, and radioactivity was measured by liquid scintillation counting using LSC Safety (Baker, Deventer, The Netherlands) with 5% water as a scintillation cocktail.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from cultivated cells grown to A_{600} ~ 2 on minimal lactate media supplemented with 5 mM Etn at 30°C was isolated by RNeasy kit (Qiagen). Reverse transcription was carried out with 2 µg of RNA after DNAse-I treatment (1 unit RQ1, Promega) in a total volume of 15 µl reverse transcription buffer (15 min at 37°C). Reverse transcriptase from Invitrogen was used, according to the manufacturer's protocol. Amplification of the PCR products was measured during exponential phase of the reaction. PCR was carried out in a final volume of 30 µl containing 3 µl 10 x GC buffer (BioTherm), 2 µl cDNA, 0.1 mM dNTP-Mix, 0.5 pM of each primer (Table 2) and 0.6 U Polymerase (BioTherm, GenXpress). For *DGA1* expression, a fragment was obtained according to the following program. After a denaturation step of 2 min at 96°C, fragments were amplified for 27 cycles of 5 s at 96°C, 10 s at 55°C, 15 s at 72°C, a final elongation step for 10 min at 72°C followed by cooling to 4°C. For *LRO1* expression, the amplification was carried out for 28 cycles of 5 s at 96°C, 10 s at 52°C, and 15 s at 72°C. As a control, the *ACT1* expression was tested by amplifying the fragments for 25 cycles of 10 s at 96°C, 60 s at 58°C and 120 s at 72°C.

Results

Growth of strains bearing defects in phosphatidylethanolamine synthesis

PE is one of the most prominent phospholipids found in yeast membranes. Previous work from our laboratory has shown that the requirement for PE in *Saccharomyces cerevisiae* is more stringent on non-fermentable carbon sources, i.e. when mitochondria are fully developed, than on fermentable carbon sources [31, 45]. Mutants defective in either one of the PS decarboxylases, Psd1p or Psd2p, grow on glucose-containing media like wild type, but lack of both enzymes leads to auxotrophy for Etn (Figure 1) or choline [31]. In contrast, strains with the *psd1*Δ mutation alone or in combination with *psd2*Δ are not viable on lactate as a carbon source unless rescued by addition of 5 mM Etn to the medium as a minimum requirement for viability. Lethality of *psd1*Δ on non-fermentable carbon sources is also prevented by supply with either serine or choline [31].

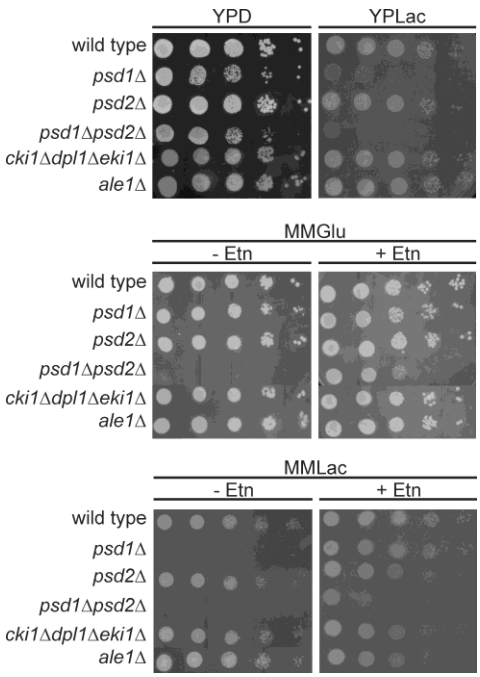

Figure 1. Growth of yeast strains with defects in phosphatidylethanolamine biosynthesis depends on the carbon sources. Cell suspensions of strains listed in the figure were spotted at dilutions (1, 1/10, 1/100, 1/1000, 1/10000) on YPD, YPLac, MMGlu and MMLac with or without 5 mM ethanolamine. Incubation was carried out at 30°C. YPD, complex glucose media; YPLac, complex lactate media; MMGlu, minimal glucose media; MMLac, minimal lactate media; Etn, ethanolamine.

The requirement of a defined PE level for yeast growth and viability raised the questions whether (i) the cellular amount/availability of PE and (ii) different routes for PE biosynthesis affect TAG formation by Lro1p. For this purpose, various strains bearing defects in PE formation, in particular *psd1*Δ, *psd2*Δ, *psd1*Δ*psd2*Δ, *ale1*Δ, and the CDP-Etn pathway mutant *cki1*Δ*dpl1*Δ*eki1*Δ, were grown on minimal lactate media supplemented with Etn creating stringent conditions for PE requirement. For blocking the CDP-Etn branch of the Kennedy pathway efficiently a *cki1*Δ*dpl1*Δ*eki1*Δ triple mutant was required. *CKI1* and *EKI1* encode kinases with overlapping specificities for the substrates Etn and choline. Furthermore, the CDP-Etn pathway is linked to sphingolipid catabolism through the action of the dihydrosphingosine phosphate lyase Dpl1p which cleaves phosphorylated sphingoid bases to long chain aldehydes and ethanolamine phosphate (Etn-P) [24, 25, 31]. In addition, two mutants defective in TAG biosynthesis, *lro1*Δ and *dga1*Δ, were used to investigate TAG and phospholipid biosynthesis under these growth conditions. As expected, growth of strains defective in *PSD1* showed the longest lag phase compared to the other mutants. Nevertheless, wild type and mutants reached nearly the same A_{600} values (3-5 units) by entering the stationary growth phase (data not shown). Since neutral lipid accumulation starts at the end of exponential growth phase and reaches its highest level in the stationary phase [3], wild type and mutant cells were analyzed at this growth stage.

Neutral lipids from mutants bearing defects in phosphatidylethanolamine or triacylglycerol synthesis

To address the question as to the link between the different PE biosynthetic routes and neutral lipid storage in the yeast, we first quantified TAG and ergosteryl esters in wild type and mutant cells grown on minimal lactate media supplemented with Etn. Previous studies had shown that in cells grown on glucose Dga1p and Lro1p contributed differentially to TAG biosynthesis with Dga1p being the more efficient enzyme [3, 9]. In lactate grown cells, deletion of *DGA1* led to a reduction of the TAG content to ~60%, whereas deletion of *LRO1* reduced the TAG level to ~80% of wild type (Figure 2). Previously, it was reported that in cells grown on glucose TAG was also synthesized by the two steryl ester synthases, Are1p and Are2p, although with minor efficiency [9, 10]. In our experiments with yeast cells grown on lactate, TAG was below the detection limit in a *dga1*Δ*lro1*Δ

double deletion strain indicating that Are1p and Are2p did not contribute to TAG formation.

Deletions of *PSD1*, *PSD2*, *PSD1PSD2* and *ALE1* had no significant effect on TAG biosynthesis (Figure 2). In contrast, a *cki1*Δ*dpl1*Δ*eki1*Δ strain bearing defects in the CDP-Etn branch of the Kennedy pathway exhibited a significant reduction of the TAG level to ~40% of wild type. This decrease was even more pronounced than in *dga1*Δ where a reduction to ~60% was observed. The striking observation that deletion of *LRO1* has less impact on TAG formation than a block in the CDP-Etn pathway can be explained by the compensatory effect of Dga1p. It has been shown that Dga1p is able to restore wild type TAG level in the *lro1*Δ mutant [9]. When the CDP-Etn pathway is blocked, both TAG biosynthetic routes are active but the availability of PE for Lro1p appears to become paramount and causes the observed decrease of TAG. To prove the hypothesis that the CDP-Etn pathway actively supports TAG formation in yeast, we grew wild type cells on minimal lactate media with or without supplementation of 5 mM Etn. In non-supplemented cultures the TAG level decreased to ~60% (38 ± 4 µg TAG/mg protein) of the control cultures supplemented with 5 mM Etn (64 ± 9 µg TAG/mg protein). These data strongly support the view that PE synthesis through the CDP-Etn pathway and TAG biosynthesis by Lro1p are linked metabolic processes. Ergosteryl ester formation was not affected in mutants devoid of PS decarboxylases. Deletion of *LRO1*, *ALE1* or the genes of the CDP-Etn pathway led to a slight decreased ergosteryl ester level compared to wild type (see Figure 2). Analysis of DAG levels in wild type and corresponding mutant strains revealed no changes in all strains tested (data not shown).

We concluded from these results that PE biosynthesis through the CDP-Etn pathway and TAG formation were tightly linked and specific. While in *cki1*Δ*dpl1*Δ*eki1*Δ accumulation of diacylglycerol (DAG) not used as a substrate for PE/PC formation through the Kennedy pathway was not detected and TAG formation was not enhanced, we hypothesized that the reduced level of PE as a donor of fatty acids might have a negative impact on Lro1p catalyzed TAG synthesis. Alternatively, gene regulatory effects could not be ruled out. Therefore, we considered both the availability of PE for channelling fatty acids to the acyl-CoA independent pathway of TAG synthesis and transcriptional control as possible reasons for the decreased amount of TAG in *cki1*Δ*dpl1*Δ*eki1*Δ.

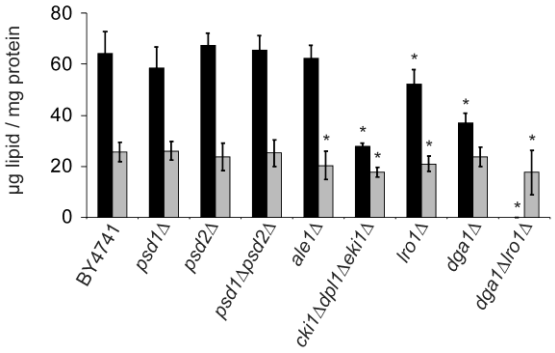

Figure 2. Neutral lipid composition of wild type and mutants defective in either phosphatidylethanolamine or triacylglycerol biosynthesis grown to stationary phase. Amounts of triacylglycerols (black bars) and ergosteryl esters (grey bars) in µg lipid per mg protein were measured in strains as indicated. Data are mean values of 3 independent experiments with error bars indicating the standard deviation. Significance was calculated by student's t-test (two tailed, unpaired). Values indicated by * correspond to $P<0.05$ and were defined to be significant.

Acyl-CoA dependent and acyl-CoA independent triacylglycerol synthase activities in mutants bearing defects in phosphatidylethanolamine synthesis

First, we investigated the activities of TAG synthesizing enzymes in mutants compromised in PE biosynthesis. Samples of cell-free homogenate and microsomes were used to analyze acyl-CoA dependent Dga1p and acyl-CoA independent Lro1p activities, respectively. Deletion of *PSD2* in *psd2*Δ and *psd1*Δ*psd2*Δ strains led to 10-20% increase, whereas blocking the CDP-Etn pathway resulted in a slightly decreased Dga1p activity to ~80% of wild type (Figure 3). A significant reduction of Dga1p activity to ~80% of wild type was only found in *psd1*Δ. Notably, Dga1p activities in *ale1*Δ and *lro1*Δ were not significantly altered.

Lro1p activity was slightly reduced in *psd1*Δ and *psd1*Δ*psd2*Δ and largely unaffected in *psd2*Δ, *ale1*Δ and *dga1*Δ (Figure 3). However, a significant decrease of Lro1p activity to ~50% of wild type was observed in the *cki1*Δ*dpl1*Δ*eki1*Δ triple mutant. Although we have to take into account that enzyme activities measured *in vitro* need not always reflect the potential of an enzyme *in vivo*, we can speculate that reduced activity of Lro1p in *cki1*Δ*dpl1*Δ*eki1*Δ may contribute to the lower TAG content of this strain.

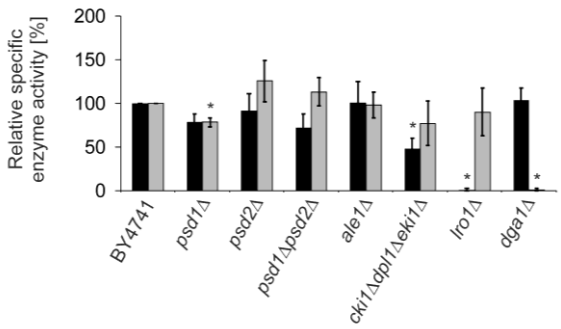

Figure 3. Relative activities of triacylglycerol synthesizing enzymes in vitro. Acyl-CoA:diacylglycerol acyltransferase activity (Dga1p) (grey bars) was measured in vitro using total cell-free homogenate. Phospholipid:diacylglycerol acyltransferase activity (Lro1p) (black bars) was measured using 100,000 x g microsomes. The specific activity of Lro1p and Dga1p in wild type was set to 100%, and data for mutant strains were calculated accordingly. As negative control, phospholipid:diacylglycerol acyltransferase was measured in *lro1Δ*, and acyl-CoA:diacylglycerol acyltransferase in *dga1Δ*. Data are mean values of 3 independent experiments with error bars indicating the standard deviation. Significance was calculated by student's t-test (two tailed, unpaired). Values indicated by * correspond to $P<0.05$ and were defined to be significant.

LRO1 and DGA1 gene expression in mutants bearing defects in phosphatidylethanolamine synthesis

One explanation for the decreased TAG levels (see Figure 2) in *cki1*Δ*dpl1*Δ*eki1*Δ may be reduced expression of *DGA1* and/or *LRO1*. Reverse transcription polymerase chain reaction (RT-PCR) was used to analyze the expression levels of these two genes in mutants defective in PE biosynthesis. As can be seen from Figure 4, expression of *LRO1* was not affected in all mutants tested. This result was surprising insofar as Lro1p activity was reduced in *cki1*Δ*dpl1*Δ*eki1*Δ (see Figure 3). It has to be taken into account, however, that the activity of this enzyme may be reduced by different effects, e.g., by translational control, post-translational modification or enzyme inhibition. In contrast, expression of *DGA1* was dramatically down-regulated in *psd1*Δ and *psd1*Δ*psd2*Δ (Figure 4). Interestingly, in both strains devoid of *PSD1*, the enzymatic activity of acyl-CoA dependent TAG formation was only slightly reduced or even increased (see Figure 3), and TAG levels were similar to wild type (see Figure 2). Altogether, these results indicate that transcriptional control of TAG synthesizing enzymes appears to have a minor impact, if any, on TAG formation *in vivo* and does not explain the low level of TAG in *cki1*Δ*dpl1*Δ*eki1*Δ. Therefore, we tested the effect of subcellular availability of the PDAT substrate PE for TAG synthesis as a possible alternative reason for our findings.

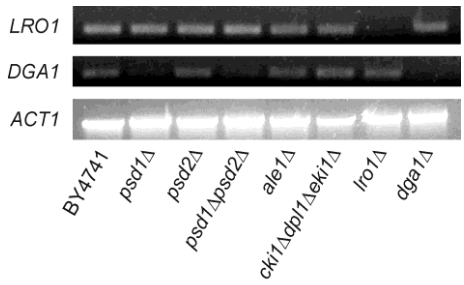

Figure 4. Gene expression levels of *LRO1*, *DGA1* and *ACT1* from wild type and strains defective in phosphatidylethanolamine and triacylglycerol biosynthesis. Strains listed in the figure were tested by RT-PCR. ACT1 (actin) was used as a loading control and *lro1Δ* and *dga1Δ* were used as negative control for *LRO1* and *DGA1* expression.

Phospholipid pattern in mutants with defects in phosphatidylethanolamine synthesis

Previous studies from our laboratory had shown that yeast cells require more PE on non-fermentable carbon sources than on fermentable carbon sources, and mitochondrial synthesis of PE by Psd1p becomes paramount [31]. Furthermore, it was concluded that the microsomal PE pool is mainly derived from the CDP-Etn branch of the Kennedy pathway whereas PE formed through Psd2p is preferentially metabolized to PC [29]. Here, we analyzed the cellular and microsomal PE levels of mutants defective in PE biosynthesis when yeast cells were grown on the non-fermentable carbon source lactate. Microsomal fractions were checked for purity by Western Blot (data not shown). Quality of subcellular fractions from all strains tested was identical. Phospholipid analysis revealed a reduction of the cellular PE content in *psd1*Δ and *psd2*Δ, and even more pronounced in *psd1*Δ*psd2*Δ as an additive effect (Table 3). Notably, the cellular PE level in *cki1*Δ*dpl1*Δ*eki1*Δ was reduced to 17% of total phospholipids compared to 15% in *psd1*Δ*psd2*Δ and 25% in wild type indicating that the CDP-Etn pathway, besides Psd1p, is also an important route for cellular PE formation under these conditions. Analysis of microsomal PE levels from mutants compromised in PE biosynthesis highlighted the importance of the CDP-Etn pathway for the supply of PE to TAG synthesis by Lro1p. The microsomal PE level in *cki1*Δ*dpl1*Δ*eki1*Δ was dramatically reduced to 12% of total phospholipids compared to 21% in wild type and was even lower than in *psd1*Δ*psd2*Δ (16%), whereas deletion of either *PSD1* or *PSD2* alone had hardly any effect (Table 3). The decrease in cellular and microsomal PE was mainly compensated by elevated levels of PC. Interestingly, an

increased level of cellular PE was found in *ale1*Δ, whereas the microsomal level of PE was similar to wild type. Since Ale1p had been identified as an efficient lyso-PE acyltransferase [26, 27] we rather expected a decrease of PE in the deletion strain. The reason for this finding may be that Ale1p has a broad substrate specificity [46-48] and/or other PE forming enzymes might compensate for the deletion of *ALE1*.

The total cellular amounts of phospholipids (Figure 5) remained nearly constant in all PE mutants and comparable to wild type. However, the cellular level of total phospholipids was significantly increased in *dga1*Δ*lro1*Δ. This result can be explained by the utilization of DAG which is not converted to TAG in this strain *via* CDP-Etn pathway for phospholipid synthesis.

Table 3. Phospholipid composition of cell-free homogenate and microsomes from cells grown on minimal lactate media supplemented with 5 mM ethanolamine. CF, cellular fraction; H, cell-free homogenate; M, microsomes (100,000 x g); LPL, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid. Mean values of at least three measurements and standard deviations are shown.

		Phospholipids in cell-free homogenate and microsomes (mol%)							
Strain	CF	LPL	PI	PS	PC	PE	CL	DMPE	PA
wild type	H	2.35 ± 1.78	11.40 ± 2.65	5.09 ± 1.57	50.95 ± 4.58	24.95 ± 2.35	3.73 ± 1.46	0.39 ± 0.54	1.14 ± 0.95
	M	1.41 ± 0.43	9.91 ± 0.82	6.30 ± 0.96	58.11 ± 2.02	20.66 ± 2.23	1.45 ± 0.30	0.16 ± 0.19	2.01 ± 0.67
psd1Δ	H	0.73 ± 0.52	11.87 ± 2.32	6.97 ± 1.17	58.83 ± 3.05	18.65 ± 1.03	2.12 ± 0.60	0.24 ± 0.26	0.59 ± 0.26
	M	1.72 ± 0.19	10.40 ± 2.24	6.90 ± 1.37	57.23 ± 4.38	20.50 ± 1.61	1.14 ± 0.24	0.67 ± 0.30	1.44 ± 0.56
psd2Δ	H	0.20 ± 0.34	8.55 ± 0.72	6.88 ± 2.16	59.73 ± 2.92	21.44 ± 4.31	2.64 ± 1.78	0.04 ± 0.07	0.52 ± 0.54
	M	1.35 ± 0.13	10.56 ± 0.55	6.12 ± 0.71	57.01 ± 1.64	21.10 ± 2.29	1.79 ± 0.25	0.42 ± 0.16	1.65 ± 0.88
psd1Δpsd2Δ	H	0.96 ± 0.73	5.12 ± 3.27	9.01 ± 2.36	66.74 ± 5.86	14.63 ± 1.05	1.97 ± 0.71	0.89 ± 1.02	0.69 ± 0.47
	M	1.85 ± 0.52	8.80 ± 2.23	9.71 ± 2.25	60.67 ± 3.90	16.01 ± 0.43	1.19 ± 0.14	0.39 ± 0.15	1.38 ± 0.40
ale1Δ	H	0.52 ± 0.45	7.35 ± 3.76	6.13 ± 2.66	51.71 ± 4.72	30.32 ± 2.35	2.55 ± 1.01	0.38 ± 0.66	1.06 ± 0.68
	M	1.61 ± 0.32	10.52 ± 1.42	5.10 ± 0.36	56.94 ± 2.67	21.58 ± 2.91	1.74 ± 0.33	0.65 ± 0.22	1.86 ± 0.54
cki1Δdpl1Δeki1Δ	H	0.77 ± 0.88	11.56 ± 3.66	5.34 ± 1.57	59.22 ± 4.57	16.93 ± 1.12	4.40 ± 0.68	0.20 ± 0.32	1.57 ± 0.58
	M	2.31 ± 1.12	10.80 ± 4.15	5.14 ± 1.36	65.62 ± 5.18	12.15 ± 0.24	1.67 ± 0.39	0.56 ± 0.41	1.75 ± 0.98

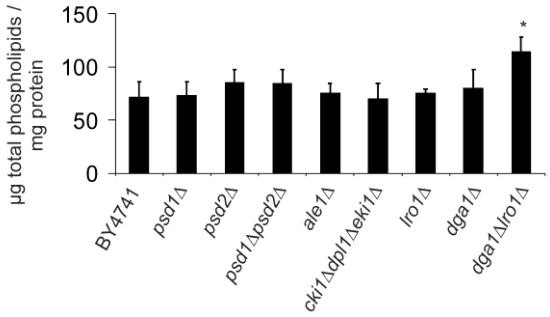

Figure 5. Amounts of total phospholipids from wild type and strains defective in phosphatidylethanolamine and triacylglycerol biosynthesis grown to the stationary phase. The amounts of total phospholipids in µg lipid per mg protein were measured in strains as indicated. Data are mean values of 3 independent experiments with error bars indicating the standard deviation. Significance was calculated by student's t-test (two tailed, unpaired). Values indicated by * correspond to $P<0.05$ and were defined to be significant.

Discussion

Here, we report a novel physiological link between phospholipid metabolism and TAG formation in the yeast *Saccharomyces cerevisiae*. At first sight, this finding appears to be not surprising, because both lipid classes recruit their DAG moiety from the same precursor phosphatidic acid. During exponential growth of the yeast, these precursors are rather channelled to phospholipid synthesis than TAG formation for maintaining membrane formation, cell growth and viability. When cells enter the stationary growth phase, phospholipid biosynthesis is decreased and the surplus of DAG and fatty acids are directed towards TAG formation leading to accumulation of this storage lipid in cytosolic LP. Similarities in the fatty acid compositions of TAG and phospholipids, but also the important role of TAG hydrolysis for sustaining the level of major yeast membrane phospholipids, support the idea of a link between TAG and phospholipid metabolism [49, 50]. Identification of phospholipid:diacylglycerol acyltransferases (PDATs) refined our understanding of the close relationship between phospholipid and TAG metabolism. Previous studies demonstrated that PE is the preferred acyl-donor for the acyl-CoA independent reaction of TAG biosynthesis catalyzed by Lro1p in yeast and plants [13-15]. Since these data were derived from *in vitro* studies, the questions as to the relevance of the Lro1p reaction *in vivo* remained. The usage of PE as a donor of fatty acids for TAG synthesis by Lro1p was of particular interest because this phospholipid is synthesized by four different routes in the yeast (see Introduction). For this purpose, we dissected the

relative contributions of the different PE biosynthetic pathways to TAG synthesis using a set of distinct yeast mutants.

To make lipid metabolic conditions for the yeast as stringent as possible, especially with respect to the requirement for PE, we cultivated cells on minimal media containing lactate as a non-fermentable carbon source. We had demonstrated before that on complex media containing lactate Psd1p became paramount as a PE synthesizing enzyme, and even exogenous supply of Etn could not restore the cellular and mitochondrial levels of PE [31]. An unexpected finding, however, was that the CDP-Etn branch of the Kennedy pathway became almost equally important as Psd1p when cells were grown on minimal lactate media (see Table 3) or on complex media containing oleic acid [33]. Thus, culture conditions and especially the use of different carbon sources affect the various PE biosynthetic pathways. Notably, the growth characteristics of *psd1*Δ and *cki1*Δ*dpl1*Δ*eki1*Δ on lactate media are different. Whereas the mutant bearing defects in the CDP-Etn branch of PE synthesis can grow very well on minimal lactate media, *psd1*Δ requires supplementation with Etn (see Figure 1). The reason for this growth defect might be the specific depletion of mitochondrial PE in the *psd1*Δ mutant strain.

The second striking effect observed with *cki1*Δ*dpl1*Δ*eki1*Δ cultivated under the given conditions was the dramatically decreased TAG level (see Figure 2). This effect was unique among all mutant strains compromised in PE formation and surprising. We had initially speculated that lack of competition for the cellular DAG pool would lead to an increase of TAG in *cki1*Δ*dpl1*Δ*eki1*Δ. This assumption was in line with results obtained with mice where elimination of the CDP-Etn pathway indeed led to an increase of neutral lipid classes [51, 52]. The phosphoethanolamine cytidylyltransferase (ECT)-deficient liver/hepatocytes of these mice displayed 10-fold elevated levels of TAG whereas cholesterol, cholesterol esters, DAG and free fatty acids were about 2-fold higher than controls. Our data, however, demonstrated a profound decrease of cellular TAG when PE formation *via* the CDP-Etn pathway was blunted. This discrepancy between mammalian cells and the yeast is most likely due to different efficiency of PE supply by the various PE biosynthetic pathways. Whereas in the yeast Psd1p is the major enzyme of cellular PE formation [29, 31], in mammalian cells the major route of PE biosynthesis strongly depends on the type of tissue and cell [53]. It was reported that in several cell lines such as CHO cells PE was predominantly supplied by PS decarboxylation, whereas in rat

liver/hepatocytes and hamster heart the CDP-Etn pathway was the major route of PE synthesis. The other marked difference is the existence of the PDAT Lro1p in yeast which has no counterpart in mammalian cells [9, 12-14].

The third striking observation was that the total cellular level of PE is not crucial for TAG synthesis in the yeast. This can be clearly seen from the lipid profiles of mutant strains (see Table 3 and Figure 2). While in *psd1*Δ*psd2*Δ and in *cki1*Δ*dpl1*Δ*eki1*Δ cellular levels of PE were comparably low, the TAG level was dramatically affected only in *cki1*Δ*dpl1*Δ*eki1*Δ. We assume that subcellular localization of the enzymes of the CDP-Etn pathway and Lro1p in the ER is more relevant for the observed effect than the total amount of PE in cells. This view is confirmed by the strongly decreased microsomal level of PE and the significant reduction of Lro1p activity in *cki1*Δ*dpl1*Δ*eki1*Δ which is associated with profound reduction of TAG (see Table 3 and Figure 3).

In summary, the CDP-Etn branch of the Kennedy pathway appears to fulfil two tasks in the yeast. First, it supplies mitochondria with PE which is underlined by the observation that the growth defect of *psd1*Δ can be rescued by Etn (see Figure 1). Secondly, the CDP-Etn branch appears to support TAG formation through the acyl-CoA independent pathway catalyzed by Lro1p. The dramatically reduced TAG level in *cki1*Δ*dpl1*Δ*eki1*Δ (see Figure 2), the reduced Lro1p activity measured *in vitro* (see Figure 3) and the low cellular and microsomal PE content (see Table 3) in this strain support this hypothesis. In summary, we demonstrate that both, subcellular localization of enzymes involved in this process in the ER and availability of PE within this compartment play an important role in the network of phospholipid and TAG metabolism.

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Chapter 3

Processing and Topology of the Yeast Mitochondrial Phosphatidylserine Decarboxylase 1

Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1

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Abstract

The inner mitochondrial membrane plays a crucial role in cellular lipid homeostasis through biosynthesis of the non-bilayer forming lipids phosphatidylethanolamine (PE) and cardiolipin (CL). In the yeast *Saccharomyces cerevisiae*, the majority of cellular PE is synthesized by the mitochondrial phosphatidylserine decarboxylase 1 (Psd1). The biogenesis of Psd1 involves several processing steps. It was speculated that the Psd1 precursor is sorted into the inner membrane and is subsequently released into the intermembrane space by proteolytic removal of a hydrophobic sorting signal. However, components involved in the maturation of the Psd1 precursor have not been identified. We show that processing of Psd1 involves the action of the mitochondrial proteases MPP and Oct1 and an autocatalytic cleavage at a highly conserved LGST motif yielding the α- and β-subunit of the enzyme. The Psd1 β-subunit (Psd1β) forms the membrane anchor, which binds the intermembrane space localized α-subunit (Psd1α). Deletion of a transmembrane segment in the β-subunit results in mislocalization of Psd1 and reduced enzymatic activity. Surprisingly, autocatalytic cleavage does not depend on proper localization to the inner mitochondrial membrane. In summary, membrane integration of Psd1 is crucial for its functionality and for maintenance of mitochondrial lipid homeostasis.

Background: Although phosphatidylserine decarboxylase 1 (Psd1) is of central importance for the generation of cellular phosphatidylethanolamine (PE), its biogenesis is only poorly understood.

Result: Biogenesis of Psd1 involves processing by MPP, Oct1 and an autocatalytic separation of Psd1α from the membrane anchored Psd1β.

Conclusion: Psd1 requires integration into the inner mitochondrial membrane for full enzymatic activity.

Significance: This study presents a new model for the biogenesis and topology of Psd1.

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Keywords: phosphatidylserine decarboxylase 1; phosphatidylethanolamine, processing, lipid, *Saccharomyces cerevisiae*, yeast, mitochondria

Abbreviations: Psd1, phosphatidylserine decarboxylase 1; Psd1α, phosphatidylserine decarboxylase 1 alpha-subunit; Psd1β, phosphatidylserine decarboxylase 1 beta-subunit; Psd1(α+β), phosphatidylserine decarboxylase 1 containing alpha- and beta-subunit; $Psd1_{MET}$, phosphatidylserine decarboxylate 1 containing six C-terminal methionines; PE, phosphatidylethanolamine; CL, cardiolipin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; LP, lysophospholipids; DMPE, dimethylphosphatidylethanolamine; TOM, translocase of the outer mitochondrial membrane; TIM, translocase of the inner mitochondrial membrane; MIA, mitochondrial intermembrane space import and assembly; Ssc1, stress-seventy subfamily C/ mitochondrial Hsp70; OM, outer mitochondrial membrane; MAM, mitochondria associated membrane; ER, endoplasmic reticulum; MPP, mitochondrial processing peptidase; HA, hemagglutinin

Introduction

Mitochondrial membranes contain a high amount of non-bilayer forming phospholipids such as phosphatidylethanolamine (PE) and cardiolipin (CL) (1-4). These lipids have a characteristic conical shape due to the relatively small head group compared to the large hydrophobic acyl chains. Non-bilayer forming phospholipids may locally induce inverted hexagonal (H_{II}) phase structures thereby increasing the tension in the lipid bilayer. This property seems to be important for vesicle formation, membrane fusion, transbilayer transport of lipids and polar solutes, protein interactions, topogenesis as well as protein function (5;6). PE and CL associate with different membrane bound proteins and protein complexes such as the respiratory chain complexes or the mitochondrial ATP/ADP carrier to maintain their stability and function (7-9). As a consequence, lack of CL affects the structural organization of the respiratory chain supercomplexes and protein translocases in both outer and inner mitochondrial membrane (2;10-17). Simultaneous loss of both major non-bilayer forming phospholipids, PE and CL, is lethal for *Saccharomyces cerevisiae* (18). Also in mammals biosynthesis of PE is essential (19), and alterations in content or acylation of CL lead to mitochondrial dysfunction and diseases like Barth syndrome (20;21).

CL synthesis is restricted to mitochondria (22-27), whereas in yeast PE biosynthesis occurs in different cellular compartments including mitochondria, the Golgi/vacuolar compartment and the endoplasmic reticulum (ER) with its mitochondria associated membrane fraction (MAM) (28-39). The mitochondrial phosphatidylserine decarboxylase (Psd1) synthesizes the majority of PE which is not only supplied to mitochondrial but also to other cellular membranes (1;40). Since the substrate of Psd1, phosphatidylserine (PS), is synthesized in the ER/MAM, transport to the site of enzymatic conversion is required. Whether this translocation process involves membrane contact sites like the ER-mitochondria tethering complex ERMES is under debate (41-43). Deletion of *PSD1* leads to ethanolamine auxotrophy during growth on non-fermentable carbon sources, reduced growth on fermentable media and morphological alterations of mitochondria (44;45). Moreover, Psd1 is required for the regulation of pleiotropic drug resistance by inducing *PDR5* gene expression (46). Although extra-mitochondrially synthesized PE can be imported into mitochondria, defects of *psd1*Δ strains cannot be completely compensated by this process indicating that PE uptake by mitochondria has limited efficiency (44;47).

Phosphatidylserine decarboxylases form a protein family, which is highly conserved from bacteria to humans (48). Psd1 from various cell types share a characteristic LGST motif, which is required for autocatalytic cleavage of the enzyme into α- and β-subunits (48-50). This process has been described in some detail for Psd1 homologs of various species (9;46;51-54). It starts with an ester bond formation between Gly-253 and Ser-254 followed by α,β-elimination and release of the mature β-subunit leaving a dehydroalanine residue at the N-terminus of the α-subunit. Hydration and subsequent elimination of ammonia lead to formation of a pyruvoyl prosthetic group at the N-terminus of the α-subunit (9;48;50;51). Mutations of the highly conserved LGST motif were shown to cause defects in Psd1 processing and formation of an inactive form of the enzyme (9;46;53). Surprisingly, such defects have no effect on the induction of *PDR5* expression for regulating multidrug resistance suggesting that this process may occur independently of PS decarboxylation (46).

Although Psd1 is of central importance for the generation of cellular PE, its biogenesis is only poorly understood. Like the vast majority of mitochondrial proteins, Psd1 is synthesized on cytosolic ribosomes as a precursor containing a cleavable signal sequence (36). Upon in vitro import into isolated yeast mitochondria three proteolytic products are formed from the Psd1 precursor indicating a stepwise processing of the polypeptide (55). Psd1 contains a hydrophobic stretch, which was proposed to target the polypeptide to the inner mitochondrial membrane (49;50;56). It was assumed that this putative inner membrane sorting sequence is removed upon import yielding the mature Psd1 localized to the mitochondrial inner membrane/intermembrane space (50;55). This view was supported by enzymatic analyses assigning the enzyme activity to the intermembrane space site of the inner membrane (31;57;58). However, experimental evidence for the functionality of the putative inner membrane sorting sequence, the import route of the Psd1 precursor and the nature of the three processed Psd1 forms were missing. Moreover, the topology of the α- and β-subunits of Psd1 remained unclear.

In a detailed biochemical study presented here we report that in yeast the Psd1 β-subunit (Psd1β) is integrated into the mitochondrial inner membrane and serves as an anchor for the intermembrane space localized α-subunit (Psd1α). Analysis of the individual processing steps of Psd1 showed involvement of the matrix-localized peptidases MPP and Oct1, which remove the N-terminal signal peptides from Psd1. We demonstrate that lack

of the hydrophobic stretch leads to mislocalization and partial inactivation of the enzyme. In contrast, self-cleavage of Psd1 into α- and β-subunits can occur upon mislocalization within mitochondria but strictly depends on the LGST motif and mitochondrial membranes. Thus, correct integration of Psd1 β-subunits into the inner membrane is essential for proper enzymatic function.

Materials and Methods

Strains and culture conditions

Psd1 mutant strains and plasmids used in this study are listed in Table 1. Deletion strains of mitochondrial proteases as well as mas1 temperature sensitive strain, $TOM70_{His}$, $TOM40_{HA}$, $TOM22_{His}$, *tom22*Δ, *tom20*Δ and *tom70*Δ strains and their corresponding wild type have been described before (59-62). The reference wild type strain for *oct1*Δ is a re-expression of *OCT1* in the *oct1*Δ background (63). Yeast cells were grown under aerobic conditions to the logarithmic growth phase at 24°C or 30°C on YPG or YPS media (1% yeast extract (Oxoid), 2% peptone (Oxoid), 3% glycerol (Roth) or 2% sucrose adjusted to pH 5.0 with HCl). The temperature sensitive phenotype of a *mas1* temperature sensitive mutant strain was induced by growth for 6 h at non-permissive temperature (61). In case of yeast strains transformed with expression plasmids, cells were grown on minimal media minus uracil containing 2% galactose (Roth), 0.67% yeast nitrogen base without amino acids (US Biological) and 0.063% amino acid mix without uracil (Roth, Fluka).

Table 1. Strains used in this study.

Strain	Genotype	Source/ Reference
wild type	BY4741 *MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
psd1Δ	BY4741 *MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4*	Euroscarf
WT+pYES2	BY4741 *MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* + pYES2	This study
psd1Δ+pYES2	BY4741 *MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4* + pYES2	This study
Psd1HA	BY4741 *MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4* + pYES2-Psd1HA	This study
Psd1S463A	BY4741 *MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4* + pYES2-Psd1S463A	This study
Psd1ΔIM	BY4741 *MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4* + pYES2-Psd1ΔIM	This study

Plasmid and strain constructions

For the generation of a yeast strain expressing a C-terminally HA-tagged Psd1, *PSD1* was amplified using gene-specific primers from genomic DNA in a standard PCR mixture containing *ExTaq* DNA polymerase (Takara). The purified PCR product was inserted *via* *Bam*HI and *Not*I (Fermentas) into the pYES2 vector (Invitrogen) leading to pYES2-PSD1HA. For the generation of Psd1S463A and Psd1ΔIM (residues from V81 to S100 were deleted), pYES2-PSD1HA was used as a template either for site-directed mutagenesis (QuickChange XL Site-directed Mutagenesis Kit; Stratagene) or overlap-extension PCR. A *psd1*Δ strain was transformed with the described constructs by lithium acetate transformation (64) generating Psd1HA, Psd1S463A, and Psd1ΔIM.

Isolation of mitochondria and submitochondrial localization of Psd1

Mitochondria were isolated by differential centrifugation according to standard procedures (4;65). Mitochondrial fractions were adjusted to a protein concentration of 10 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS-KOH (pH 7.2)), aliquots were shock-frozen in liquid nitrogen and stored at -80°C. Enrichment of marker proteins and cross-contamination of subcellular fractions were assessed as described (4).

The submitochondrial localization of Psd1 was determined by accessibility of Psd1 to externally added proteinase K in intact, hypotonically swollen or lysed mitochondria. For hypo-osmotic swelling of mitochondria, mitoplasts were generated by treating mitochondria with a 9:1 ratio of EM buffer (10 mM MOPS-KOH (pH 7.2) and 1 mM EDTA) and SEM buffer for 10 min on ice. Subsequently, samples were treated with 20-50 µg/ml proteinase K for 15 min on ice. For lysis, mitochondria were treated with Triton X-100 at a final concentration of 0.5% (v/v) prior to addition of proteinase K. In general, proteinase K activity was stopped by addition of 2 mM PMSF and incubation for 10 min on ice. Subsequently, mitochondria were re-isolated by centrifugation (16,000xg, 10 min, 4°C) and washed with SEM buffer. Samples were subjected to SDS-PAGE and Western Blot analysis. To determine membrane association of proteins carbonate extraction was used as described previously (65-68). In brief, isolated mitochondria were resuspended in freshly prepared 0.1 M sodium carbonate buffer at pH 10.8 or pH 11.5 and incubated on ice for 30 min. Mitochondrial membranes were re-isolated by ultracentrifugation (100,000xg, 40 min, 4°C). The pellet was solubilized in SDS-PAGE loading dye whereas

proteins remaining in the supernatant were precipitated by trichloroacetic acid. Samples were subjected to SDS-PAGE and Western Blot analysis.

Import of precursor proteins into isolated mitochondria and microsomes

For *in vitro* transcription, a PCR generated template containing the SP6 promoter was used. RNA was purified (MEGAclear kit; Invitrogen) and used for *in vitro* translation (TNT kit; Promega) in the presence of ^{35}S-labeled methionine. Import of ^{35}S-labeled precursor proteins into isolated yeast mitochondria (corresponding to 50 µg protein content) was performed at 25°C in the presence of 2 mM NADH, 2 mM ATP and an ATP-regenerating system (5 mM creatine phosphate and 0.1 mg/ml creatine kinase) in import buffer (3% BSA (w/v), 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 10 mM MOPS-KOH (pH 7.2)) (65). Import reactions were stopped on ice and by dissipation of the membrane potential with 8 µM antimycin A, 1 µM valinomycin and 20 µM oligomycin. For protease treatment, samples were incubated with 50 µg/ml proteinase K for 15 min on ice. Mitochondria were re-isolated by centrifugation (16,000xg, 10 min, 4°C) and washed with SEM buffer. For pulse-chase experiments, ^{35}S-labeled Psd1 was imported into mitochondria for 5 min under standard import conditions (pulse). Subsequently, mitochondria were re-isolated, washed and incubated a second time under import conditions but without addition of new ^{35}S-labeled Psd1 precursor (chase). Samples were subjected to SDS-PAGE and ^{35}S-labeled proteins were detected by autoradiography. For blue-native electrophoresis, mitochondria were solubilized with digitonin buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 10% glycerol) containing 1% (w/v) digitonin. After a clarifying spin, mitochondrial protein complexes were separated by blue-native electrophoresis as described (69;70).

The import of ^{35}S-labeled precursor proteins into dog pancreatic microsomes (Promega) was carried out following the standard import protocol for isolated mitochondria. Membranes were re-isolated by centrifugation (125,000xg, 30 min, 4°C) and washed with SEM buffer. Membranes were lysed under denaturing conditions and subjected to SDS-PAGE analysis.

Affinity purification of tagged proteins

Mitochondria were solubilized in digitonin buffer containing 1% (w/v) digitonin at a protein concentration of 1 mg/ml for 20 min on ice. After a clarifying spin, the supernatant was incubated with an anti-HA matrix (Roche) under constant rotation for 1 h at 4°C. Following several washing steps with digitonin buffer containing 0.3% (w/v) digitonin, proteins were eluted and subjected to SDS-PAGE, Western Blot analysis and immunodetection with the indicated antisera. For affinity purification of arrested precursor, ^{35}S-labeled Psd1 was imported in the absence of a membrane potential into mitochondria containing $Tom22_{His}$, $Tom40_{HA}$ or $Tom70_{His}$. Subsequently, pull down experiments were performed as described (59-62).

Phospholipid Analysis

Lipids were extracted from total cell free homogenate and mitochondria by the procedure of Folch *et al.* (1957) using chloroform/methanol (2:1; v/v) (71). Remaining contaminants, e. g. proteins and salts were removed by additional washing steps of the organic phase with 0.034% $MgCl_2$ solution (w/v), 2 N KCl/methanol (4:1; v/v), and methanol/water/chloroform (48:47:3; per vol), respectively. Individual phospholipids were separated by two-dimensional thin-layer chromatography on Silica gel 60 plates (Merck) by using chloroform/methanol/25% NH_3 (68:35:5; per vol) as the first and chloroform/acetone/methanol/acetic acid/water (53:20:10:10:5; per vol) as the second developing solvent. Phospholipids were visualized on thin-layer chromatography plates by staining with iodine vapour, scraped off and quantified as described (72).

Psd1 Activity Assay

The enzymatic activity of Psd1 was determined *in vitro* by measuring the conversion of ^{3}H-labeled PS to ^{3}H-labeled PE in isolated mitochondria from yeast cells grown to the logarithmic growth phase as reported by Kuchler *et al.* (1986) (31). Radioactively labeled ^{3}H-PS was synthesized *in vitro* by incubating isolated yeast microsomes (8 mg protein) with 0.02/0.2 mM CDP-DAG, 5 mM hydroxylamine, 0.6 mM $MnCl_2$, 0.2% Triton X-100, 0.5 mM L-serine, 0.01 µCi L-^{3}H-serine (specific activity 21.99 Ci/mmol), and 0.1 M Tris-HCl (pH 8.0) in a total volume of 2 ml for 2 h at 30°C. The reaction was stopped by addition of 3 ml chloroform/methanol (2:1; v/v) and lipids were extracted as previously

described (71). The Psd1 activity assay was performed in a final volume of 1.5 ml containing 100 nmol ^{3}H-PS (specific activity: 0.64 nCi/nmol), 0.1 M Tris-HCl (pH 7.2), 10 mM EDTA and 1 mg protein from isolated mitochondria (31;47). Incubations were carried out for 7 min at 30°C and terminated by addition of 3 ml chloroform/methanol (2:1; v/v). Lipids were extracted and individual phospholipids were separated as described above. After visualization with iodine vapour, PE was scraped off, and radioactivity was measured by liquid scintillation counting using LSC Safety (Baker) with 5% water as scintillation cocktail.

Results

The β-subunit of Psd1 anchors the α-subunit to the inner mitochondrial membrane

A special feature of Psd1 maturation is its structural arrangement in the form of an α- and a β-subunit (Figure 1A; Figure 1B, lanes 1, 4). Interestingly, the precise topology of Psd1, especially of the two non-identical subunits within mitochondrial compartments, has not yet been determined. To address this issue in detail, we used a specific antibody recognizing the β-subunit of the protein (Figure 1B, lane 1) and generated a yeast strain expressing Psd1 with an HA-tag C-terminally fused to the α-subunit (Figure 1A). HA-tagging did not affect maturation of Psd1 because the mature β-subunit of Psd1-HA behaved like wild type Psd1 (data not shown). Use of an HA-specific antibody allowed us to detect a 4 kDa α-subunit of Psd1 in isolated mitochondria (Figure 1B, lane 4). The appearance of the α-subunit was not affected by deletion of the predicted inner membrane sorting signal of Psd1 (Psd1ΔIM) (Figure 1B, lane 6). To further test the specificity of both antibodies, we constructed a yeast strain expressing a Psd1S463A variant with a mutation at the LGST cleavage site, which cannot undergo autocatalytic processing (Figure 1A) (9;46;53). As expected, both antibodies recognized unprocessed Psd1 (Figure 1B, lanes 2, 5) and could therefore be used to determine the submitochondrial localization of both Psd1 subunits.

For this purpose, we treated intact, hypotonically swollen or lysed mitochondria with proteinase K (Figure 1C). Both α- and β-subunits were protected in intact mitochondria but accessible to the protease after rupture of the outer membrane by osmotic swelling (Figure 1C, lanes 2, 4). Similarly, the inner membrane protein Tim23 with a domain exposed to the

intermembrane space was degraded after rupture of the outer membrane (Figure 1C, lanes 2, 4). The outer membrane protein Tom70 was proteolytically cleaved on intact mitochondria, whereas the matrix localized Tim44 was digested only after lysis of mitochondria with detergent (Figure 1C, lane 6). These results indicate that both subunits of Psd1 face the intermembrane space, which is in line with the reported detection of Psd1 activity (31;57;58).

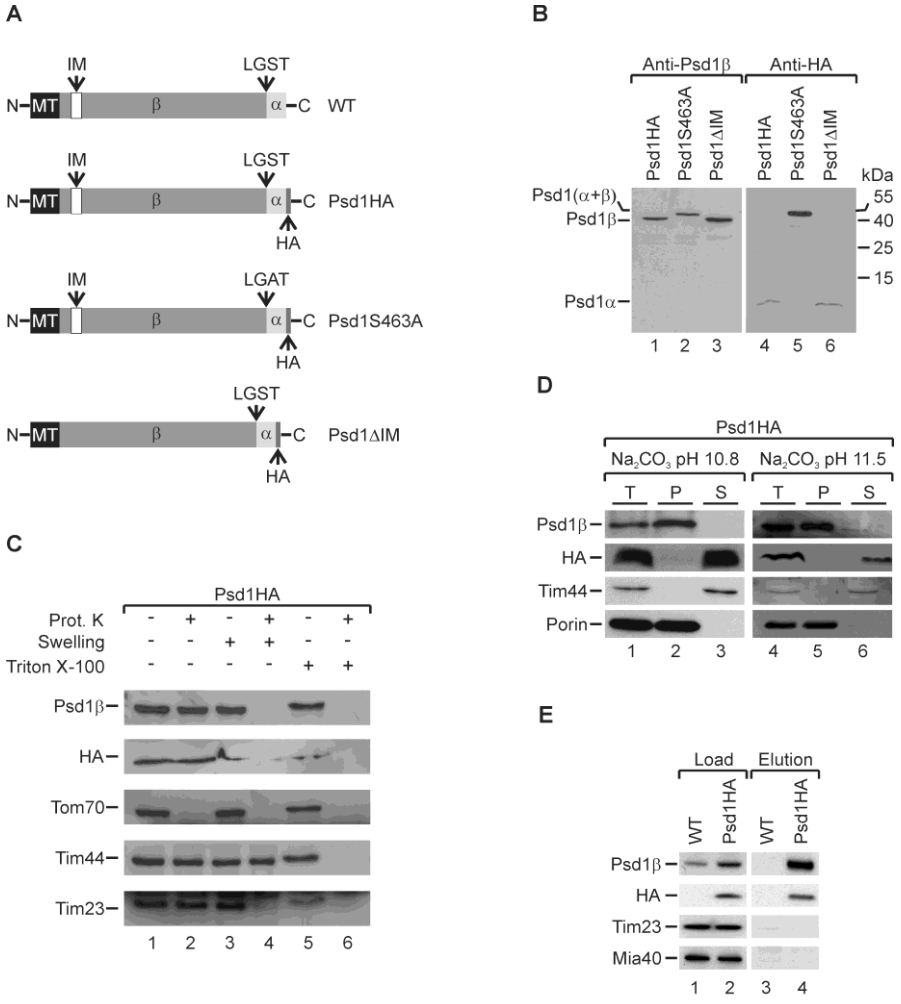

Figure 1. The β-subunit of Psd1 tethers the Psd1 α-subunit to the inner membrane. (A) The used Psd1 constructs are shown schematically. MT, predicted mitochondrial targeting sequence; IM, putative inner membrane sorting signal. (B) Mitochondrial proteins from yeast strains expressing Psd1HA, Psd1S463A or Psd1ΔIM were separated by SDS-PAGE and visualized by immunodetection with the indicated antibodies. (C) Intact, swollen or with Triton X-100 lysed mitochondria were incubated with or without proteinase K. Subsequently, samples were subjected to SDS-PAGE and the indicated components were visualized by immunodetection with the respective antisera. (D) Mitochondria were subjected to carbonate extraction. Total (T), membrane bound (P) and soluble proteins (S) were separated by SDS-PAGE and components were visualized by immunodetection with the indicated antisera. (E) Psd1HA was precipitated with an anti-HA affinity matrix. Load (5%) and eluted proteins (100%) were separated by SDS-PAGE and proteins were visualized by immunodetection with the indicated antisera.

Previously, it was speculated that Psd1 might be released into the intermembrane space by cleavage of a proposed inner membrane sorting signal (50;55), although experimental evidence for this hypothesis was missing. To test a possible membrane association of Psd1 we used a well established alkaline extraction method (66;67). This approach revealed that the Psd1 β-subunit (Psd1β) remained membrane associated after carbonate extraction at pH 10.8 and pH 11.5 similar to the outer membrane protein porin, whereas the peripheral membrane protein Tim44 was efficiently removed (Figure 1D). We concluded from this result that the β-subunit was integrated into the inner mitochondrial membrane. In contrast, the α-subunit was extracted under all tested conditions and therefore localized to the intermembrane space (Figure 1D). Furthermore, we tested attachment of both subunits to each other after autocatalytic processing in the $PSD1_{HA}$ strain by pull-down experiments utilizing an anti-HA affinity matrix (Figure 1E). The Psd1 β-subunit was efficiently co-purified with the HA-tagged α-subunit (Figure 1E, lane 4) indicating stable association of both Psd1 subunits. Thus, the β-subunit of Psd1 contains a membrane anchor tethering the soluble α-subunit to the inner mitochondrial membrane.

Tom70 and Tom22 are the main mitochondrial receptors for the Psd1 precursor

Localization of Psd1 to the inner membrane raises the question about the import mechanism of this protein into mitochondria. In general, the majority of mitochondrial proteins are translated on cytosolic ribosomes as precursors with an N-terminal signal sequence, which is sufficient for targeting and import into mitochondria. The translocase of the outer mitochondrial membrane (TOM complex) is crucial for the translocation of precursor proteins across the outer membrane (73-77). First, we tested whether Psd1 was imported *via* the TOM complex at all by importing ^{35}S-labeled Psd1 precursor into mitochondria isolated from a yeast strain lacking Tom22 (Figure 2A). Deletion of Tom22 results in destabilization of the TOM complex and blocks the import of the majority of TOM-dependent substrates into mitochondria (78). Upon import into isolated mitochondria three forms of Psd1 were detected (Figure 2A, lanes 1-3), which were absent in the reticulocyte lysate showing that processing does not occur spontaneously (data not shown). The three fragments represent intermediates 1 and 2 (i_1 and i_2) formed by unknown processing steps and presumably the mature Psd1 β-subunit (m) (Figure 2A, lanes 1-3) (55). Intermediate 1 was not completely taken up by mitochondria since it was accessible

to externally added proteinase K and predominantly formed in the absence of a membrane potential (Figure 2A, lanes 1-4). In contrast, intermediate 2 and the mature Psd1β were only formed in the presence of the membrane potential and showed protease resistance (Figure 2A, lanes 1-3). Thus, we conclude that intermediate 2 and the mature Psd1β were fully imported into mitochondria. In the absence of Tom22, import of the precursor into isolated mitochondria was strongly reduced indicating the general requirement of the TOM complex for biogenesis of Psd1 (Figure 2A). The TOM complex has two peripheral receptors, Tom20 and Tom70. Tom20 was reported to recognize precursor proteins with cleavable signal information whereas Tom70 mainly interacts with hydrophobic precursors harboring internal non-cleavable targeting information (3;73-79).

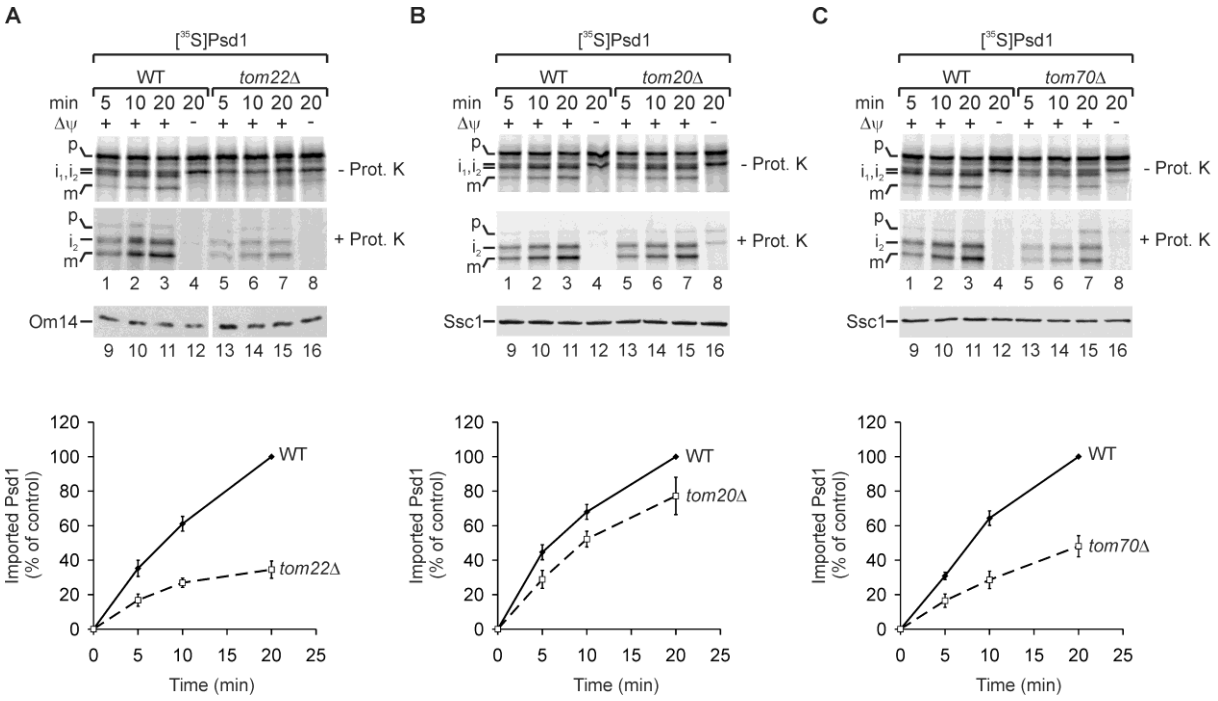

Figure 2. Tom70 and Tom22 are the main receptors for the import of Psd1 precursor into mitochondria. (A-C) ^{35}S-labeled Psd1 precursor was imported in the presence or absence of a membrane potential (Δψ) into mitochondria from wild type, *tom22*Δ (A), *tom20*Δ (B) or *tom70*Δ (C). Subsequently, mitochondria were incubated with or without proteinase K. ^{35}S-labeled proteins were detected by autoradiography. The time dependent formation of the mature form of Psd1β (m) in mitochondria was quantified by using ImageJ. The longest import time point of Psd1β (m) into wild type mitochondria was set to 100%. Mean values of four measurements with standard error of the mean are shown. Immunodetection with the indicated antisera was used as a loading control. p, precursor; i_1,i_2, intermediate 1 and 2; m, mature Psd1β.

To test which peripheral receptor was involved in the import of Psd1 precursor, we performed import experiments with mitochondria from yeast strains lacking either Tom20 or Tom70. Surprisingly, import of Psd1 into mitochondria lacking Tom20 was only mildly affected (Figure 2B) but significantly reduced in the absence of Tom70 (Figure 2C). Thus, although Psd1 harbors a cleavable signal sequence, Tom70 and Tom22 are the main receptors for the import of Psd1 precursor into mitochondria.

MPP and Oct1 cleave the signal sequence of Psd1

The import of ^{35}S-labeled Psd1 precursor into mitochondria leads to formation of three fragments (see Figure 2A) (53;55). However, the molecular mechanism of proteolytic cleavage and the nature of these fragments remained unclear. Previously, it was speculated that the inner membrane sorting sequence might be removed by a protease like Yme1 (50;55). However, the observed membrane integration of the β-subunit point to a different processing mode. To identify the processing proteases required for Psd1 maturation, we analyzed the protein pattern of Psd1 in mitochondria from strains lacking described or predicted mitochondrial proteases (61) using the antibody specific for Psd1 β-subunit (Figure 3A). Surprisingly, deletion of Yme1 did not affect the size of mature Psd1 in mitochondria at all (Figure 3A, lane 4). Also processing of freshly imported Psd1 was not affected in the absence of Yme1 (data not shown). Similarly, the inner membrane proteases Imp1 and Imp2, both required for maturation of mitochondrial proteins destined to the intermembrane space (74;76;80;81), had no effect on Psd1 maturation (data not shown).

Whereas deletion of several other mitochondrial proteases did not affect the formation of Psd1β, a small size shift of Psd1 was observed in mitochondria lacking Oct1 (octapeptidyl aminopeptidase) (Figure 3A, lane 8). It is known that the matrix localized Oct1 typically removes an octapeptide from a precursor intermediate after cleavage by the mitochondrial processing peptidase (MPP) to stabilize the client protein (63). To confirm processing of the Psd1 precursor by Oct1, we imported ^{35}S-labeled Psd1 into mitochondria lacking this protease (Figure 3B). We observed a size shift of intermediate 2 (i_2) and of the mature Psd1 β-subunit (m) indicating that Oct1 indeed removed a peptide from the Psd1 precursor (Figure 3B, lanes 3, 7).

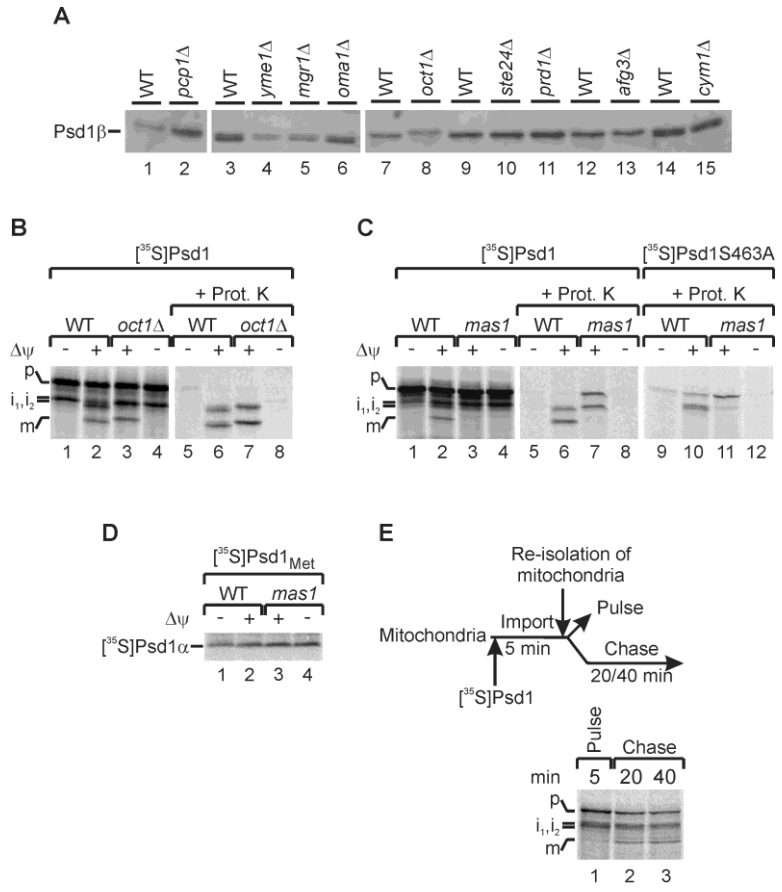

Figure 3. MPP and Oct1 process the Psd1 precursor. (A) Mitochondrial proteins from several deletion strains were analyzed on SDS-PAGE. The β-subunit of Psd1 was visualized by immunodetection with a Psd1-specific antibody. (B) ^{35}S-labeled Psd1 precursor was imported in the presence or absence of a membrane potential (Δψ) into mitochondria from wild type or *oct1*Δ. Subsequently, mitochondria were incubated with or without proteinase K. ^{35}S-labeled proteins were detected by autoradiography. p, precursor; i_1,i_2, intermediate 1 and 2; m, mature Psd1β. (C) ^{35}S-labeled Psd1 and Psd1S463A precursor were imported in the presence or absence of a membrane potential (Δψ) into mitochondria from wild type or *mas1ts* mutant strain. Subsequently, mitochondria were incubated with or without proteinase K. ^{35}S-labeled proteins were detected by autoradiography. p, precursor; i_1,i_2, intermediate 1 and 2; m, mature Psd1β. (D) ^{35}S-labeled $Psd1_{Met}$ containing 6 methionines at the C-terminus of Psd1 was imported in the presence or absence of a membrane potential (Δψ) into mitochondria from wild type or *mas1ts* mutant strain. ^{35}S-labeled Psd1α was detected by autoradiography. (E) Experimental scheme and pulse-chase experiment of ^{35}S-labeled Psd1 into wild type mitochondria. ^{35}S-labeled Psd1 was imported into wild type mitochondria for 5 min at 25°C under standard import conditions (lane 1; pulse). After re-isolation and washing steps, mitochondria were further incubated under import conditions but without addition of new ^{35}S-labeled precursor (lane 2, 3; chase). ^{35}S-labeled proteins were detected by autoradiography. p, precursor; i_1,i_2, intermediate 1 and 2; m, mature Psd1β.

The amino acid sequence of the Psd1 precursor suggests the presence of a classical N-terminal mitochondrial signal sequence (36). Such signal sequences are typically removed by MPP of the mitochondrial matrix, which consists of the two essential subunits Mas1 and Mas2 (74;80;81). To address the role of MPP in processing of Psd1 we imported the Psd1 precursor into mitochondria isolated from a temperature sensitive Mas1 mutant (61;62). *Mas1ts* was inactivated *in vivo* by shifting the culture to non-permissive growth conditions. In isolated mitochondria of the *in vivo* heat-shocked *mas1ts* cells removal of the signal peptide from intermediate 2 and the mature Psd1β was blocked (Figure 3C, lanes 3, 7). Thus, the N-terminal presequence of the Psd1 precursor is cleaved by the matrix located proteases MPP and Oct1 yielding Psd1β (m).

So far, we only speculated that the mature Psd1β is formed by self-processing, which leads to the formation of two protease protected bands upon import of Psd1 in *mas1ts* (Figure 3C, lane 7). To test this notion experimentally, we imported a Psd1 variant into wild type and *mas1ts* mitochondria which contains a point mutation in the conserved LGST motif to prevent autocatalytic cleavage (9;46;53). This Psd1S463A construct was imported with the same efficiency as wild type but cleavage into α- and β-subunit was blocked (Figure 3C, lane 10). In *mas1ts* mitochondria only the upper band was formed indicating that the mature Psd1β emerged upon self-cleavage in a MPP independent manner (Figure 3C, lane 11). To confirm this conclusion we analyzed formation of the α-subunit of Psd1 in *mas1ts* mitochondria directly. The C-terminus of Psd1 was tagged with six methionines to allow detection of ^{35}S-labeled Psd1α. This $Psd1_{Met}$ construct was imported like wild type Psd1 (data not shown) and the formation of Psd1α with a size of 4 kDa was not affected in *mas1ts* mitochondria (Figure 3D, lanes 3, 4). To determine at which import step autocatalysis occurred, we performed a pulse-chase experiment with ^{35}S-labeled Psd1 (Figure 3E). ^{35}S-labeled Psd1 was imported for a short time period (pulse: Figure 3E, lane 1). Subsequently, mitochondria were re-isolated and further incubated under import conditions but without addition of new ^{35}S-labeled precursor (chase: Figure 3E, lanes 2, 3). Interestingly, the mature Psd1β was formed more efficiently after the chase but only to a minor extent during the short pulse period (Figure 3E). This observation indicated that self-cleavage of Psd1 occurs at late import time points and follows processing by MPP and Oct1 under normal import conditions.

TOM complex-arrested Psd1 precursor is processed into α- and β-subunit.

Strikingly, Psd1α is also formed in the absence of a membrane potential which indicates that self-cleavage of Psd1 can occur independently of its import into mitochondria (Figure 3D, lane 1). Since intermediate 1 is also formed in the absence of a membrane potential (Figure 3B, lane 1) we wondered whether intermediate 1 represented a self-processed Psd1 precursor. To address this issue we performed import experiments with the Psd1S463A precursor in wild type mitochondria (Figure 4A, lanes 5-8). Interestingly, intermediate 1 was not generated during import of the Psd1S463A precursor demonstrating that intermediate 1 is formed by self-processing of Psd1 into α- and β-subunit. To assess the efficiency of the self-cleavage by arrested Psd1, we quantified the formation of Psd1α in the presence and absence of a membrane potential (Figure 4B). We found that albeit import into mitochondria favors self-processing, a marked amount of arrested Psd1 precursor undergoes autocatalysis as well (Figure 4B, lane 3).

It has been shown before that upon depletion of the membrane potential some hydrophobic precursors accumulate at the TOM complex (12;82). To test whether or not the arrested Psd1 precursor binds to the TOM complex as well, we incubated ^{35}S-labeled Psd1 precursor with isolated mitochondria in the absence of a membrane potential (Figure 4C). After the import, mitochondria were lysed using the mild detergent digitonin, and samples were analyzed by blue-native electrophoresis to detect the TOM-bound intermediate. In the absence of a membrane potential, the Psd1 precursor was stably bound to the TOM complex as shown by a size shift of the intermediate after adding antibodies against Tom5 or Tom22 to mitochondria (Figure 4C, lanes 3, 6). To analyze whether both precursor and intermediate 1 bind to the TOM complex, we arrested ^{35}S-labeled Psd1 at the translocases in mitochondria containing His-tagged Tom22, Tom70 or HA-tagged Tom40 and performed affinity purification (Figure 4D). These experiments revealed that intermediate 1 and unprocessed precursors were co-purified with tagged TOM subunits (Figure 4D, lanes 4, 8, 12).

To determine if Tom receptors play a role in self-cleavage of Psd1, we analyzed the formation of Psd1α in mutants lacking one Tom receptor (Figure 4E). Only the absence of Tom70 slightly affected Psd1α formation (Figure 4E, upper panel). This observation indicated that Tom70 is involved in the initial recognition of the Psd1 precursor whereas Tom22 is involved in later import steps. Altogether, in the absence of a membrane

potential autocatalytic cleavage of the Psd1 precursor into α- and β-subunits occurred when the precursor is exposed on the outer membrane and binds to the TOM complex.

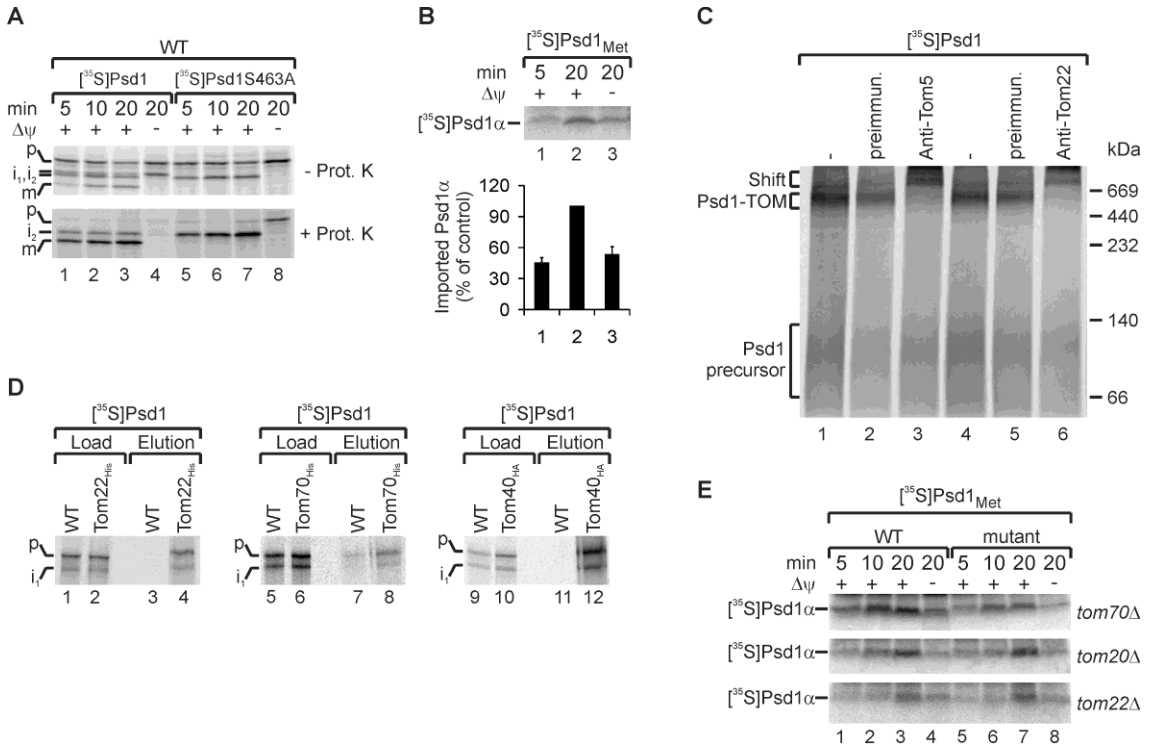

Figure 4. TOM complex-arrested Psd1 precursor undergoes autocatalytic processing. (A) ^{35}S-labeled Psd1 and Psd1S463A precursor were imported in the presence or absence of a membrane potential (Δψ) into wild type mitochondria. Subsequently, mitochondria were incubated with or without proteinase K. ^{35}S-labeled proteins were detected by autoradiography. p, precursor; i_1,i_2, intermediate 1 and 2; m, mature Psd1β. (B) ^{35}S-labeled $Psd1_{Met}$ containing 6 methionines at the C-terminus of Psd1 was imported in the presence or absence of a membrane potential (Δψ) into mitochondria from wild type. ^{35}S-labeled Psd1α was detected by autoradiography. The time dependent formation of Psd1α in mitochondria was quantified by using ImageJ. The longest import time point of Psd1α into wild type mitochondria in the presence of a membrane potential was set to 100%. Mean values of three measurements with standard error of the mean are shown. (C) ^{35}S-labeled Psd1 precursor was imported in the absence of a membrane potential into wild type mitochondria. Mitochondria were incubated with or without antibodies against Tom5 or Tom22, or pre-immune sera as a control. Subsequently, protein complexes of lysed mitochondria were separated by blue-native gel electrophoresis. ^{35}S-labeled proteins were detected by autoradiography. (D) ^{35}S-labeled Psd1 precursor was imported in the absence of a membrane potential into wild type, $Tom22_{His}$ $Tom70_{His}$ and $Tom40_{HA}$ mitochondria. Subsequently, mitochondria were lysed and subjected to affinity purification utilizing the His- and HA-tag. Load (4%) and eluted proteins (100%) were separated by SDS-PAGE and analyzed by autoradiography. p, precursor; i_1, intermediate 1. (E) ^{35}S-labeled $Psd1_{Met}$ precursor was imported in the presence or absence of a membrane potential (Δψ) into mitochondria from wild type, *tom70*Δ, *tom20*Δ and *tom22*Δ. ^{35}S-labeled Psd1α was detected by autoradiography.

Psd1 requires integration into the inner mitochondrial membrane for full enzymatic activity

The surprising finding that an arrested Psd1 precursor likely undergoes self-processing at the outer membrane tempted us to speculate that separation of α- and β-subunit may also occur in other mitochondrial compartments or even on other membranes. To test this hypothesis we followed the processing of ^{35}S-labeled Psd1 precursor after incubation with mitochondria and microsomes under the same import conditions. We could not detect any cleavage of the Psd1 precursor when canine pancreatic microsomes were added (Figure 5A, lanes 3, 4). This result indicates that Psd1 maturation depends on mitochondrial membranes which is consistent with the results obtained with *Plasmodium knowlesi* (PkPSD) (54). We also tested whether sorting of Psd1 to the inner membrane is required for self-processing and function. To address this issue, we generated a yeast strain expressing a Psd1 variant that lacks the predicted inner membrane anchor (V81-S100; Psd1ΔIM) (Figure 1A) (46). Detection of both α- and β-subunits using the subunit specific antibodies described above demonstrated that Psd1ΔIM undergoes autocatalytic processing (Figure 1B, lane 3, 6; Figure 5B). In contrast to Psd1HA, the Psd1ΔIM α- and β-subunit were not accessible to proteases after rupturing the outer membrane by osmotic swelling (Figure 5B, lane 4). Like the matrix localized Tim44, Psd1ΔIM was only degraded by proteinase K when membranes were lysed with detergent (Figure 5B, lane 6).

This result indicated that Psd1ΔIM was fully transported to the matrix site of the inner membrane and that the predicted inner membrane anchor was crucial for correct topology. To compare the processing efficiency of both constructs, import experiments with ^{35}S-labeled Psd1ΔIM and wild type Psd1 into isolated mitochondria were performed (Figure 5C). Deletion of the putative inner membrane anchor in Psd1ΔIM did even stimulate import and processing steps of the precursor by MPP and Oct1 (Figure 5C, lanes 5-8).

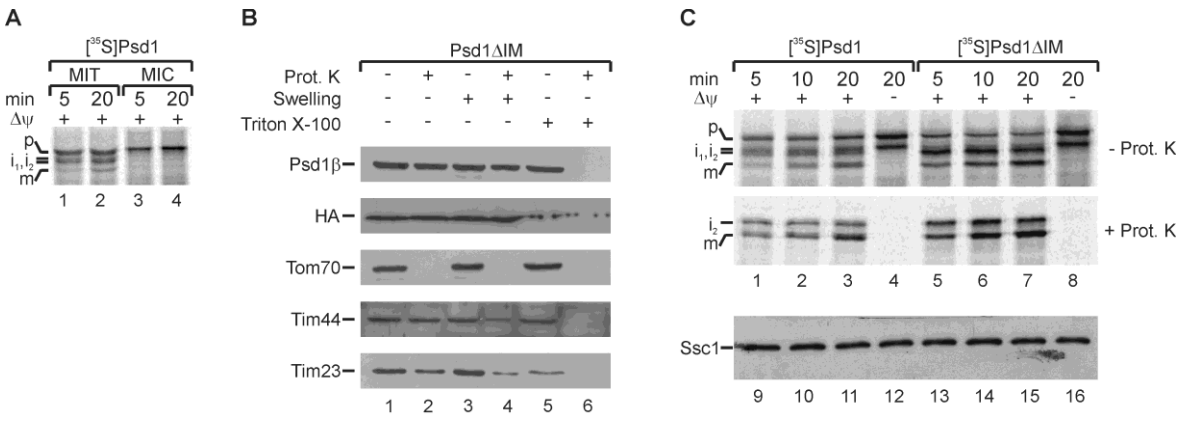

Figure 5. Autocatalytic processing of Psd1 is restricted to mitochondria but does not depend on the proper sorting of Psd1. (A) ^{35}S-labeled Psd1 precursor was imported in the presence of a membrane potential ($\Delta\psi$) into wild type mitochondria (MIT) and canine pancreatic microsomes (MIC). p, precursor; i_1,i_2, intermediate 1 and 2; m, mature Psd1β. (B) Intact, swollen or with Triton X-100 lysed mitochondria from a yeast strain expressing Psd1ΔIM were incubated with or without proteinase K. Subsequently, samples were subjected to SDS-PAGE and proteins were visualized by immunodetection with the indicated antisera. (C) ^{35}S-labeled Psd1 or Psd1ΔIM was imported in the presence or absence of a membrane potential ($\Delta\psi$) into wild type mitochondria. Subsequently, mitochondria were incubated with or without proteinase K. ^{35}S-labeled proteins were detected by autoradiography. For loading controls the mitochondrial Hsp70 (Ssc1) was immunodetected with specific antibodies. p, precursor; i_1,i_2, intermediate 1 and 2; m, mature Psd1.

Finally, we determined the effect of Psd1ΔIM mislocalization on the activity of the enzyme (Figure 6A) and on the mitochondrial phospholipid pattern (Figure 6B). Mitochondria from a strain expressing Psd1S463A did not display detectable decarboxylase activity due to the inability to generate the active site of the enzyme. In contrast, mislocalization of Psd1 by removing the membrane anchor of the β-subunit (Psd1ΔIM) led to a reduced enzymatic activity compared with wild type and HA-tagged Psd1 (Figure 6A). The loss of Psd1 activity in Psd1S463A led to a typical *psd1*Δ phospholipid pattern which is characterized by an enrichment of PC at the expense of PE in mitochondria and homogenate (Figures 6B and 6C) (18;47). The level of PS within mitochondria was not increased as it is used as a substrate for Psd2, which is localized to the Golgi/vacuolar compartment and responsible for extramitochondrial synthesized PE (37;38). Interestingly, the phospholipid pattern of Psd1ΔIM in homogenate resembled that of Psd1S463A (Figure 6C). These findings indicated that mislocalization of Psd1 to the mitochondrial matrix leads to a reduced activity of the enzyme (Figure 6A) and consequently to insufficient supply of mitochondrially synthesized PE to the total cellular PE pool (Figure 6C).

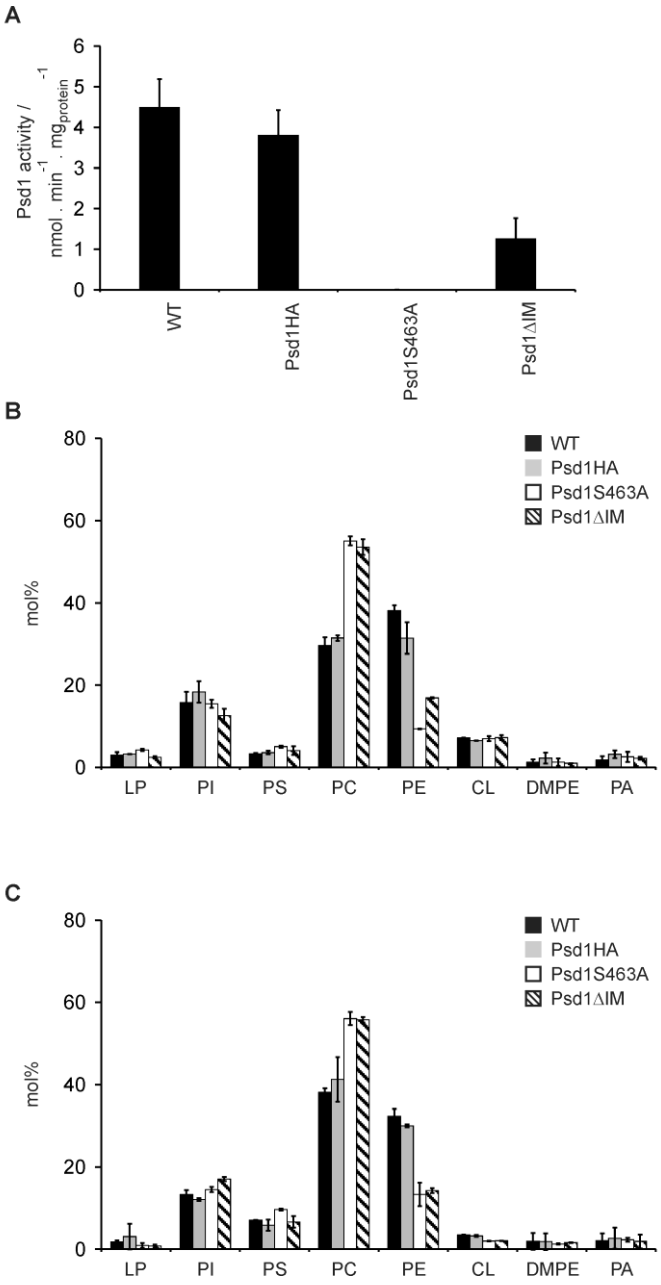

Figure 6. Inner membrane localization of Psd1 is required for optimal enzymatic activity. (A) Phosphatidylserine decarboxylase activity was measured using isolated mitochondria from wild type and indicated Psd1 mutants. Strains were grown on minimal galactose media at 30°C. Mean values of three independent measurements and standard deviations are shown. (B) – (C) Phospholipids were extracted from mitochondria (B) and homogenate (C) isolated from wild type and the indicated Psd1 mutants. Strains were grown on minimal galactose media at 30°C. LP, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid. Mean values of two independent measurements and standard deviations are shown.

Discussion

In yeast, Psd1 catalyzes formation of the majority of cellular PE by decarboxylation of PS (1;28;31;37-39;83). Deletion of *PSD1* leads to severe cell damage including alterations of mitochondrial morphology and reduced growth under respiratory conditions caused by dramatically reduced PE levels in mitochondrial and other cellular membranes (44;45). For this reason, functional Psd1 is highly important to maintain cell integrity and function.

Here we show that correct import and sorting is required for the enzymatic activity of Psd1. In previous experiments it was shown that GFP-tagged Psd1 localizes to the inner mitochondrial membrane (47). However, the question remained open whether the protein was integrated into the membrane or sorted into the intermembrane space, especially due to the occurrence of two non-identical subunits (46;50;53;55). In the present study we demonstrate that the β-subunit of Psd1 is tightly anchored into the inner membrane, most likely *via* a single transmembrane segment. This conclusion is supported by the prediction of at least one transmembrane region as reported previously (53;55). Deletion of this hydrophobic segment (amino acids V81 to S100) (46;54). leads to mislocalization of Psd1 to the matrix side of the inner membrane, partial loss of enzymatic activity and consequently to reduced PE levels (Figure 5B and 6). In such a construct, however, cleavage of the proenzyme to its non-identical subunits was not inhibited (Figure 1B). This result suggests that not only correct processing of Psd1 but also the membrane environment plays a crucial role for the formation of an active enzyme. Since the soluble α-subunit harbors the active site of the enzyme or at least an essential part of this domain, the recruitment to the inner membrane surface with orientation to the intermembrane space appears to be crucial for the ability of Psd1 to decarboxylate the polar head group of PS. The soluble α-subunit present in the intermembrane space interacts with the membrane bound β-subunit (Figure 1D and 1E).

To support the topological model of Psd1 presented in this study, we investigated the mitochondrial import route of this protein and identified components required for its processing. Here, we show that the two matrix-localized processing peptidases MPP and Oct1 remove the targeting signals from the Psd1 precursor (Figure 3C). Finally, the processed Psd1 undergoes self-cleavage into α- and β-subunit (Figure 3E). MPP usually cleaves the mitochondrial signal sequence, and subsequently Oct1 removes a small peptide, typically an octapeptide (80;81). The predicted MPP cleavage site is after leucine

48 of Psd1. Moreover, the Psd1 precursor contains a sequence typical for an Oct1 cleavage motif, namely arginine at position -10 and phenylalanine at position -8 of the putative Oct1 cleavage site after serine at position 56. In general, the second processing step catalyzed by Oct1 is considered to stabilize the N-terminus (63). Sequential cleavage by MPP and Oct1 presumably leads to a stabilizing glycine at the N-terminus of the mature Psd1 (61;63). Proteases, which typically remove inner membrane sorting sequences, like Imp1 and Imp2 (80;81) had no effect on Psd1 processing. This finding is in line with the observation that the hydrophobic inner membrane sorting sequence is not proteolytically removed and can therefore serve as a membrane anchor for Psd1. Thus, the yeast mitochondrial Psd1 β-subunit is embedded into the inner membrane like its bacterial counterpart (48).

Surprisingly, we discovered that cleavage of the Psd1 precursor into α- and β-subunits, which is a crucial step in the formation of the active center of the enzyme, can also take place when the precursor is arrested at the TOM complex of the outer mitochondrial membrane by depletion of the membrane potential (Figure 4B and 4D). Even mislocalization of the protein to the matrix site of the inner membrane did not affect its self-cleavage (Figure 5B and 5C). Thus, separation of the two subunits does not depend on proper maturation and targeting of Psd1 within mitochondria. Interestingly, self-processing of the Psd1 precursor into Psd1α at the outer membrane is only mildly affected by deletion of Tom70 whereas deletion of other Tom receptors had no effect on this processing step (Figure 4E). Thus, Tom70 provides an initial docking site for the incoming Psd1 precursor but is not strictly required for autocatalysis of Psd1. Other components of the mitochondrial surface or specific membrane properties may be important for the autocatalytic processing of Psd1 as well. Choi *et al.* (2012) reported that processing of Psd from *Plasmodium knowlesi* (PkPSD) into α- and β-subunits was influenced by its lipid surrounding (54). *In vitro* experiments using liposomes demonstrated that processing of PkPSD to the mature enzyme form and Psd activity were enhanced by increasing concentrations of DOPS (dioleoyl phosphatidylserine), whereas other anionic phospholipids such as DOPA (dioleoyl phosphatidic acid) or DOPG (dioleoyl phosphatidylglycerol) displayed an inhibitory effect (54). Future studies will be necessary to reveal whether a mitochondria-specific lipid composition contributes to induce the self-processing of Psd1 because this step does not occur when the precursor is incubated with microsomal membranes (Figure 5A).

In summary, our studies demonstrate clearly that insertion of Psd1 into the inner membrane/intermembrane space is paramount for the formation of an enzymatically active protein. The specific membrane environment may contribute to this process. Correct topology of Psd1 at the inner membrane-intermembrane space interface appears to be important for proper access of the amphipathic substrate PS with its polar head group and hydrophobic tails to the active center of the enzyme.

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Chapter 4

Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins

Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins

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Abstract

The mitochondrial outer membrane contains proteinaceous machineries for the import and assembly of proteins, including the translocase of the outer membrane (TOM) and the sorting and assembly machinery (SAM). It has been shown that the dimeric phospholipid cardiolipin (CL) is required for the stability of TOM and SAM complexes and thus for the efficient import and assembly of β-barrel proteins and some α-helical proteins of the outer membrane. Here we report that mitochondria deficient in phosphatidylethanolamine (PE), the second non-bilayer forming phospholipid, are selectively impaired in the biogenesis of β-barrel proteins, but not of α-helical outer membrane proteins. The stability of TOM and SAM complexes is not disturbed by the lack of PE. By dissecting the import steps of β-barrel proteins, we show that an early import stage involving translocation through the TOM complex is affected. In PE-depleted mitochondria, the TOM complex binds precursor proteins with reduced efficiency. We conclude that PE is required for the proper function of the TOM complex but unlike CL is not important for the structural integrity of the translocase.

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Introduction

Mitochondrial outer membrane proteins fulfill essential functions in the eukaryotic cell, including transport of metabolites and communication with the cytosol and other organelles. Porin, also termed voltage-dependent anion channel (VDAC), mediates the transport of metabolites (Colombini, 2012). The translocase of the outer membrane (TOM) forms the main entry gate for mitochondrial precursor proteins that are synthesized on cytosolic ribosomes (Koehler et al., 2004; Dolezal et al., 2006; Neupert and Herrmann, 2007; Baker et al., 2007; Mihara, 2000; Endo and Yamano, 2009; Becker et al., 2012). The endoplasmatic reticulum (ER)-mitochondria encounter structure (ERMES) tethers mitochondria to the ER (Kormann et al., 2009) and the outer membrane harbors components important for mitochondrial fusion and fission (Okamoto and Shaw, 2005; Hoppins et al., 2007; Westermann, 2010). Additionally, the outer membrane plays an important role in apoptosis (Taylor et al., 2008). Thus, the biogenesis of outer membrane proteins and their correct assembly into protein machineries is essential for mitochondrial function.

The outer membrane contains two types of integral membrane proteins: proteins with α-helical transmembrane segments and β-barrel proteins. All outer membrane proteins are synthesized on cytosolic ribosomes and are targeted to the mitochondrial surface. β-barrel precursor proteins are transported via the protein-conducting channel Tom40 of the TOM complex (Wiedemann et al., 2003; Paschen et al., 2003). Subsequently, small Tim chaperones of the intermembrane space guide the β-barrel precursors to the sorting and assembly machinery (SAM) (Paschen et al., 2003; Wiedemann et al., 2003, 2004; Hoppins and Nargang, 2004; Gentle et al., 2004). The SAM complex, also termed TOB (topogenesis of mitochondrial outer membrane β-barrel proteins) complex, binds to a conserved β-signal of the precursor protein and mediates the insertion into the outer membrane (Paschen et al., 2003; Wiedemann et al., 2003; Gentle et al., 2004; Ishikawa et al., 2004; Chan and Lithgow, 2008; Kutik et al., 2008a; Walther et al., 2009). For α-helical outer membrane proteins, a common import pathway has not been found. The receptor protein Tom70 and the mitochondrial import proteins Mim1 and Mim2 play a crucial role in the biogenesis of multi-spanning outer membrane proteins like Ugo1, whereas TOM core subunits or the SAM complex are not involved (Otera et al., 2007; Becker et al., 2011a; Papic et al., 2011; Dimmer et al., 2012). Additionally, Mim1 is also involved in the

import of single-spanning outer membrane proteins like Tom20 and small Tom proteins (Becker et al., 2008; Hullet et al., 2008; Popov-Celecetic et al., 2008; Becker et al., 2011a; Papic et al., 2011; Dimmer et al., 2012). A specialized SAM form containing the mitochondrial distribution and morphology protein 10 (Mdm10) mediates biogenesis of Tom22, whereas other single-spanning precursor proteins insert independently of known proteinaceous factors but can be impaired by elevated levels of ergosterol (Meisinger et al., 2004; Setoguchi et al., 2006; Stojanovski et al., 2007a; Meineke et al., 2008; Kemper et al., 2008; Becker et al., 2011b; Krumpe et al., 2012; Merklinger et al., 2012).

The mitochondrial outer membrane contains two non-bilayer forming phospholipids, cardiolipin (CL) and phosphatidylethanolamine (PE) (Sperka-Gottlieb, 1988; Zinser et al., 1991; Gebert et al., 2009). In yeast mitochondria, CL synthase (Crd1) and phosphatidylserine decarboxylase 1 (Psd1) catalyze the formation of CL and PE, respectively (Trotter et al., 1993; Clancey et al., 1993; Jiang et al., 1997; Tuller et al., 1998; Chang et al., 1998; Birner et al., 2001; Horvath et al., 2012). Minor amounts of mitochondrial PE can also be provided by the activity of Psd2 at the vacuole/Golgi membranes, the acyl-transferases Tgl3 and Ale1 and the CDP-ethanolamine pathway (Trotter and Voelker, 1995; Bürgermeister et al., 2004; Riekhof et al., 2006, 2007; Rajakumari and Daum, 2010). Additionally, several mitochondrial proteins were reported to regulate the levels of CL and PE (Birner et al., 2003; Tamura et al., 2006, 2009, 2012; Kutik et al., 2008b; Kornmann et al., 2009; Osman et al., 2009, 2011; Kuroda et al., 2011; Ngyuen et al., 2012). Lack of CL affects stability and function of various mitochondrial membrane protein complexes, including the respiratory chain of the inner membrane (Jiang et al., 2000; Pfeiffer et al., 2003; Zhang et al., 2005; van der Laan et al., 2007; Claypool et al., 2008; Kutik et al., 2008b; Mileykovskaya and Dowhan, 2009; Tamura et al., 2006, 2009; Gallas et al., 2006; Gonzalvez et al., 2008; DeVay et al., 2009). Although the outer membrane contains only small amounts of CL, lack of CL causes destabilization of and reduced precursor binding by the TOM and SAM complexes. The biogenesis of β-barrel proteins and the assembly of some α-helical outer membrane proteins are impaired in CL-deficient mitochondria (Gebert et al., 2009). A double deletion of *PSD1* and *CRD1* is lethal, suggesting overlapping functions of PE and CL that are essential for cell viability (Gohil et al., 2005). Depletion of CL as well as of PE lead to a decreased activity of the respiratory chain, which in turn impairs preprotein import into the inner membrane and

matrix due to a reduced inner membrane potential (Jiang et al., 2000; Kutik et al., 2008b; Böttinger et al., 2012). CL and PE also have overlapping functions in the fusion of mitochondria (Joshi et al., 2012). However, since neither respiratory activity nor mitochondrial fusion are strictly essential for cell viability of yeast, the effects of CL and PE on respiration and fusion cannot explain the synthetic lethality of the double deletion.

The role of PE in biogenesis of mitochondrial outer membrane proteins has not been addressed so far, although PE represents about 33% of the outer membrane phospholipids (Sperka-Gottlieb et al., 1988; Zinser et al., 1991). For this report, we analyzed the biogenesis of outer membrane proteins in PE-depleted mitochondria. While no defect in the biogenesis of α-helical proteins was observed, the import of β-barrel proteins was impaired at the stage of translocation through the TOM complex. The stability of TOM and SAM complexes was not affected, yet the TOM complex bound precursor proteins with reduced efficiency. We conclude that PE is required for the efficient function of the TOM machinery.

Results and Discussion

Biogenesis of β-barrel proteins is impaired in PE-deficient mitochondria

To study the role of PE in the biogenesis of mitochondrial outer membrane proteins we isolated mitochondria from a *psd1*Δ yeast strain and a *psd1*Δ*psd2*Δ double deletion strain (Birner et al., 2001; Horvath et al., 2011; Böttinger et al., 2012). As expected, the levels of PE were strongly reduced (supplementary material Fig. S1A). The levels of phosphatidylcholine, phosphatidylinositol and phosphatidylserine were increased in the mutant mitochondria, whereas the CL content was unchanged (supplementary material Fig. S1A) (Birner et al., 2001). To study biogenesis of β-barrel proteins we imported the ^{35}S-labeled precursor of Tom40 into isolated wild-type and mutant mitochondria. The import of this model β-barrel precursor allows the analysis of distinct assembly steps, which can be visualized by blue native gel electrophoresis (Fig. 1A, lanes 1-3) (Model et al., 2001; Wiedemann et al., 2003; Paschen et al., 2003; Ishikawa et al., 2004; Humphries et al., 2005; Chan and Lithgow, 2008; Dukanovic et al., 2009; Becker et al., 2010). Upon short import times the Tom40 precursor binds to the SAM complex. Subsequently, the precursor is released to form a second intermediate (Int-II) and finally assembles into the mature

TOM complex of about 450 kDa (Wiedemann et al., 2003). In the PE-depleted mutant mitochondria, all assembly steps of Tom40 were reduced (Fig. 1A, lanes 4-9).

We analyzed the biogenesis of three outer membrane proteins that contain α-helical transmembrane segments: Tom22, Tom20 and Ugo1. In contrast to the strong defect in Tom40 assembly, the assembly of ^{35}S-labeled Tom22 and Tom20 into the TOM complex was not inhibited in *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria (Fig. 1B, C). Similarly, import of Ugo1, determined by formation of the mature dimer (Becker et al., 2011a; Papic et al., 2001), was not decreased in the mutant mitochondria, but even enhanced (Fig. 1D). We conclude that PE-depleted mitochondria are impaired in the import of β-barrel proteins but not in the biogenesis of several α-helical outer membrane proteins.

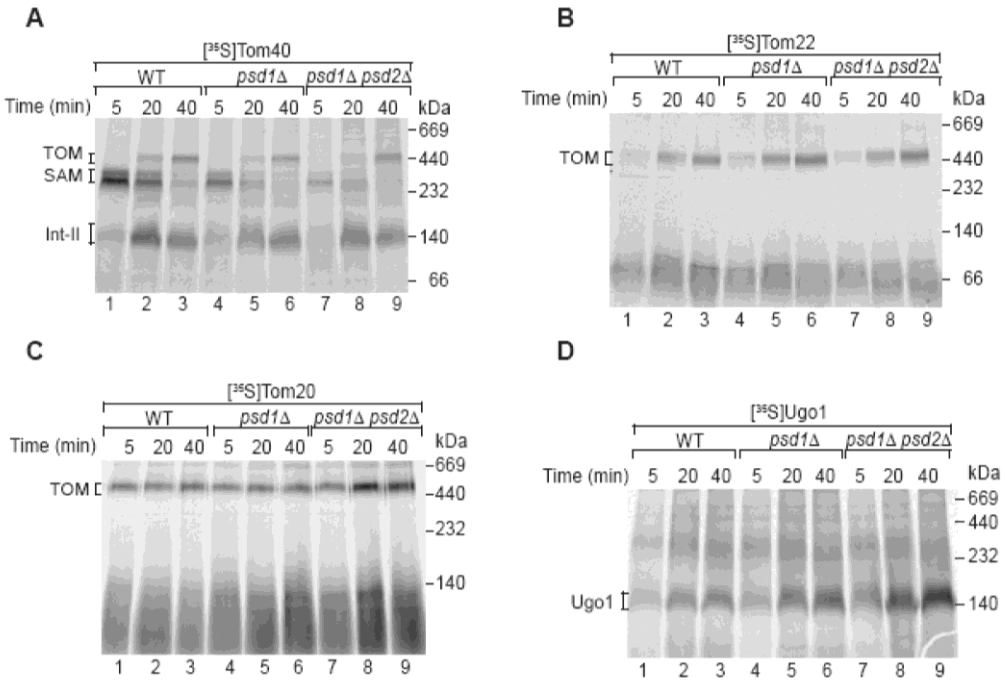

Figure 1. The biogenesis of β-barrel proteins is impaired in PE-depleted mitochondria. ^{35}S-labeled Tom40 (A), Tom22 (B), Tom20 (C) or Ugo1 (D) were imported into isolated wild-type, *psd1*Δ or *psd1*Δ*psd2*Δ mitochondria at 25°C for the indicated periods. The mitochondria were lysed with digitonin and analyzed by blue native electrophoresis and digital autoradiography.

Transport of β-barrel proteins across the TOM complex is impaired in PE-depleted mitochondria

To address how PE-depletion may impair β-barrel biogenesis, we first analyzed the steady state levels of mitochondrial proteins, including subunits of TOM, SAM and Mim1, as well as control proteins of internal mitochondrial compartments. The protein levels were not altered to a major extent and thus could not explain the strong defect in Tom40 assembly (supplementary material Fig. S1B).

In *crd1*Δ mitochondria lacking CL, the assembly of Tom40 is reduced; here, the assembly defect is at least partially caused by a destabilized SAM complex (Gebert et al., 2009). We asked if the SAM complex was affected in PE-depleted mitochondria. We lysed mitochondria with the non-ionic detergent digitonin and studied outer membrane protein complexes by blue native electrophoresis (Fig. 2A). The two SAM complexes, SAM_{core} and SAM-Mdm10 (Meisinger et al., 2004; Meisinger et al., 2007; Wideman et al., 2010 Thornton et al., 2010, Yamano et al., 2010a; Yamano et al., 2010b; Becker et al., 2011b), as well as further outer membrane protein complexes, Mim1, porin and Om45, were not or only mildly affected by depletion of PE (Fig. 2A).

In order to identify the PE-dependent stage of β-barrel biogenesis, we dissected the biogenesis of Tom40 into distinct steps. To analyze binding of the β-barrel precursor to the SAM complex, we imported a mutant form of Tom40, which is blocked in release from the SAM complex due to a single amino acid exchange in the β-signal (Kutik et al., 2008a). The mutant Tom40 precursor efficiently accumulates at the SAM complex of wild-type mitochondria (Fig. 2B, lanes 1-3). Binding of the Tom40 precursor to the SAM complex was impaired in *psd1*Δ mitochondria and strongly inhibited in *psd1*Δ*psd2*Δ mitochondria (Fig. 2B, lanes 4-9). Thus, PE is involved in an early step of the Tom40 assembly pathway, at the stage of binding to the SAM complex or at an earlier stage leading to the SAM complex.

Precursors of β-barrel proteins are initially imported by the TOM complex to the intermembrane space side (Model et al., 2001; Krimmer et al., 2001; Wiedemann et al., 2003; Paschen et al., 2003; Hoppins and Nargang, 2004; Wiedemann et al., 2004; Habib et al., 2005). This initial import step cannot be resolved by blue native electrophoresis as no blue native-stable intermediate is formed, but can be analyzed by the protection against externally added protease (Wiedemann et al., 2003; Paschen et al., 2003; Wiedemann et

al., 2004; Hoppins and Nargang, 2004; Chan and Lithgow, 2008; Dukanovic et al., 2009). We imported Tom40 into the mutant mitochondria and determined the accessibility to added proteinase K. The amount of protease-protected Tom40 precursor was reduced in the PE-depleted mitochondria (Fig. 2C, lower panel). Moreover, the binding of Tom40 precursor to the mitochondrial surface was moderately decreased (Fig. 2C, upper panel). We conclude that PE is required at an early stage of Tom40 import into mitochondria that takes place before binding to the SAM complex, i.e. for the initial translocation of the precursor across the outer membrane by the TOM complex. The analysis of Tom22 assembly supports the conclusion that the SAM complex was not generally (unspecifically) damaged by lack of PE since the biogenesis of Tom22 depends on each SAM subunit (Meisinger et al., 2004; Stojanovski et al., 2007a; Dukanovic et al., 2009; Thornton et al., 2010; Becker et al., 2011b; Lackey et al., 2011) but is not altered in PE-depleted mitochondria (Fig. 1B).

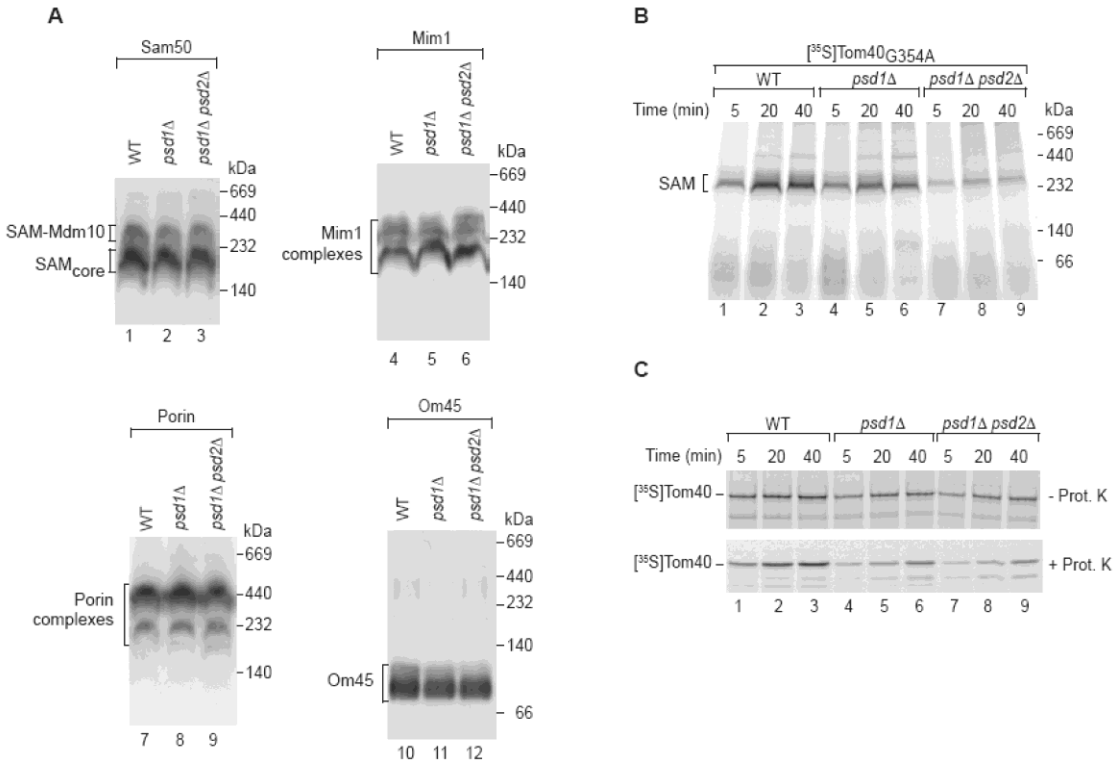

Figure 2. Impaired transport of β-barrel precursors across the outer membrane of PE-depleted mitochondria. (A) Wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria were lysed with digitonin and separated by blue native electrophoresis. Protein complexes were detected by immunodecoration with the indicated antisera. (B) ^{35}S-labeled Tom40$_{G354A}$ was imported into wild-type, *psd1*Δ or *psd1*Δ*psd2*Δ. The imported proteins were analyzed by blue native electrophoresis and autoradiography. (C) ^{35}S-labeled Tom40 was imported into wild-type, *psd1*Δ or *psd1*Δ*psd2*Δ with or without subsequent treatment with proteinase K. Proteins were separated by SDS-PAGE.

PE is required for the function but not for the stability of the TOM complex

We asked if PE was required for the integrity and/or function of the TOM complex. We resolved the TOM complex by blue native electrophoresis. For *crd1*Δ mitochondria, it has been shown that the interaction of Tom20 with the TOM complex is disturbed (Fig. 3A, lane 11) (Gebert et al., 2009). In contrast, the TOM complex of *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria was not altered on blue native electrophoresis. Tom40, Tom22 and Tom20 were present in the mature TOM complex in both mutants to the same extent as in wild-type mitochondria (Fig. 3A, lanes 1-9).

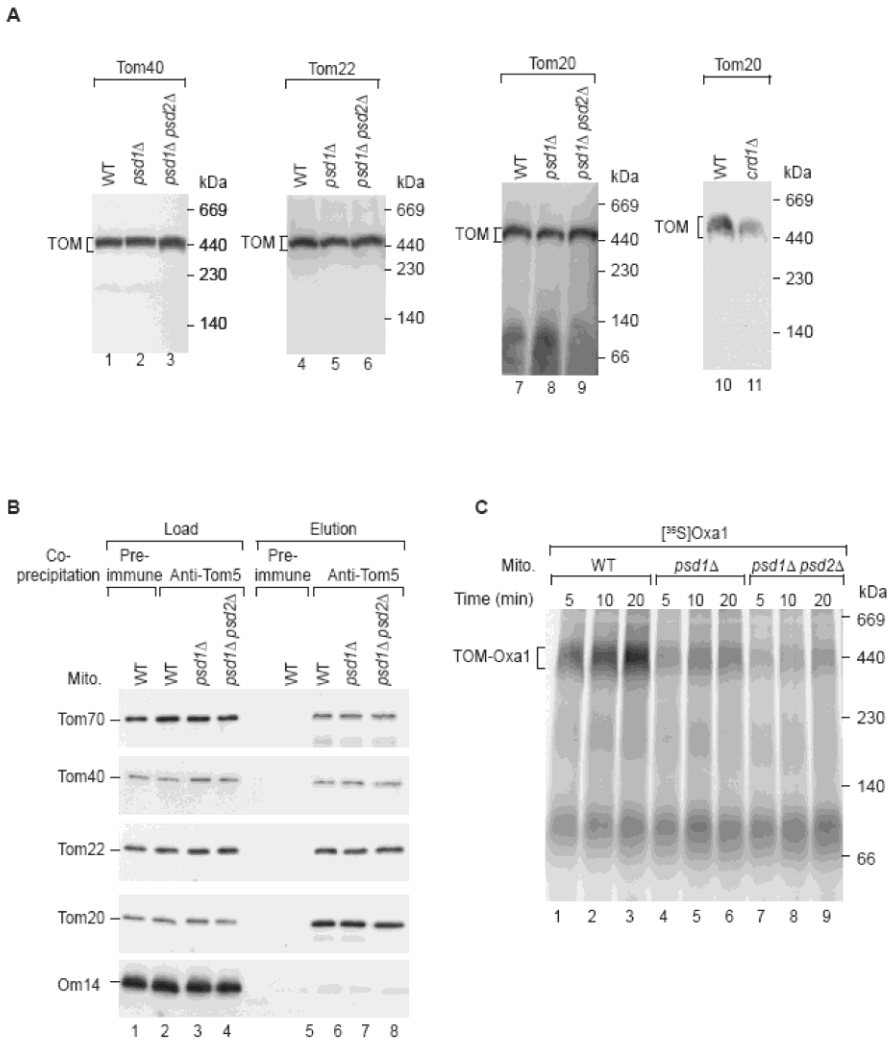

Figure 3. PE is required for precursor binding to the TOM complex. (A) Wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria were lysed with digitonin and separated by blue native electrophoresis. Protein complexes were detected by immunodecoration with the indicated antisera. (B) Wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria were lysed with digitonin and subjected to co-immunoprecipitation with the indicated antisera. Proteins were eluted, separated by SDS-PAGE and detected by immunodecoration with the indicated antisera. Load 4%; elution 100%. (C) ^{35}S-labeled Oxa1 was imported into wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria in the absence of a membrane potential. The samples were analyzed by blue native electrophoresis and autoradiography.

Furthermore, co-immunoprecipitation with Tom5-specific antibodies revealed that the reduced PE content did not disturb the association of the receptors Tom20, Tom22 and Tom70 with the Tom40-Tom5 core of the TOM complex (Fig. 3B) (van Wilpe et al., 1999). Thus, the stability of the TOM translocon was not altered in *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria. To directly analyze the activity of the TOM complex in the interaction with preproteins, we used the inner membrane-targeted Oxa1 precursor. In the absence of an inner membrane potential, Oxa1 is efficiently arrested at the TOM complex of the outer membrane and forms a blue native-stable intermediate (Frazier et al., 2003; Chacinska et al., 2005; Gebert et al., 2009). The interaction of ^{35}S-labeled Oxa1 with the TOM complex was strongly decreased in PE-depleted mitochondria (Fig. 3C). In the *psd1*Δ*psd2*Δ mitochondria, the formation of the Oxa1-TOM intermediate was virtually blocked (Fig. 3C), demonstrating that PE is required for proper function of the TOM complex.

Conclusions

We report that PE is involved in the biogenesis of mitochondrial β-barrel proteins. PE is required for the initial translocation of the precursor protein across the outer membrane via the TOM complex. The capability of the TOM complex to interact with precursor proteins is strongly compromised in PE-depleted mitochondria. The biogenesis of outer membrane proteins with α-helical membrane anchors, which are not transported through the TOM channel (Becker et al., 2011a; Papic et al., 2011), is not inhibited by depletion of PE, demonstrating that the protein import activity of the outer membrane is not generally impaired. PE is not required for the structural integrity of the TOM and SAM complexes, whereas lack of CL results in destabilization of both TOM and SAM (Gebert et al., 2009). Thus, both non-bilayer forming phospholipids CL and PE are required for the biogenesis of mitochondrial β-barrel proteins, but play distinct roles. Whereas CL affects the stability and thus also the function of several translocase complexes, PE plays a selective role for the activity of the TOM complex. Since Tom40 is essential for cell viability, its biogenesis is rate-limiting for the growth of yeast (Dolezal et al., 2006; Neupert and Herrmann, 2007; Endo and Yamano, 2009; Chacinska et al., 2009; Dukanovic and Rapaport, 2010; Becker et al., 2012). The involvement of both CL and PE in the biogenesis pathway of Tom40 provides a possible explanation for the synthetic lethality of a double deletion of *PSD1* and *CRD1* (Gohil et al., 2005).

Materials and Methods

Yeast strains, growth conditions and isolation of mitochondria

The yeast strains *crd1*Δ, *psd1*Δ, *psd1*Δ*psd2*Δ and the corresponding wild-type BY4741 have been described (Gebert et al., 2009; Horvath et al., 2011). Cells were grown in YPLac or in YPG medium (Kutik et al., 2008b) at 21 – 30°C to an early logarithmic growth phase. Mitochondria were isolated, adjusted to a protein concentration of 10 mg/ml, aliquoted and shock frozen with liquid nitrogen as described (Stojanovski et al., 2007b).

Protein import into mitochondria

For import studies, ^{35}S-labeled precursor proteins were synthesized with the coupled transcription/translation kit (TNT Kit, Promega, Madison, USA). The import was performed as described (Stojanovski et al., 2007b). For protease treatment, proteinase K was added to a final concentration of 50 µg/ml and the samples were incubated for 15 min on ice. The activity of the protease was blocked by addition of PMSF to a final concentration of 2 mM. For blue native electrophoresis, mitochondria were solubilized with 1% (w/v) digitonin in digitonin buffer (20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol) for 15 min on ice. After a clarifying spin (16,100xg, 10 min, 4°C) samples were loaded on a blue native gel. The blue native gel was prepared as described (Stojanovski et al., 2007b).

Co-immunoprecipitation

For co-immunoprecipitation, mitochondria were solubilized with 1% digitonin in digitonin buffer and incubated with protein-A sepharose (GE Healthcare, Chalfont St. Gilles, UK) coupled to Tom5-specific antibodies or preimmune antibodies. Binding was performed for 1 h at 4°C under constant rotation. After excessive washing, bound proteins were eluted with 0.1 M glycine, pH 2.5, and subjected to SDS-PAGE.

Determination of mitochondrial phospholipid distribution

Lipids were extracted from isolated mitochondria with chloroform/methanol (2:1; v/v) as described (Folch et al., 1957). Subsequently, washing steps of the organic phase with 0.034% (w/v) $MgCl_2$ solution, 2 N KCl/methanol (4:1; v/v) and methanol/water/chloroform (48:47:3; per vol.) were performed. Phospholipids were separated by thin-layer chromatography as desbribed (Horvath et al., 2012). Phospholipids were detected by iodine vapour, scrapped off and quantified (Broekhuyse, 1968).

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Supplementary Material

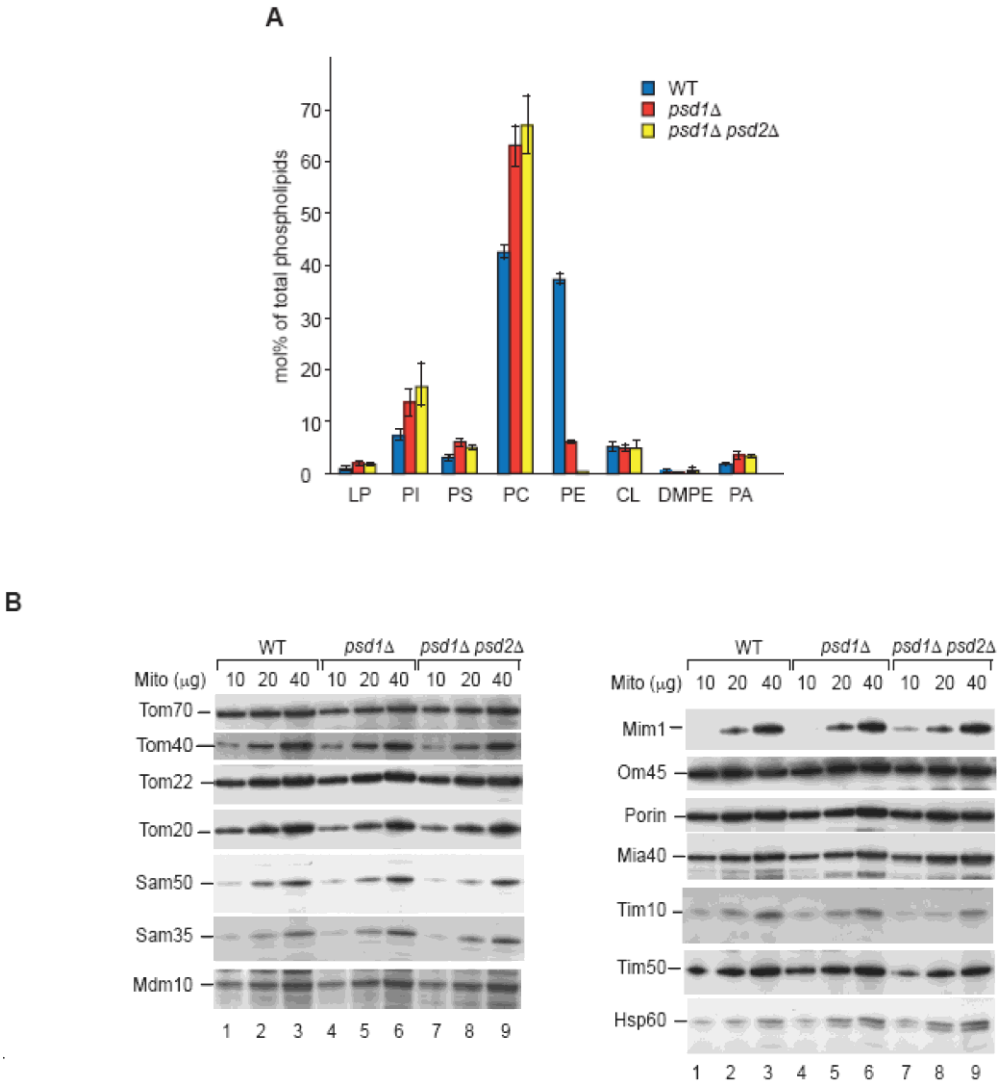

Figure S1. Analysis of phospholipids and proteins of *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria. (A) The phospholipid distribution of isolated wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria was determined. Mean values and standard deviation are shown (n = 4). CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LP, lysophospholipids. (B) Proteins of isolated wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria were analyzed by SDS-PAGE and detected by immundecoration with the indicated antisera.

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Chapter 5

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

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

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Abstract

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 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: AAC, ADP/ATP carrier; CL, cardiolipin; Crd1, Cardiolipin synthase; $\Delta\psi$, mitochondrial membrane potential; $F_1\beta$, β subunit of F_1F_o-ATP synthase; PE, phosphatidylethanolamine; Psd1, phosphatidylserine decarboxylase 1; TIM22, carrier translocase of inner mitochondrial membrane; TIM23, presequence translocase of inner mitochondrial membrane

Communication

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 comparatively 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 the CDP-ethanolamine pathway and the acyl-transferases 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 the 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 bc1 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 (TOM complex) 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 $\Delta\psi$, and thus the decrease of $\Delta\psi$ in CL-deficient mitochondria is a main reason for the decrease of protein import.[35,39,57] In addition, the stability and function of protein translocases like the TIM23 complex are affected when CL is absent and 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. 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.

Phosphatidylethanolamine-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 the 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 nearly absent in the double deletion mutant (1%) (Fig. S1b).[6] We determined the phospholipid content of purified inner membrane vesicles in these mutants and observed a moderate reduction of the phospholipid/protein ratio in comparison to wild-type (Fig. S1c).

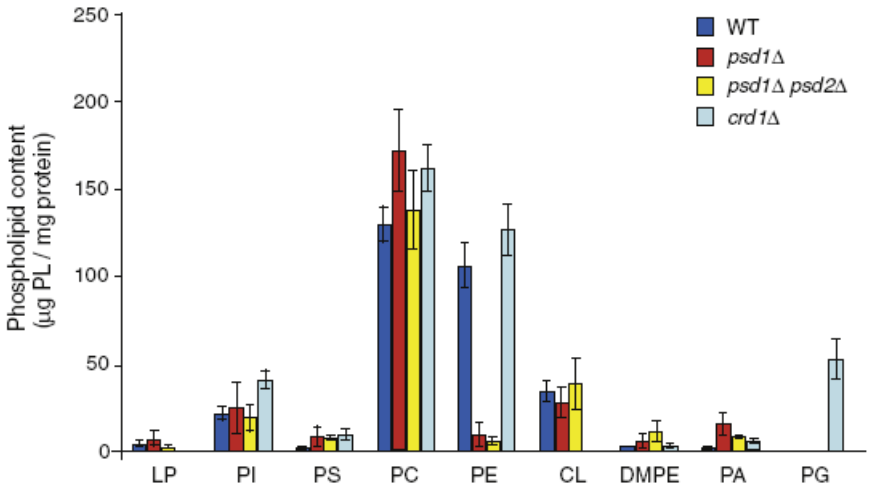

Figure 1. PE is selectively depleted in inner membrane vesicles from *psd1*Δ and *psd1*Δ*psd2*Δ. The *S. cerevisiae* strains *crd1*Δ, *psd1*Δ and *psd1*Δ*psd2*Δ in the BY4741 background[69] were grown in YPLac medium[39] at 30°C to early logarithmic growth phase. Mitochondria were isolated by differential centrifugation, the protein concentrations were adjusted and inner membrane vesicles were isolated by sucrose gradient centrifugation as described.[4,92] Phospholipids were extracted, separated by thin-layer chromatography and analyzed as reported previously.[69,90,91] The absolute amounts of the single phospholipid species were determined with the help of a calibration curve that was generated with a standard phosphor solution containing 1 mg/ml phosphor.[93] 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.

Moreover, we determined the absolute amounts of individual phospholipid classes in inner membrane vesicles from wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria. Fig. 1 shows that only the PE content was strongly reduced in the mutants, whereas the abundance of other phospholipids remained largely unaffected. In comparison, the levels of PE in cell extracts and mitochondrial inner membrane vesicels from *crd1*Δ mutants were similar to wild-type levels, whereas the levels of phosphatidylglycerol were strongly increased; Figs. 1 and 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.[61,70-73] 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.[75,76] 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 between *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).

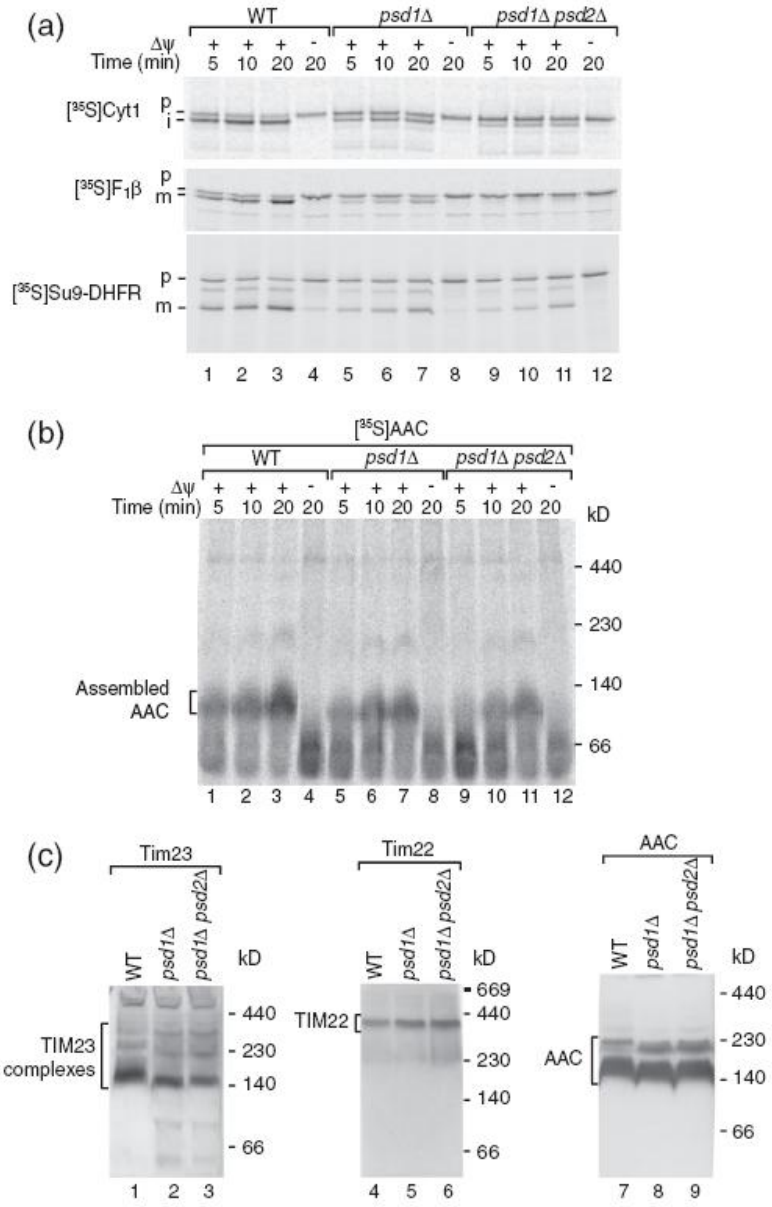

Figure 2. PE-depleted mitochondria are impaired in import of preproteins into and across the inner membrane. (a) Isolated mitochondria from wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ yeast strains were incubated with the ^{35}S-labeled precursors of cytochrome c_1 (Cyt1), $F_1\beta$ and Su9-DHFR in import buffer (3% [w/v] BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 5 mM methionine, 2 mM KH_2PO_4, 10 mM MOPS/KOH, pH 7.2, 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 EDTA, 10 mM MOPS/KOH, pH 7.2), 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 presence or absence of $\Delta\psi$. The mitochondria were lysed with 1% (w/v) digitonin in digitonin buffer (20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol) and protein complexes were separated by blue native electrophoresis.[77] ^{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 and immunodecoration with the indicated antisera.

The TIM23 translocase forms several blue native-stable complexes,[39,58,73] which were not dissociated but only slightly shifted to faster migrating forms in *psd1*Δ and in *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, are not dissociated when PE is depleted, indicating that PE is not crucial for the stability of these complexes.

Phosphatidylethanolamine 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 the other preproteins analyzed (Fig. 2a). It was previously shown that the import of the precursor of $F_1\beta$ requires a higher membrane potential and is thus more sensitive to a reduction of $\Delta\psi$ than the import of the precursors of Su9-DHFR,[70] 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.[39,72,78] $\Delta\psi$ was partially reduced in *psd1Δ* 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 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.

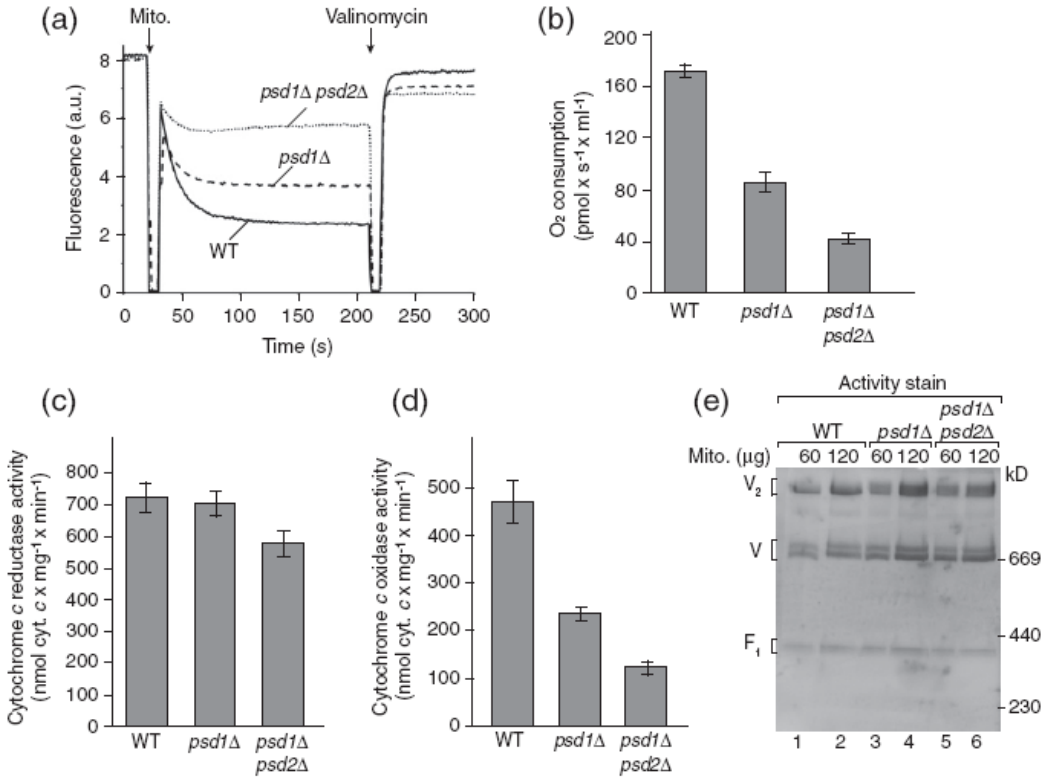

Figure 3. PE is required for the activity of the respiratory chain. (a) The membrane potential ($\Delta\psi$) of wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ yeast mitochondria was assessed at 25°C by fluorescence quenching using the $\Delta\psi$-dependent dye $DiSC_3(5)$ (3,3'-dipropylthiadicarbocyanine iodide) in membrane potential buffer (0.6 M sorbitol, 0.1% [w/v] BSA, 10 mM $MgCl_2$, 0.5 mM EDTA, and 20 mM KP_i, pH 7.2) as described previously.[39,78] (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, pH 7.2, 250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl, 5 mM KP_i, 1 mM ADP, 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 pmol x s^{-1} x ml^{-1}. 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.[79,80] Ubiquinol-dependent cytochrome *c* reduction was measured as described by Palsdottir & Hunte[80] 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, pH 7.4, 1 mM NaN_3, 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.*[81] 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, pH 7.4, 1 mM antimycin A, 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.[82,83] 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, pH 8.4, 5 mM $MgCl_2$, 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.

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,[82,83] revealing comparable activities in wild-type and *psd* mutant mitochondria (Fig. 3e).

Taken together, the step-wise decrease of PE levels in *psd1*Δ and *psd1*Δ*psd2*Δ mutants correlates with the step-wise 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.[70,72]

Lack of phosphatidylethanolamine 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; the association of the cytochrome bc_1 complex and cytochrome *c* oxidase is destabilized (Fig. 4a, lane 5; Fig. 4b, lane 8).[38,39,47,48,78,84,85] 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 CL[48,49] are depleted. However, PE and CL showed different effects on the molecular level when we analyzed the stability of mitochondrial protein complexes. 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.[42,43,48,64,86,87] CL and PE are both non-bilayer forming phospholipids. Their opposite effects on protein complex stability may provide an explanation why CL does not full 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.*[87] indeed showed that the negative charge of CL is important for maintaining the structural integrity of respiratory supercomplexes. Wittig & Schägger[88] and Bultema *et al.*[89] proposed that the cytochrome bc_1 complex and 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.

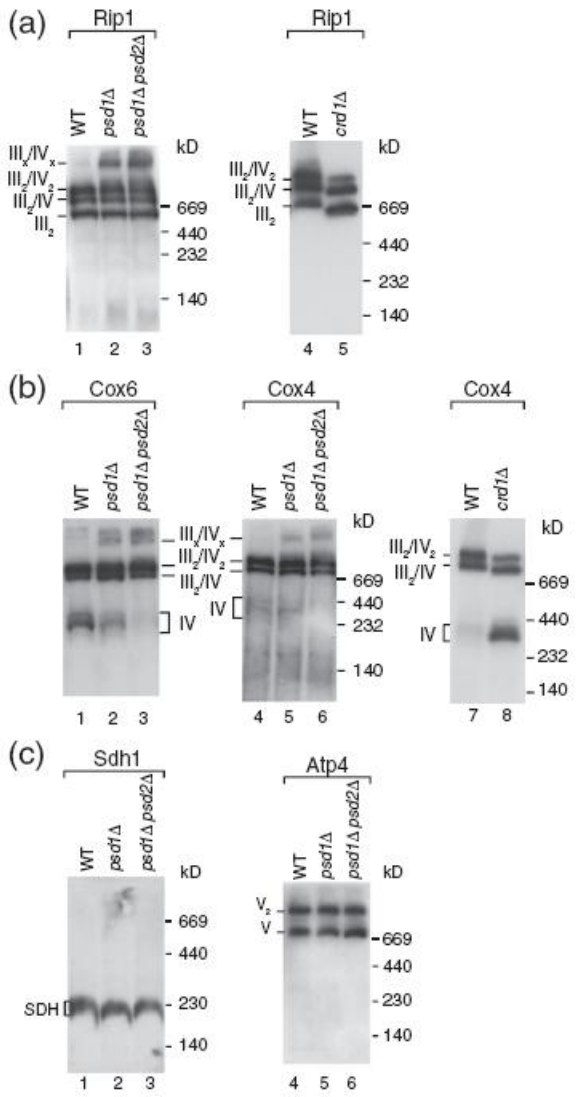

Figure 4. Depletion of PE stabilizes respiratory chain supercomplexes. (a) – (c) Wild-type, *psd1*Δ, *psd1*Δ*psd2*Δ and *crd1*Δ mitochondria were lysed with 1% (w/v) digitonin in digitonin buffer and subjected to blue native electrophoresis, followed by Western blotting and immunodecoration with specific antisera as indicated. 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).

Supplementary Material

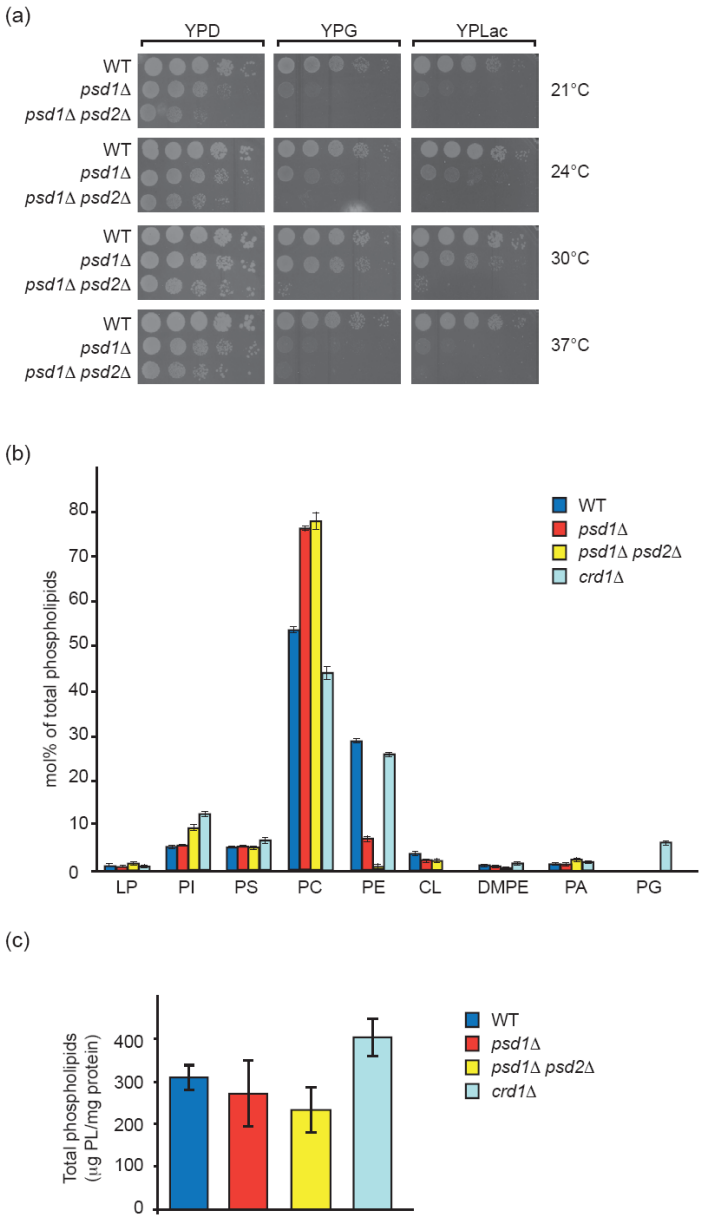

Figure S1. Growth tests and phospholipid distribution of wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ yeast cells. (a) Growth of wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ yeast cells (serial dilutions) was analyzed on the indicated growth media and temperatures. YPD: 1% (w/v) yeast extract, 2% (w/v) Bacto Peptone and 2% (w/v) glucose; YPG: 1% (w/v) yeast extract, 2% (w/v) Bacto Peptone and 3% (v/v) glycerol; YPLac: 1% (w/v) yeast extract, 2% (w/v) Bacto Peptone, 2.2% (v/v) lactic acid, 0.05% (w/v) glucose, 0.05% (w/v) $CaCl_2$, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) NH_4Cl, 0.05% (w/v) NaCl, 0.8% (w/v) NaOH, pH 5.0. (b) The phospholipid composition of wild-type, *psd1*Δ, *psd1*Δ*psd2*Δ and *crd1*Δ cell extracts was determined as described.[69,90,91] Mean values with standard error of the mean are shown (n = 4). DMPE, dimethylphosphatidylethanolamine; LP, lysophospholipids; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. (c) Inner membrane vesicles from of wild-type, *psd1*Δ, *psd1*Δ*psd2*Δ and *crd1*Δ mitochondria were isolated by sucrose gradient centrifugation.[92] Phospholipids were extracted and the total phospholipid content was determined as described[93]. Shown are the mean values of two determinations with range.

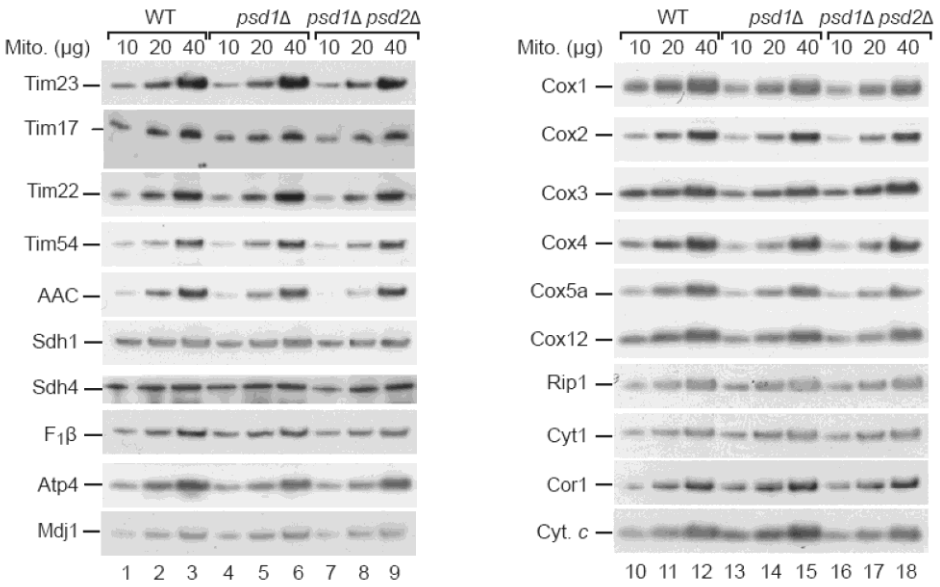

Figure S2. Steady-state levels of proteins in wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ yeast mitochondria. The indicated amounts (total protein) of wild-type, *psd1*Δ and *psd1*Δ*psd2*Δ mitochondria were subjected to SDS-PAGE. Proteins were detected by Western blotting and immunodecoration with the indicated antibodies.

Acknowledgments

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Chapter 6

Role of mitochondrial inner membrane organizing system in protein biogenesis of the mitochondrial outer membrane

Role of mitochondrial inner membrane organizing system in protein biogenesis of the mitochondrial outer membrane

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Abstract

Mitochondria contain two membranes, the outer membrane and the inner membrane with folded cristae. The mitochondrial inner membrane organizing system (MINOS) is a large protein complex required for maintaining inner membrane architecture. MINOS interacts with both preprotein transport machineries of the outer membrane, the translocase of the outer membrane (TOM) and the sorting and assembly machinery (SAM). It is unknown, however, if MINOS plays a role in the biogenesis of outer membrane proteins. We have dissected the interaction of MINOS with TOM and SAM and report that MINOS binds to both translocases independently. MINOS binds to the SAM complex via the conserved polypeptide transport-associated (POTRA) domain of Sam50. Mitochondria lacking mitofilin, the large core subunit of MINOS, are impaired in the biogenesis of β-barrel proteins of the outer membrane, whereas mutant mitochondria lacking any of the other five MINOS subunits import β-barrel proteins like wild-type mitochondria. We show that mitofilin is required at an early stage of β-barrel biogenesis that includes the initial translocation through the TOM complex. We conclude that MINOS interacts with TOM and SAM independently and that the core subunit mitofilin is involved in biogenesis of outer membrane β-barrel proteins.

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Abbreviations: Fcj1, formation of crista junction protein 1 (mitofilin); MINOS, mitochondrial inner membrane organizing system; POTRA, polypeptide transport-associated; SAM, sorting and assembly machinery; TEV, tobacco etch virus; TOM, translocase of outer mitochondrial membrane.

Introduction

Mitochondria consist of two membranes and two aqueous compartments, intermembrane space and matrix. The inner membrane is folded into tubular invaginations, termed cristae. Crista junctions connect the cristae membranes with the remainder of the inner membrane that is adjacent to the outer membrane and is termed inner boundary membrane (Frey and Mannella 2000; Mannella, 2006; Zick *et al.*, 2009). Recent studies led to the identification of a large protein complex of the inner membrane that plays a crucial role in the maintenance of inner membrane architecture. The complex was termed mitochondrial inner membrane organizing system (MINOS), mitochondrial contact site (MICOS) complex or mitochondrial organizing structure (MitOS) (von der Malsburg *et al.*, 2011; Harner *et al.*, 2011; Hoppins *et al.*, 2011; Herrmann, 2011; Alkhaja *et al.*, 2012; van der Laan *et al.*, 2012). MINOS consists of six subunits that are all inner membrane proteins exposed to the intermembrane space. Two core proteins, mitofilin (Fcj1) and Mio10 (Mcs10/Mos1/MINOS1), are essential for keeping the cristae membranes attached to the inner boundary membrane (John *et al.*, 2005; Rabl *et al.*, 2009; Mun *et al.*, 2010; Harner *et al.*, 2011; Head *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Zerbes *et al.*, 2012). The further subunits, Aim5 (Mcs12), Aim13 (Mcs19/MINOS3), Aim37 (Mcs27) and Mio27 (Mcs29/Mos2), contribute to the integrity of the MINOS complex and maintenance of cristae architecture.

In addition to its role in inner membrane architecture, MINOS was found to interact with protein complexes of the outer mitochondrial membrane, including the two essential preprotein transport machineries (Xie *et al.*, 2007; Darshi *et al.*, 2011; Harner *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Körner *et al.*, 2012; Ott *et al.*, 2012; Zerbes *et al.*, 2012). The translocase of the outer membrane (TOM) forms the main entry gate for most nuclear-encoded mitochondrial precursor proteins, whereas the sorting and assembly machinery (SAM or TOB) mediates the insertion of □-barrel proteins into the mitochondrial outer membrane (Dolezal *et al.*, 2006; Neupert and Herrmann, 2007; Chacinska *et al.*, 2009; Dukanovic and Rapaport, 2011; Endo *et al.*, 2011; Becker *et al.*, 2012). These contact sites between inner and outer membranes are involved in the maintenance of cristae morphology (Körner *et al.*, 2012; Ott *et al.*, 2012). In addition, it was shown that mitofilin/Fcj1 supports the transport of small precursor

proteins into the intermembrane space (von der Malsburg *et al.*, 2011). Mitofilin contains a large intermembrane space domain that interacts with the TOM complex and the receptor Mia40 of the mitochondrial intermembrane space assembly (MIA) machinery (Chacinska *et al.*, 2004; Mesecke *et al.*, 2005; Dabir *et al.*, 2007; Grumbt *et al.*, 2007; Stojanovski *et al.*, 2008; Banci *et al.*, 2009; Kawano *et al.*, 2009; Koehler and Tienson, 2009; Bien *et al.*, 2010). The transient interaction of mitofilin with Mia40 helps to position this intermembrane space receptor in close vicinity of the TOM complex and thus precursor proteins passing through the TOM channel can be immediately captured by Mia40 (von der Malsburg *et al.*, 2011).

The mitochondrial outer membrane contains two major protein types: proteins with α-helical transmembrane segments and β-barrel proteins. The precursors of β-barrel proteins are initially imported via the TOM complex and translocated to the intermembrane space (Model *et al.*, 2001; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003, 2004; Mihara, 2003). Chaperone complexes of the small TIM-type help in transfer of the hydrophobic precursors to the SAM complex that mediates insertion of the proteins into the outer membrane (Paschen *et al.*, 2003, 2005; Wiedemann *et al.*, 2003, 2004; Gentle *et al.*, 2004; Hoppins and Nargang, 2004; Habib *et al.*, 2005; Chan and Lithgow, 2008; Kutik *et al.*, 2008). For α-helical precursor proteins, several import pathways have been described that can involve TOM receptors, SAM and further outer membrane proteins (Stojanovski *et al.*, 2007a; Becker *et al.*, 2008, 2009, 2011; Hulett *et al.*, 2008; Kemper *et al.*, 2008; Popov-Celeketic *et al.*, 2008; Thornton *et al.*, 2010; Dukanovic and Rapaport, 2011; Papić *et al.*, 2011; Dimmer *et al.*, 2012). Since MINOS interacts with both outer membrane translocases, it may potentially be connected to protein import into the outer membrane. However, different views on the relation of MINOS to the biogenesis of outer membrane proteins have been reported (Darshi *et al.*, 2011; Körner *et al.*, 2012).

For this report, we analyzed the interaction of MINOS with TOM and SAM. We show that MINOS binds to TOM and SAM in an independent manner. Mutant mitochondria lacking mitofilin/Fcj1 were impaired in the biogenesis of β-barrel proteins. Mitofilin is involved in an early stage of β-barrel import that includes the translocation of precursor proteins through the TOM complex. We conclude that mitofilin not only promotes the import of small proteins into the intermembrane space (von der Malsburg *et al.*, 2011) but also the biogenesis of β-barrel proteins of the outer membrane.

Results

Differential co-purification of TOM and SAM with MINOS subunits

We asked whether MINOS interacts with TOM and SAM simultaneously or whether distinct outer membrane contacts exist. In a first approach, we performed pull-down experiments with Protein A-tagged MINOS components. We used a *Saccharomyces cerevisiae* strain expressing mitofilin/Fcj1 with a C-terminal Protein A-tag (von der Malsburg *et al.*, 2011) and generated a yeast strain that expressed Mio27 with a C-terminal Protein A-tag. The Protein A-tags were attached to the MINOS components via a linker containing a tobacco etch virus (TEV) protease cleavage site. Extracts of the yeast cells were prepared using the non-ionic detergent digitonin and subjected to IgG affinity chromatography. Bound proteins were eluted by cleavage with TEV protease. $Fcj1_{ProtA}$ and $Mio27_{ProtA}$ co-purified the other five MINOS subunits with comparable efficiency (Figure 1, lanes 5 and 6), whereas control proteins of the inner and outer membranes were not found in the eluate (Figure 1, lanes 5, 6, 11 and 12).

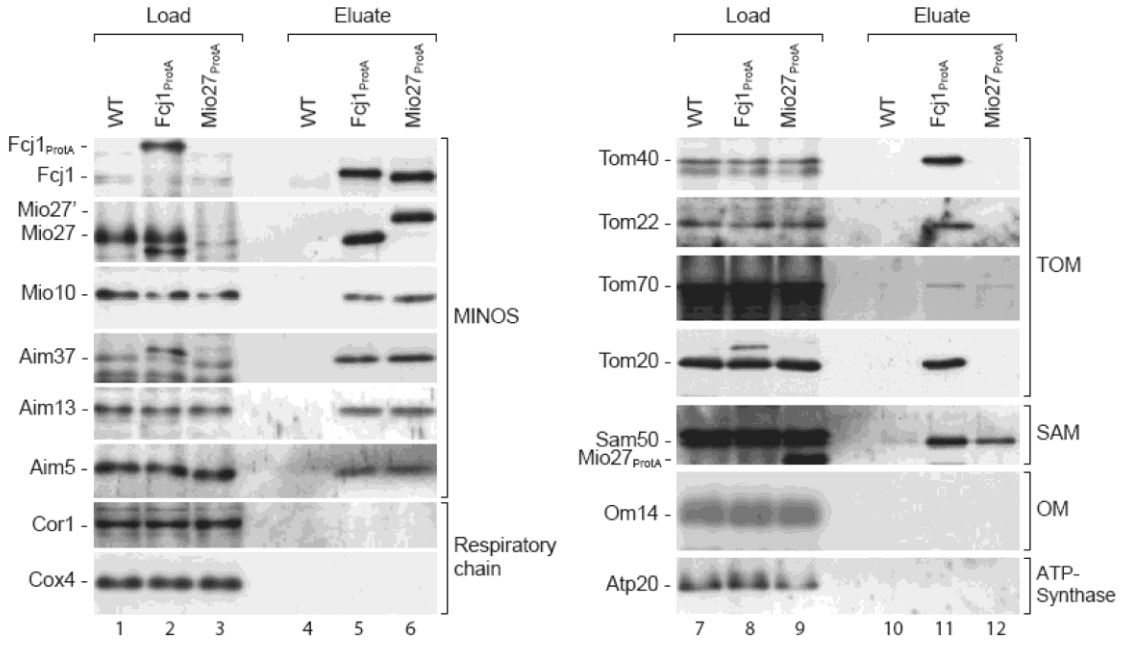

Figure 1. The mitochondrial inner membrane organizing system (MINOS) interacts with outer membrane protein complexes. Whole cell digitonin extracts from wild-type (WT) cells and cells expressing Protein A fusion constructs ($Fcj1_{ProtA}$ and $Mio27_{ProtA}$) were subjected to IgG affinity chromatography, elution with TEV protease, SDS-PAGE and immunoblotting. Load, 1.5%; elution, 100%. Mio27', TEV-cleaved form of $Mio27_{ProtA}$; OM, outer mitochondrial membrane.

In addition to the MINOS subunits, $Fcj1_{ProtA}$ co-purified TOM and SAM subunits (Figure 1, lane 11) (the receptor Tom70 is only loosely associated with the yeast TOM complex and thus co-purified in minor amounts; Meisinger *et al.*, 2001). In contrast, $Mio27_{ProtA}$ neither co-purified the channel-forming protein Tom40 nor the receptors Tom20, Tom22 and Tom70 (Figure 1, lane 12), demonstrating that the TOM complex was not pulled-down with tagged Mio27. However, $Mio27_{ProtA}$ co-purified Sam50, the core component of the SAM complex, though with a reduced efficiency compared to the co-purification with $Fcj1_{ProtA}$ (Figure 1, lanes 11 and 12). These findings raised the possibility that binding of TOM and SAM to MINOS is not coupled but can be separated.

Requirement of the POTRA domain of Sam50 for MINOS-SAM interaction but not for MINOS-TOM interaction

To obtain direct evidence for an independent interaction of MINOS with the outer membrane translocases we asked for determinants that are required for formation of the interactions. The SAM complex contains one large hydrophilic domain that is exposed to the intermembrane space, the polypeptide transport-associated (POTRA) domain at the N-terminus of Sam50 (Kozjak *et al.*, 2003; Paschen *et al.*, 2003; Sánchez-Pulido *et al.*, 2003; Gentle *et al.*, 2004; Habib *et al.*, 2007; Knowles *et al.*, 2008; Kutik *et al.*, 2008; Stroud *et al.*, 2011a), whereas Sam35 and Sam37 expose domains to the cytosolic side (Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004; Chan and Lithgow, 2008; Kutik *et al.*, 2008). We used a yeast strain that expressed Sam37 with a Protein A-tag (Kozjak *et al.*, 2003). From a digitonin extract of the cells, tagged Sam37 pulled-down the further SAM proteins Sam35 and Sam50 as expected (Figure 2, lane 5; Kozjak *et al.*, 2003), but also the subunits of MINOS (Figure 2, lane 11; Aim5, Aim13 and Aim37 were co-purified with lower yield than Fcj1, Mio10 and Mio27). Control proteins of outer and inner membranes were not co-purified (Figure 2, lane 5). We generated a $Sam37_{ProtA}$ strain, in which the N-terminal 120 residues of Sam50, including the entire POTRA domain, were deleted ($Sam50_{\Delta 120}$) (Kutik *et al.*, 2008; Stroud *et al.*, 2011a). Co-purification of the subunits of the SAM complex was not affected by the lack of the POTRA domain (Figure 2, lane 6; Habib *et al.*, 2007; Stroud *et al.*, 2011a).

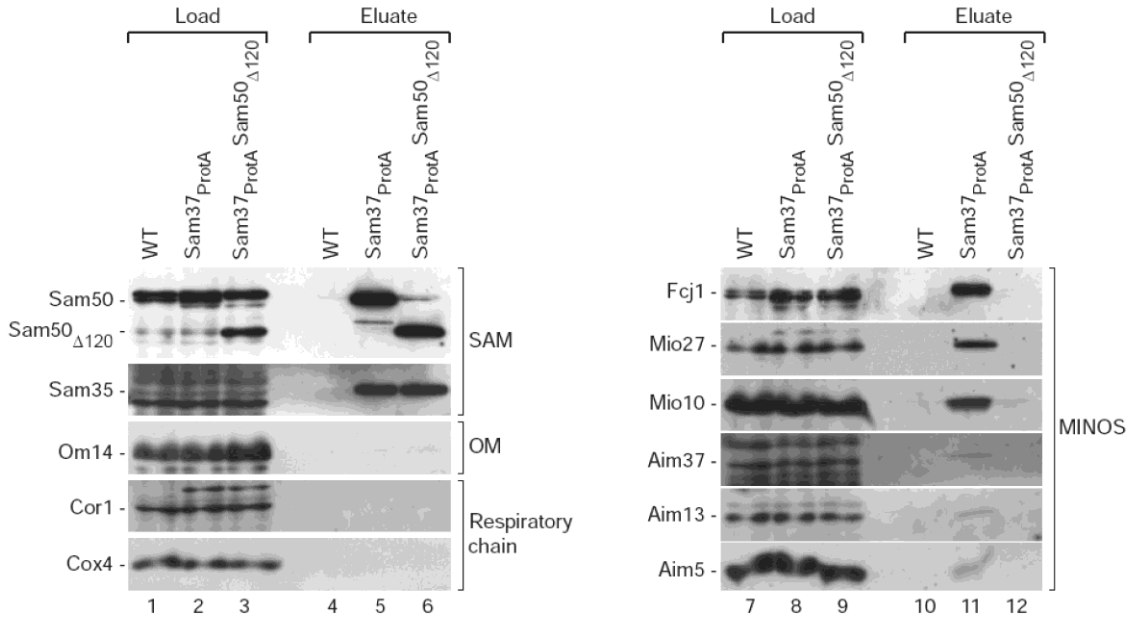

Figure 2. Interaction between MINOS and SAM depends on the POTRA domain of Sam50. Whole cell powder from wild-type (WT), $Sam37_{ProtA}$, and $Sam37_{ProtA}$ $Sam50_{\Delta 120}$ cells was solubilized in digitonin-containing buffer, subjected to IgG affinity chromatography and analyzed by SDS-PAGE and immunoblotting. Load, 1%; elution, 100%. OM, outer mitochondrial membrane.

However, the pull-down of MINOS subunits with tagged Sam37 was strongly inhibited when the POTRA domain of Sam50 was lacking (Figure 2, lane 12). These results indicate that the POTRA domain is required for the interaction of the SAM complex with MINOS.

To address if the POTRA domain was required for the interaction of TOM with MINOS we generated an $Fcj1_{ProtA}$ strain, in which the POTRA domain of Sam50 was deleted (Figure 3). Tagged Fcj1 pulled-down the other five MINOS subunits independently of the presence or absence of the POTRA domain (Figure 3, lanes 5 and 6). The interaction of Fcj1 with Sam50 and Sam35 was strongly inhibited by the lack of the POTRA domain (Figure 3, lane 12) (the steady-state levels of Sam50 and Sam35 were not affected; Figure 3, lanes 8 and 9). However, Tom22 and Tom40 were efficiently co-purified with tagged Fcj1 and did not require the presence of the Sam50 POTRA domain (Figure 3, lanes 11 and 12). The mitochondrial ultrastructure analyzed by electron microscopy was not altered when mitofilin/Fcj1 carried a Protein A-tag and was only mildly affected by the lack of the Sam50 POTRA domain (Supplemental Figure S1), indicating that the POTRA-mediated MINOS-SAM interaction is not strictly essential for maintaining the architecture of the mitochondrial inner membrane.

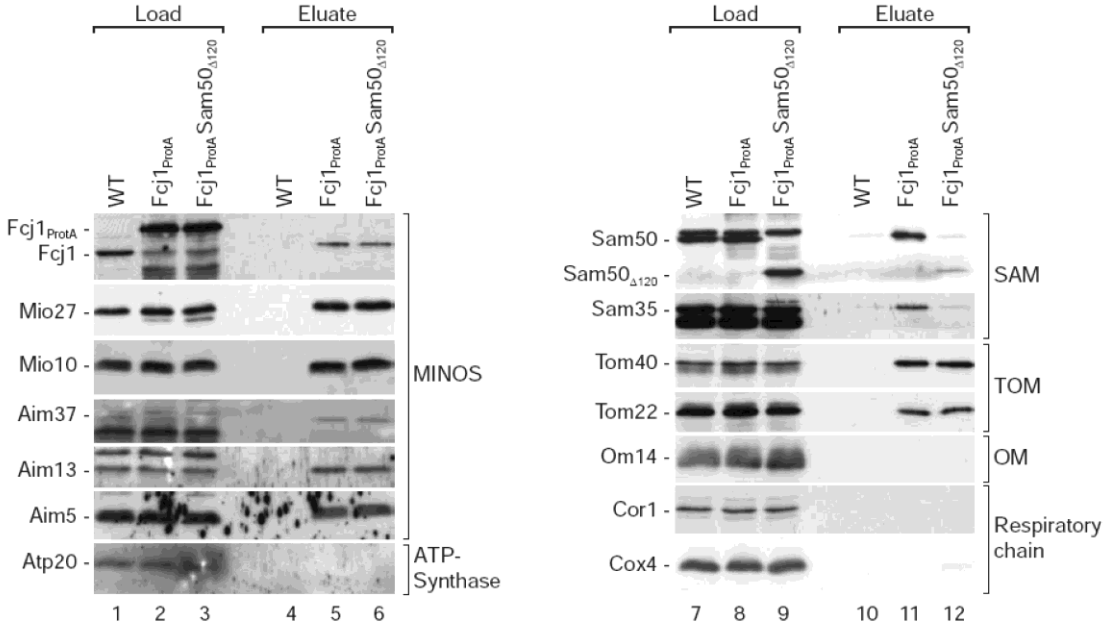

Figure 3. MINOS independently interacts with outer membrane TOM and SAM complexes. Whole cell digitonin extracts from wild-type (WT), $Fcj1_{ProtA}$, and $Fcj1_{ProtA}$ $Sam50_{\Delta120}$ cells were subjected to IgG affinity chromatography and analyzed by SDS-PAGE and immunoblotting. Load, 1.5%; elution, 100%. OM, outer mitochondrial membrane.

We conclude that mitofilin/Fcj1 can bind TOM despite an impaired interaction between mitofilin/Fcj1 and SAM. In Figure 1 we showed that tagged Mio27 pulled-down SAM but not the TOM complex, indicating that the MINOS-SAM interaction does not require the interaction with the TOM complex. Taken together, these findings demonstrate that TOM and SAM independently interact with components of the MINOS machinery of the inner membrane.

Biogenesis of outer membrane proteins in MINOS mutant mitochondria

Biogenesis of mitochondrial β-barrel proteins requires both TOM and SAM (Endo and Yamano, 2009; Dukanovic and Rapaport, 2011; Becker *et al.*, 2012). To study if MINOS was involved in outer membrane protein biogenesis, we used the radiolabeled precursor of Tom40 as model substrate. For this precursor, three assembly stages can be resolved by blue native electrophoresis of digitonin-lysed mitochondria (Model *et al.*, 2001; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Chan and Lithgow, 2008; Dukanovic *et al.*, 2009). Upon incubation with isolated mitochondria, Tom40 forms the intermediate I that represents interaction of the precursor with the SAM complex (Model *et*

al., 2001; Wiedemann *et al.*, 2003; Becker *et al.*, 2010). Subsequently the precursor forms a smaller intermediate II before it is assembled into the mature TOM complex (Figure 4, lanes 4-6). We isolated mitochondria from single deletion yeast strains of the six MINOS subunits and imported the precursor of Tom40. Most of the mutant mitochondria imported and assembled Tom40 like wild-type mitochondria (Figure 4, lanes 4-24). The only exception were mitochondria lacking mitofilin/Fcj1 that were considerably impaired in all three assembly stages of Tom40 (Figure 4, lanes 1-3).

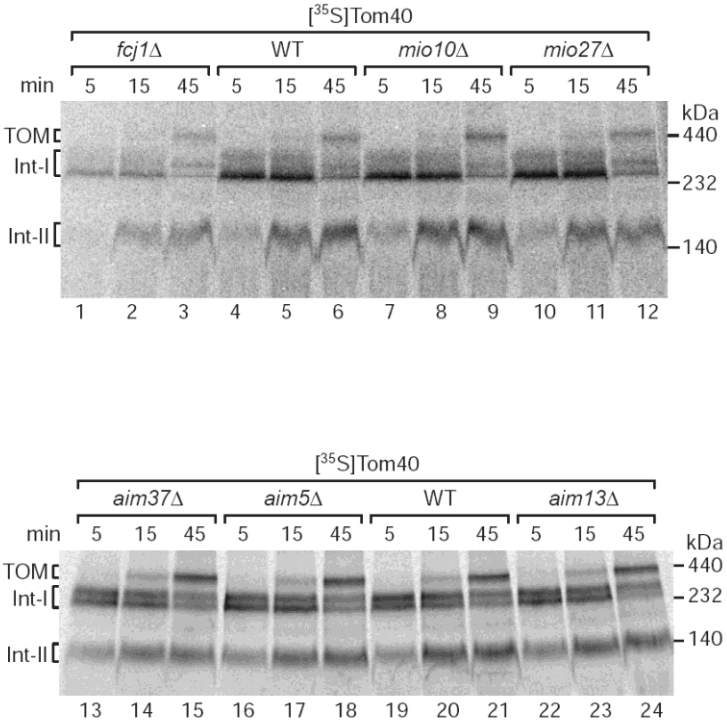

Figure 4. Deletion of *FCJ1*, but not of other MINOS components, leads to impaired Tom40 biogenesis. ^{35}S-labeled Tom40 was imported into mitochondria isolated from wild-type (WT), *fcj1*Δ, *mio10*Δ, *mio27*Δ, *aim37*Δ, *aim5*Δ, and *aim13*Δ cells. Mitochondria were solubilized with digitonin and subjected to blue native electrophoresis and digital autoradiography. Int-I, precursor-SAM assembly intermediate-I; Int-II, assembly intermediate-II.

We analyzed the steady-state levels of TOM, SAM and TIM proteins and did not observe any substantial differences between *fcj1*Δ and wild-type mitochondria that would explain the defect in Tom40 biogenesis (Supplemental Figure S2A). Moreover, the stability of neither the TOM complex nor the SAM complex, as analyzed by blue native electrophoresis, was affected by the lack of mitofilin/Fcj1 (Supplemental Figure S2B). The biogenesis of further β-barrel proteins of the outer mitochondrial membrane, porin and

Mdm10, was analyzed by monitoring assembly of the radiolabeled precursors. These precursors are also imported via the TOM and SAM complexes but with faster kinetics than Tom40 and thus the wild-type precursors do not form stable SAM-intermediates in considerable amounts (Wiedemann *et al.*, 2003; Kutik *et al.*, 2008; Stroud *et al.*, 2011a). Biogenesis of porin and Mdm10 was retarded in *fcj1*Δ mitochondria but not in *mio10*Δ mitochondria (Supplemental Figure S2, C and D), supporting the view that mitofilin/Fcj1 but not Mio10 is involved in the efficient import of outer membrane β-barrel proteins.

We performed several control experiments to exclude possible indirect effects. (i) Major alterations of mitochondrial phospholipid levels such as lack of cardiolipin can impair the biogenesis of outer membrane proteins (Gebert *et al.*, 2009). We compared the phospholipid composition of mitochondria from *fcj1*Δ and wild-type yeast. In addition, mitochondria lacking Mio10 were also analyzed. Supplemental Figure S3 shows that the phospholipid composition was not substantially changed by the lack of the central MINOS components mitofilin/Fcj1 or Mio10. (ii) We generated a yeast strain where *FCJ1* was expressed under the control of a galactose-inducible promoter. Upon shift of the cells to glucose-containing medium, the levels of Fcj1 were decreased (the levels of TOM, SAM and TIM components as well as the stability of TOM and SAM complexes were not affected; Supplemental Figure S4, A and B). We selected conditions under which Fcj1 was strongly depleted (Supplemental Figure S4A), yet the inner membrane potential $\Delta\psi$ was comparable to that of wild-type mitochondria (Figure 5A). For *fcj1*Δ mitochondria, it has been reported that $\Delta\psi$ was partially decreased and thus also the $\Delta\psi$-dependent import of proteins into or across the inner membrane was partially reduced (von der Malsburg *et al.*, 2011). In contrast, the Fcj1-depleted mitochondria imported the matrix protein F_1-ATPase subunit β and the inner membrane proteins cytochrome c_1 and ADP/ATP carrier with an efficiency close to that of wild-type mitochondria (Figure 5, B and C). Though the import of mitochondrial outer membrane proteins does not require the inner membrane potential (Chacinska *et al.*, 2009), use of the Fcj1-depleted mitochondria offered the opportunity to minimize pleiotropic effects. The assembly of the precursor of Tom40 was impaired in Fcj1-depleted mitochondria (Figure 5D), supporting the view of a specific role of mitofilin/Fcj1 in this process.

We conclude that lack of mitofilin/Fcj1 impairs the biogenesis pathway of β-barrel precursors, whereas other MINOS components are not required for β-barrel assembly.

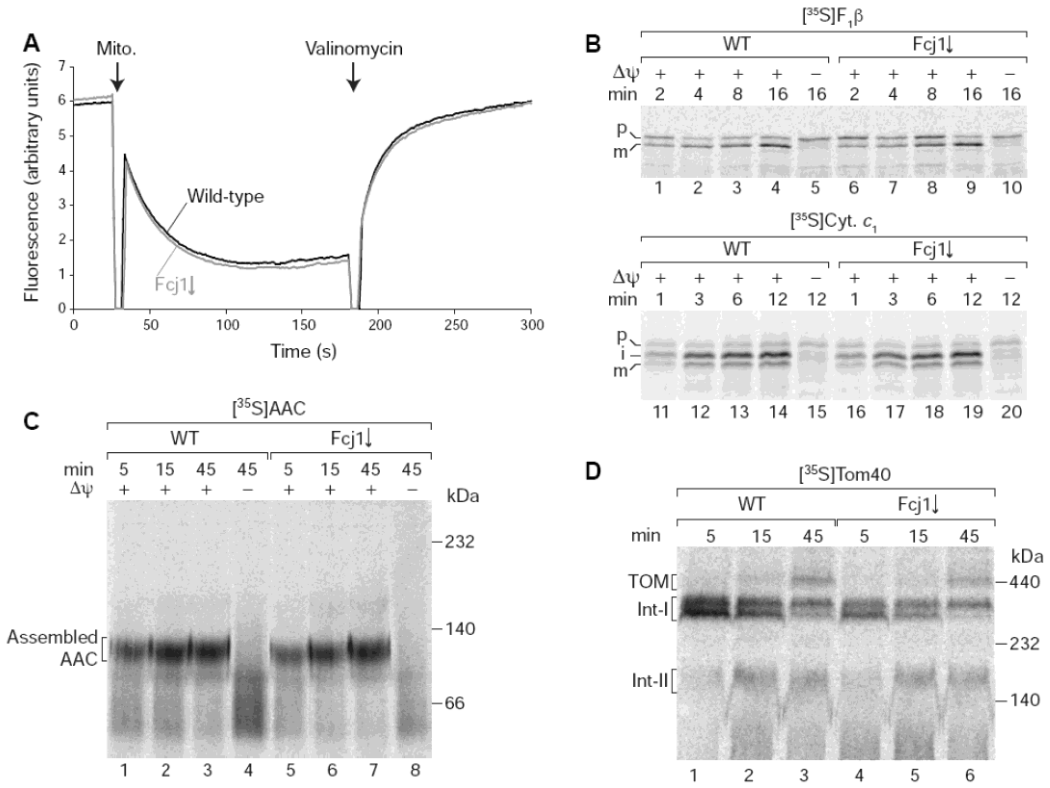

Figure 5. Biogenesis of Tom40 is impaired upon depletion of Fcj1. (A) Fcj1↓ (YPH499 *fcj1::kanMX6*, *PGAL1-FCJ1*) and wild-type control cells were pre-cultured in the presence of 2% galactose. Subsequently, expression of *PGAL1-FCJ1* was inhibited with 1% glucose, mitochondria were isolated and the mitochondrial membrane potential was assessed using the potential-sensitive dye dipropylthiadicarbocyanine iodide [$DiSC_3(5)$]. (B) The ^{35}S-labeled precursors of F_1-ATPase subunit β ($F_1β$) and cytochrome c_1 (Cyt. c_1) were imported into isolated mitochondria for the indicated periods. After proteinase K treatment to remove non-imported precursors, samples were analyzed by SDS-PAGE and digital autoradiography. p, precursor; i, intermediate; m, mature. (C) [^{35}S]ADP/ATP carrier (AAC) or (D) [^{35}S]Tom40 were imported into isolated mitochondria as indicated and analyzed by blue native electrophoresis and digital autoradiography. Int-I, precursor-SAM assembly intermediate-I; Int-II, assembly intermediate-II.

Lack of mitofilin/Fcj1 impairs biogenesis of Tom40 at a stage before the SAM complex

At which stage of Tom40 assembly is mitofilin/Fcj1 involved? Since the formation of the SAM-bound state (assembly intermediate I on native gels) is strongly impaired in *fcj1*Δ mitochondria, either the SAM complex itself or a step leading to the SAM complex is compromised. We imported the radiolabeled precursor of Tom22 that uses TOM receptors and each subunit of the SAM complex before its assembly into the TOM complex (Keil and Pfanner, 1993; Meisinger *et al.*, 2004; Stojanovski *et al.*, 2007a; Dukanovic *et al.*, 2009; Thornton *et al.*, 2010). Assembly of radiolabeled Tom22 into the TOM complex occurred with similar efficiency in *fcj1*Δ mitochondria, *mio10*Δ mitochondria and wild-type mitochondria (Supplemental Figure S5A). Thus, the SAM complex as well as TOM

receptors are functional in *fcj1*Δ mitochondria (the precursor of Tom22 is not translocated through the TOM channel to the intermembrane space but is directly inserted into the outer membrane by the SAM complex; Stojanovski *et al.*, 2007a; Thornton *et al.*, 2010). Import and assembly of the precursor of Tom5 into the TOM complex was also not affected by the lack of Fcj1 or Mio10 (Supplemental Figure S5B).

Since the POTRA domain is required for the MINOS-SAM interaction, we compared Tom40 assembly in $Sam50_{\Delta 120}$ mitochondria to Tom40 assembly in *fcj1*Δ mitochondria. It has been reported that lack of the POTRA domain only mildly affects the biogenesis of radiochemical amounts of Tom40 (Kutik *et al.*, 2008; Stroud *et al.*, 2011a) and thus *fcj1*Δ mitochondria apparently show a much stronger defect in Tom40 biogenesis. To directly compare this to the pull-down experiments that depended on the presence of the POTRA domain (Figure 2), we studied the import of Tom40 into $Sam37_{ProtA}$ mitochondria lacking the POTRA domain of Sam50. Tom40 assembly was not inhibited in the POTRA-deficient mutant mitochondria (Figure 6A). Since the POTRA domain is required for a stable MINOS-SAM interaction, the biogenesis pathway of Tom40 is not inhibited when the MINOS-SAM interaction is disturbed. Taken together with the full activity of the SAM complex in the assembly of Tom22, these results suggest that the Tom40 assembly defect in *fcj1*Δ mitochondria may occur at a step preceding formation of the SAM-precursor intermediate.

The early steps in Tom40 biogenesis involve initial translocation across the outer membrane to the intermembrane space and binding to the small TIM chaperones (Model *et al.*, 2001; Mihara, 2003; Wiedemann *et al.*, 2003, 2004; Hoppins and Nargang, 2004). These early steps do not involve blue native-stable intermediates and thus cannot be directly visualized by native gel electrophoresis (Wiedemann *et al.*, 2004; Rao *et al.*, 2012). Upon a short import time, the precursor of Tom40 was associated with tagged Fcj1 (Figure S5C), suggesting an involvement of mitofilin/Fcj1 at an early import stage. The translocation of the Tom40 precursor across the outer membrane can be assessed by its protection against externally added protease (Wiedemann *et al.*, 2003, 2004; Paschen *et al.*, 2003; Chan and Lithgow, 2008; Dukanovic *et al.*, 2009). We imported radiolabeled Tom40 precursor into isolated mitochondria. The efficiency of Tom40 translocation to a protease-protected location was significantly reduced in *fcj1*Δ mitochondria compared to wild-type

mitochondria (Figure 6, B and C), indicating that this initial import step of Tom40 is affected by the lack of mitofilin/Fcj1.

Taken together, mitochondria lacking mitofilin/Fcj1 are impaired in an early step of Tom40 biogenesis that precedes the SAM complex and includes the initial translocation across the outer membrane.

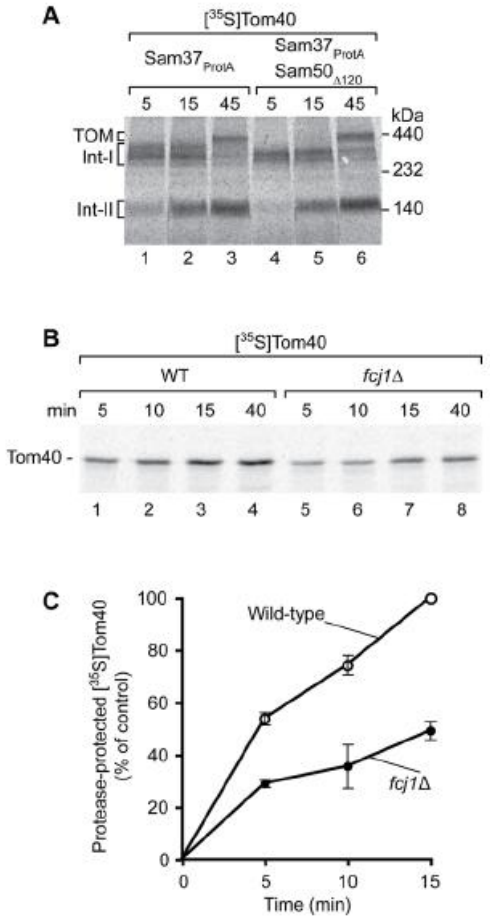

Figure 6. Mitofilin/Fcj1 is required at an early stage of Tom40 biogenesis. (A) ^{35}S-labeled Tom40 was imported into $Sam37_{ProtA}$ and $Sam37_{ProtA}$ $Sam50_{\Delta 120}$ mitochondria followed by solubilization in digitonin buffer, blue native electrophoresis and autoradiography. Int-I, precursor-SAM assembly intermediate-I; Int-II, assembly intermediate-II. (B) Radiolabeled Tom40 was incubated with wild-type (WT) and *fcj1*Δ mitochondria. Non-imported precursor was removed by proteinase K treatment and mitochondria were subjected to SDS-PAGE and digital autoradiography. (C) Quantification of Tom40 import experiments performed as described in (B). Data are represented as mean +/- standard error of the mean (n = 3; n = 2 and range for the 10 min time point). The amount of protease-protected [^{35}S]Tom40 after 15 min import into wild-type mitochondria was set to 100% (control).

Discussion

We report a new function for mitofilin/Fcj1 and the MINOS machinery of the mitochondrial inner membrane. This membrane organizing system is not only involved in the maintenance of mitochondrial cristae morphology (Harner *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Körner *et al.*, 2012; Ott *et al.*, 2012; Zerbes *et al.*, 2012) and protein import via the MIA pathway into the intermembrane space (von der Malsburg *et al.*, 2011), but also in the biogenesis of outer membrane proteins with β-barrel topology.

Both protein translocases of the outer membrane, TOM and SAM, independently bind to MINOS. (i) We observed for the SAM complex that the conserved POTRA domain on the intermembrane space side of Sam50 is required for the stable interaction with MINOS, whereas the binding of TOM to MINOS occurs independently of the POTRA domain. (ii) The TOM complex is efficiently pulled-down only by tagged mitofilin/Fcj1 but not by other tagged subunits of MINOS such as Mio27 and Aim5, although these components co-purify all other MINOS subunits (von der Malsburg *et al.*, 2011; this study). In contrast, the SAM complex is not only co-purified with mitofilin/Fcj1 but also with several other MINOS components (Xie *et al.*, 2007; Darshi *et al.*, 2011; Harner *et al.*, 2011; Alkhaja *et al.*, 2012; this study). Thus, MINOS can be found in association with the SAM complex independently of the MINOS-TOM interaction. (iii) When the MINOS complex is fully or partially disrupted by deletion of *MIO10*, *AIM5* or *AIM13*, the interaction of mitofilin/Fcj1 with the TOM complex is not disturbed but occurs with wild-type efficiency (von der Malsburg *et al.*, 2011), demonstrating that an intact MINOS complex is not required for the mitofilin/Fcj1-TOM interaction. Taken together, two distinct forms of contact sites between outer membrane translocases and the inner membrane organizing system are formed: a MINOS-SAM contact that requires the POTRA domain and likely includes the entire MINOS complex; and a mitofilin/Fcj1-TOM contact that does not depend on the other MINOS components.

We analyzed mitochondria, which were isolated from yeast single deletion mutants of each of the six MINOS genes, for the biogenesis of outer membrane proteins. Remarkably, only mitochondria lacking mitofilin/Fcj1 were impaired in the assembly pathway of β-barrel proteins, as assessed with the model substrate Tom40. All other mutant mitochondria imported Tom40 with wild-type efficiency. Since *fcj1*Δ and *mio10*Δ mutants show a

comparably strong degree of morphological alteration of the mitochondrial inner membrane (Harner *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012), it can be excluded that the β-barrel assembly defect is indirectly caused by the morphological defect. Moreover, we observed that the phospholipid profiles of *fcj1*Δ and *mio10*Δ mitochondria are similar to that of wild-type mitochondria. We dissected the biogenesis pathway of Tom40 into distinct stages and observed that the lack of mitofilin/Fcj1 affected an early import step that includes translocation of the Tom40 precursor through the TOM complex to the intermembrane space. The activity of the SAM complex itself was not compromised in *fcj1*Δ mitochondria since the SAM-dependent precursor of Tom22 was efficiently assembled in the mutant mitochondria and the POTRA-dependent MINOS-SAM interaction was not required for Tom40 assembly.

Taken together, these findings suggest a model in that mitofilin/Fcj1 is present in at least two pools. On the one hand mitofilin/Fcj1 is a subunit of the MINOS complex. This complex is crucial for maintenance of inner membrane morphology and also mediates the interaction with the SAM complex. On the other hand a fraction of mitofilin/Fcj1 molecules are also functional without the other MINOS components. These mitofilin/Fcj1 molecules interact with the TOM complex and are involved in the biogenesis of β-barrel proteins of the outer membrane. Interestingly, the import of intermembrane space proteins via the MIA pathway is only impaired in *fcj1*Δ mitochondria but not in deletion mutants of other MINOS components (von der Malsburg *et al.*, 2011), supporting the view that the roles of mitofilin/Fcj1 in promoting protein biogenesis via the β-barrel and MIA pathways are performed by the mitofilin/Fcj1 pool that does not depend on an intact MINOS complex. MINOS, SAM and TOM have been suggested to be part of a large endoplasmic reticulum-mitochondria organizing network (ERMIONE) that is involved in controlling mitochondrial architecture and biogenesis (van der Laan *et al.*, 2012). The findings reported here support the view that ERMIONE functions as a dynamic network (Zerbes *et al.*, 2012).

Materials and Methods

Yeast strains

S. cerevisiae strains used in this study are derivatives of YPH499 (*MAT*a, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801*) (Sikorski and Hieter, 1989). YPH499 strains *fcj1*Δ, *mio10*Δ, *mio27*Δ, *aim5*Δ, *aim13*Δ, and *aim37*Δ were generated by homologous recombination using *kanMX4* cassettes amplified from genomic DNA from strains *fcj1*Δ (BY4741), *mio10*Δ (BY4741), *mio27*Δ (BY4741), *aim5*Δ (BY4741), *aim13*Δ (BY4741), and *aim37*Δ (BY4741) obtained from Euroscarf, Frankfurt (Brachmann et al., 1998). The strains $Sam37_{ProtA}$, $Oxa1_{ProtA}$ and $Fcj1_{ProtA}$ have been described (Kozjak *et al.,* 2003; Frazier *et al.,* 2006; von der Malsburg *et al.,* 2011). A strain expressing Mio27 fused to a C-terminal Protein A-tag for affinity chromatography was generated by homologous recombination using a cassette consisting of a TEV protease cleavage site, a Protein A moiety and a *HIS3* marker gene (Knop *et al.,* 1999). A similar cassette was transformed into $Sam50_{\Delta 120}$ cells (Kutik *et al.,* 2008) to generate the strain $Fcj1_{ProtA}$ $Sam50_{\Delta 120}$. A fragment encoding a *HIS3* marker gene, a *NOP1* promoter, a Protein A moiety and a TEV protease cleavage site was amplified from genomic DNA derived from $Sam37_{ProtA}$ cells and transformed into $Sam50_{\Delta 120}$ cells to generate the strain $Sam37_{ProtA}$ $Sam50_{\Delta 120}$. A cassette encoding a *kanMX6* module and a *GAL1* promoter was integrated 5' of the *FCJ1* open reading frame by homologous recombination to generate strain Fcj1↓ (YPH499 *fcj1::kanMX6, PGAL1-FCJ1*) (Longtine *et al.,* 1998).

Growth conditions, isolation of mitochondria and analysis of protein content

For isolation of mitochondria, cells were grown at 30°C. Typically, cells were cultured in YPG medium (1% [w/v] yeast extract, 2% [w/v] bacto-peptone, 3% [v/v] glycerol). For depletion of Fcj1, the strain Fcj1↓ (YPH499 *fcj1::kanMX6, PGAL1-FCJ1*) and the corresponding wild-type strain were precultured in YPGal medium (1% [w/v] yeast extract, 2% [w/v] bacto-peptone, 2% [w/v] galactose) for 6 hours and transferred on YPG medium. After approximately three doubling times, 1% glucose was added to the medium to block expression of the *PGAL1-FCJ1* gene and cells were harvested after 11 hours. Mitochondria were isolated by sequential centrifugation as described (Meisinger *et al.,* 2006). Mitochondrial protein content was analyzed by SDS-PAGE and Western blotting.

Alternatively, protein complexes were analyzed by solubilization in digitonin buffer (1% [w/v] digitonin, 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [v/v] glycerol, 2 mM phenylmethylsulfonyl fluoride [PMSF]), blue native electrophoresis (Stojanovski *et al.*, 2007b) and Western blotting.

Protein import into isolated mitochondria

In vitro import reactions typically contained 50 - 80 µg mitochondria (protein amount) diluted in 100 µl import buffer (3% [w/v] bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 10 mM MOPS-KOH, pH 7.2, 4 mM ATP, 4 mM NADH, 5 - 10 mM creatine phosphate, 100 - 200 µg/ml creatine kinase) (Ryan *et al.*, 2001; Stojanovski *et al.*, 2007b). Radiolabeled precursor proteins generated by in vitro translation in the presence of [^{35}S]methionine (TNT SP6 Quick Coupled kit, Promega) were added to pre-warmed import reactions (20°C / 25°C). Samples were transferred on ice after different time points to terminate import reactions. Import of the precursors of ADP/ATP carrier, F_1-ATPase subunit β and cytochrome c_1 was terminated by addition of an AVO mix (8 µM antimycin A, 1 µM valinomycin, 20 µM oligomycin). Where indicated, non-imported precursor proteins were removed by incubation with 50 µg/ml proteinase K on ice for 15 min. Proteinase K was subsequently inactivated by addition of 2 mM PMSF. Mitochondria were washed with SEM buffer (250 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EDTA) and analyzed by SDS-PAGE or blue native electrophoresis, followed by digital autoradiography.

Preparation of yeast whole cell extracts and affinity chromatography

Yeast cells were cultured in YPG medium at 30°C. Cells were harvested by centrifugation and washed twice with demineralized water and twice with washing buffer (20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [v/v] glycerol) (Stroud *et al.*, 2011b; Zerbes *et al.*, 2012). Cells were frozen in liquid nitrogen and ground using a cryomill (20 min, 25 Hz). The resulting whole cell powder was solubilized in solubilization buffer (20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [v/v] glycerol, 1% [w/v] digitonin, 2 mM PMSF, 1x EDTA free proteinase inhibitor (Roche), 30 µg/ml DNAse I) followed by a clarifying spin. Protein extracts were subsequently applied to IgG affinity chromatography. Unspecifically bound proteins were removed by extensive washing (20

mM Tris/HCl, pH 7.4, 0.5 mM EDTA, 60 mM NaCl, 10% [v/v] glycerol, 0.3% [w/v] digitonin, 2 mM PMSF). Bound proteins were eluted by TEV protease cleavage, applied to SDS-PAGE and visualized by Western blotting.

Phospholipid analysis

Isolated mitochondria were subjected to lipid extraction using chloroform/methanol (2:1; v/v) as described (Folch *et al.*, 1957). The organic phase was subsequently washed with 0.034% $MgCl_2$ solution (w/v), 2 N KCl/methanol (4:1; v/v) and methanol/water/chloroform (48:47:3; per vol). To separate individual phospholipids, two-dimensional thin-layer chromatography using Silica gel 60 plates (Merck) was applied (first developing solvent: chloroform/methanol/25% NH_3 [68:35:5; per vol]; second developing solvent: chloroform/acetone/methanol/acetic acid/water [53:20:10:10:5; per vol]). After iodine vapour staining of thin-layer chromatography plates, phospholipids were scraped off and quantified according to Broekhuyse (1968).

Miscellaneous

For assessment of the mitochondrial membrane potential, the potential-sensitive dye dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] was used (Geissler *et al.*, 2000). For electron microscopy analysis, diaminobenzidine (DAB) staining and imaging of cells were performed as described (von der Malsburg *et al.*, 2011; Zerbes *et al.*, 2012).

Acknowledgments

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Supplementary Material

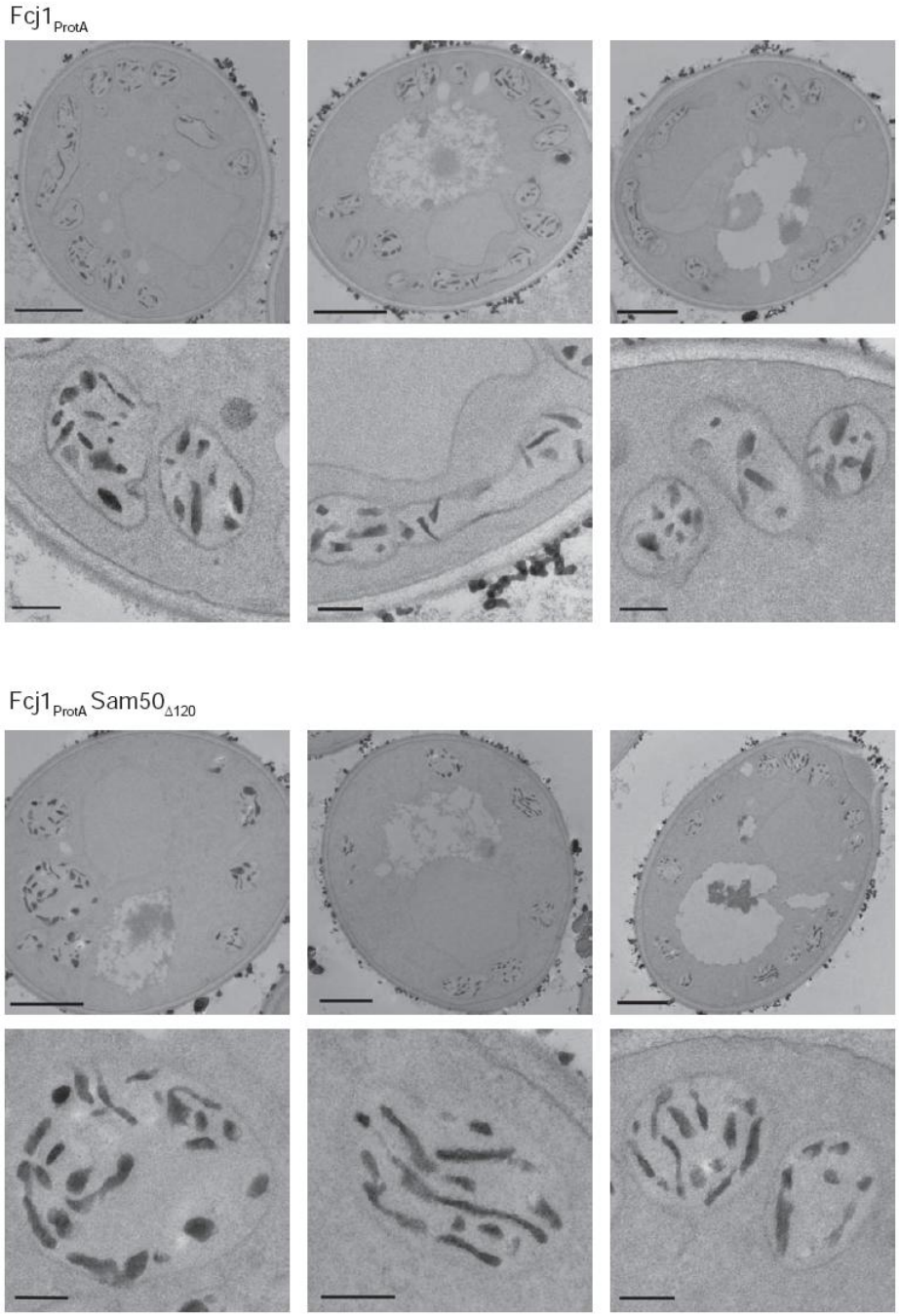

Figure S1. Mitochondrial ultrastructure of yeast cells lacking the POTRA domain of Sam50. Representative electron microscopy images of $Fcj1_{ProtA}$ cells and $Fcj1_{ProtA}\,Sam50_{\Delta 120}$ cells lacking the N-terminal POTRA domain of Sam50 are shown (mitochondria were stained with diaminobenzidine (DAB)). Bars in the first and third rows represent 1 µM; bars in the second and fourth rows represent 200 nm.

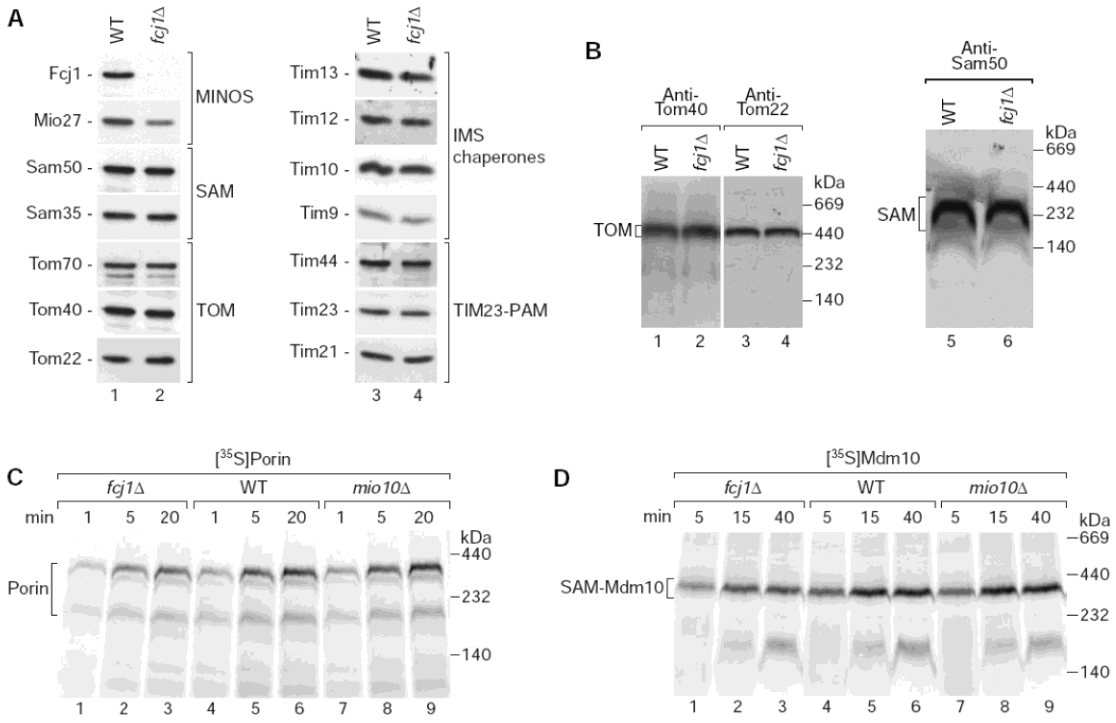

Figure S2. Steady-state levels and protein import in *fcj1*Δ mitochondria. Mitochondria isolated from wild-type (WT) and *fcj1*Δ cells were subjected to SDS-PAGE (A) or blue native electrophoresis (B) and mitochondrial protein content was analyzed by immunoblotting. IMS, intermembrane space; PAM, presequence translocase-associated motor; TIM, translocase of the inner mitochondrial membrane. (C) [^{35}S]Porin or (D) [^{35}S]Mdm10 were incubated with isolated wild-type, *fcj1*Δ and *mio10*Δ mitochondria for the indicated periods. The mitochondria were analyzed by blue native electrophoresis and digital autoradiography.

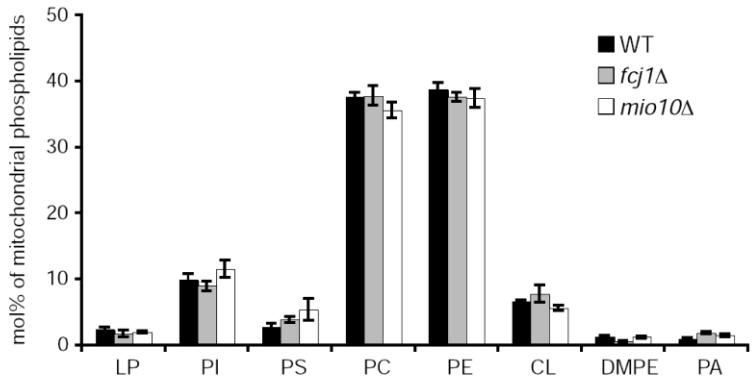

Figure S3. Phospholipid composition of MINOS mutant mitochondria. Mitochondria were isolated from wild-type (WT), *fcj1*Δ, and *mio10*Δ cells and mitochondrial phospholipids were extracted and quantified. Mean values of four measurements with standard error of the mean are shown. LP, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid.

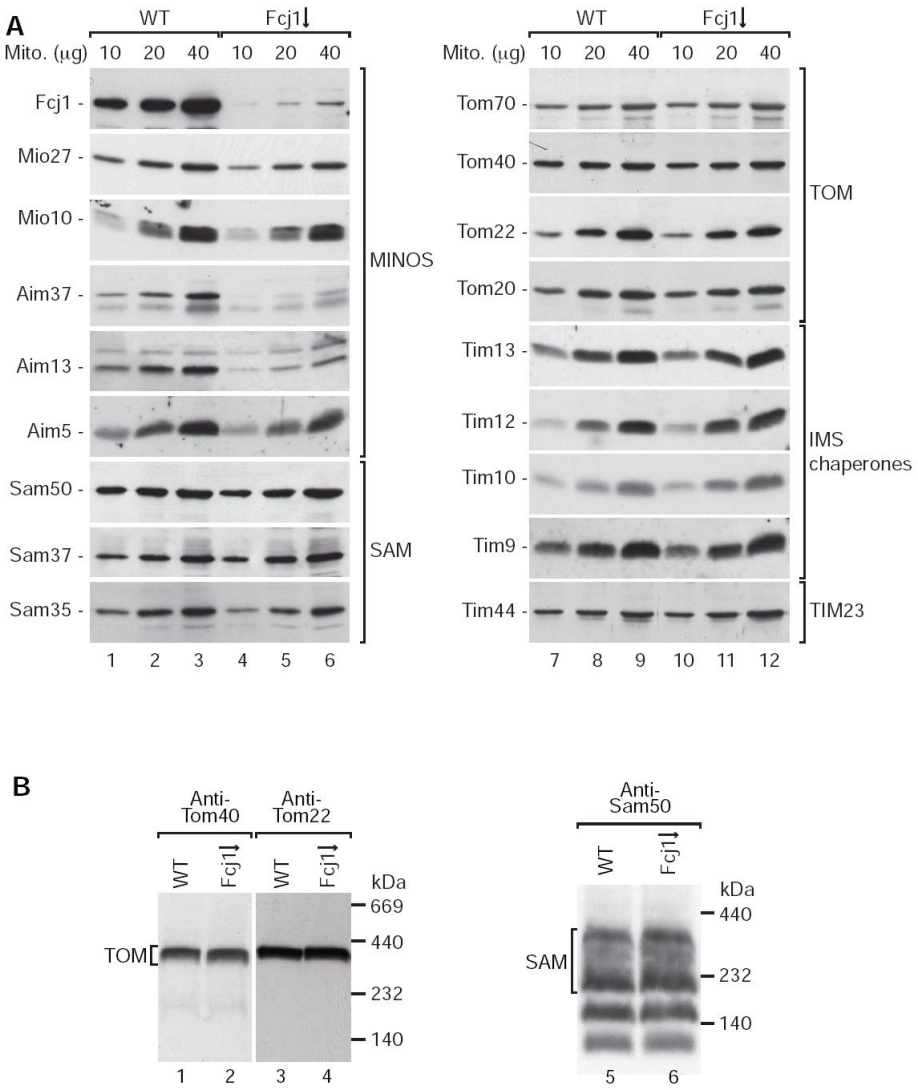

Figure S4. Mitochondrial protein content upon depletion of mitofilin/Fcj1 in yeast. Mitochondria (µg protein) isolated from wild-type (WT) or Fcj1-depleted (Fcj1↓) cells were subjected to SDS-PAGE (A) or blue native electrophoresis (B) and the protein content was analyzed by Western blotting.

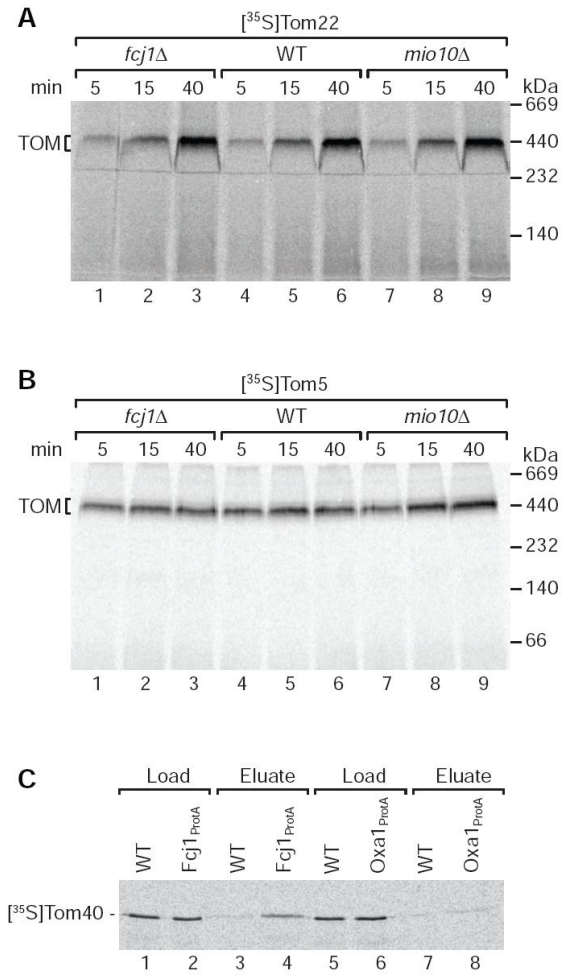

Figure S5. Biogenesis of outer membrane proteins in *fcj1*Δ mitochondria. (A) ^{35}S-labeled Tom22 or (B) ^{35}S-labeled Tom5 were imported into wild-type (WT), *fcj1*Δ and *mio10*Δ mitochondria for the indicated periods. Upon solubilization in digitonin-containing buffer, blue native electrophoresis and digital autoradiography were applied. (C) [^{35}S]Tom40 was imported into wild-type, Fcj1$_{ProtA}$ and Oxa1$_{ProtA}$ (control) mitochondria for five minutes. Mitochondria were re-isolated, lysed with digitonin-containing buffer and subjected to IgG affinity chromatography, elution with TEV protease, SDS-PAGE and digital autoradiography. Load, 0.5%; elution, 100%.

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General Discussion and Summary

Phospholipid metabolism and triacylglycerol (TAG) formation in the yeast *Saccharomyces cerevisiae* are dynamic processes depending on the substrate availability and the growth state. Identification of Lro1 as a phospholipid:diacylglycerol acyltransferase (PDAT) preferentially utilizing fatty acid moieties from phosphatidylethanolamine (PE) for the acyl-CoA independent TAG synthesis, suggested a close relationship between the biosynthesis of both lipid classes [1-3]. Here, we showed a link between TAG and PE metabolism and demonstrated that the CDP-ethanolamine (CDP-Etn) pathway contributed most to the cellular TAG level (Figure 1). Disruption of the CDP-Etn pathway decreased not only the Lro1 activity but also the cellular and microsomal PE levels whereas transcription of *LRO1* was not affected. Our findings attributed a dual function to the CDP-Etn pathway. On one hand, it produces PE for supply to mitochondria thereby rescuing growth defects of *psd1Δ* strains. On the other hand, it supports TAG formation through the acyl-CoA independent pathway catalyzed by Lro1. Furthermore, local availability of PE formed by the CDP-Etn pathway in microsomes appears to be crucial for TAG synthesis *via* Lro1, since enzymes of both biosynthetic pathways reside in the same subcellular compartment. Thus, the PE pool in micorosmes appears to be an important tool to regulate cellular TAG levels.

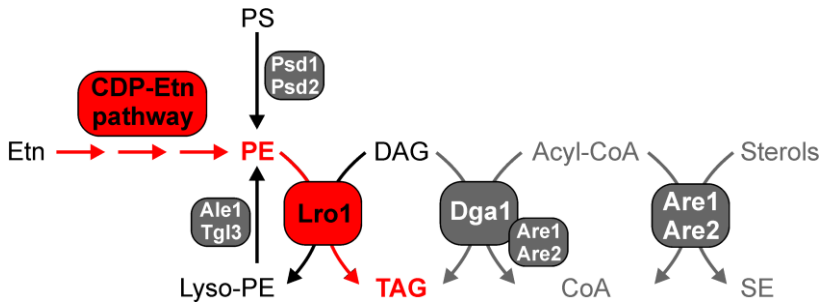

Figure 1. Metabolic link between PE biosynthesis and TAG formation. Lro1 preferentially uses PE from the CDP-Etn pathway as a substrate for the acyl-CoA independent TAG synthesis. DAG, diacylglycerols; Etn, ethanolamine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SE, sterol esters; TAG, triacylglycerols.

Although Psd1 is not primarily involved in PE supply to the acyl-CoA independent TAG formation, it represents the major source of PE biosynthesis in the yeast *Saccharomyces cerevisiae* [4]. Like the vast majority of mitochondrial proteins, Psd1 is synthesized on cytosolic ribosomes and translocated into mitochondria where processing/protein maturation occurs. Psd1 in the precursor form contains a mitochondrial targeting sequence, an internal sorting sequence, and an α- and a β-subunit which are linked by an LGST cleavage site. Cleavage at this site leads to the mature and active form of the enzyme which contains a pyruvoyl group at the N-terminus of the α-subunit [5-8]. One aim of this Thesis was to study (i) the mechanisms triggering Psd1 import into mitochondria, (ii) effects of mitochondrial processing peptidases on protein maturation and (iii) the topology of Psd1, in particular localization of α- and β-subunits within mitochondrial compartments. We showed that Psd1 is preferentially recognized by the outer mitochondrial membrane receptors Tom70 and Tom22 thereby guiding the precursor to the general import pore Tom40 (Figure 2).

During this recognition and translocation process at the outer mitochondrial membrane an autocatalytic cleavage into α- and β-subunits occurs. Interestingly, both subunits are still connected to each other as confirmed by pull-down assays and further handed over to the translocase of the inner mitochondrial membrane.

The N-terminal targeting sequences of the β-subunit are cleaved by the matrix located processing peptidases MPP and Oct1. The mature Psd1 β-subunit is laterally released into the inner mitochondrial membrane and anchors the soluble α-subunit to the intermembrane space. Deletion of the transmembrane segment within the β-subunit caused a mislocalization of Psd1 into the mitochondrial matrix and reduced the enzymatic activity, but autocatalytic cleavage into both subunits was not impaired. Additionally, we observed that cleavage of α- and β-subunit was prevented by replacing serine with alanine at the highly conserved LGST motif leading to an inactive enzyme although import into mitochondria was not impaired. In summary, membrane integration of Psd1 and correct processing are crucial for enzymatic function and maintaining mitochondrial lipid homeostasis.

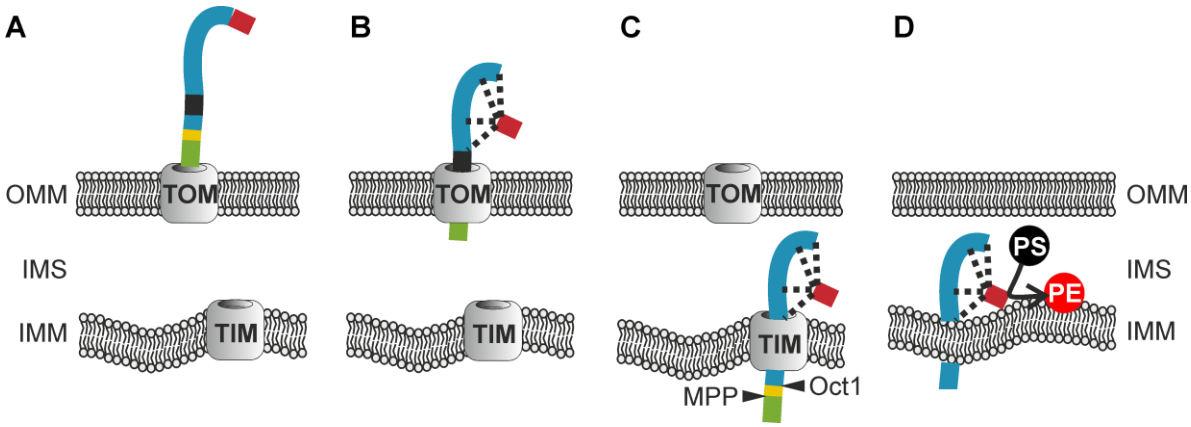

Figure 2. Import, processing and topology of the mitochondrial phosphatidylserine decarboxylase 1 from yeast. (A) Psd1 in the precursor form binds to the translocase of the outer mitochondrial membrane (TOM complex). (B) Autocatalytic processing into α-(red) and β-subunits (blue) occurs at the outer membrane with both subunits still attached to each other. (C) N-terminal targeting sequences (yellow, green) are recognized by the translocase of the inner mitochondrial membrane (TIM complex) and cleaved by the matrix located peptidases MPP and Oct1. (D) The β-subunit is localized to the inner mitochondrial membrane and anchors the soluble α-subunit to the intermembrane space-inner membrane interface. IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; PE, phosphatidylethanolamine; PS, phosphatidylserine.

As a final goal of this Thesis effects of *PSD1* deletion on mitochondrial function were studied. These investigations were performed in close collaboration with N. Pfanner (Freiburg, Germany). Depletion of PE impaired biogenesis of β-barrel proteins into the outer mitochondrial membrane at an early stage which involves the binding of the precursor to the translocase of the outer membrane (TOM complex) (Figure 3). In contrast to CL, PE is not important for the structural integrity of the TOM complex but required for its proper function in binding precursor proteins efficiently [9]. In the inner mitochondrial membrane, depletion of PE causes an impaired respiratory capacity, and reduced cytochrome c oxidase activity thereby decreasing the membrane potential of the inner mitochondrial membrane and affecting the import of precursor proteins into and across this submitochondrial compartment. Furthermore, PE favors the formation of larger respiratory chain supercomplexes between cytochrome bc_1 and cytochrome c oxidase.

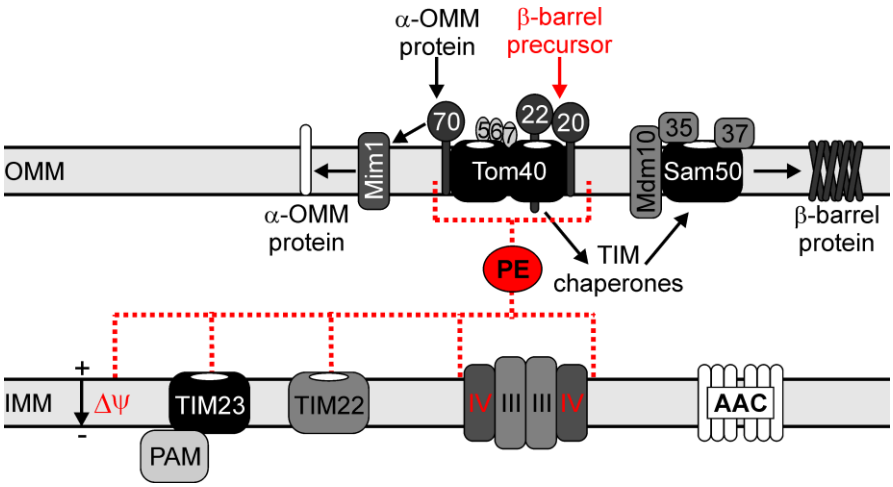

Figure 3. The interaction of PE with outer and inner mitochondrial protein complexes. PE affects the import of β-barrel precursor proteins at the stage of the TOM complex, decrease the inner membrane potential (Δψ), the import efficiency in and across the inner mitochondrial membrane (TIM23 and TIM22 complexes) and the cytochrome c oxidase activity (IV). PE favours the formation of larger respiratory chain supercomplexes between cytochrome bc_1 (III) and cytochrome c oxidase (IV). AAC, ADP/ATP carrier; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; PE, phosphatidylethanolamine; SAM, sorting and assembly machinery

Recently, MINOS a large protein complex required for maintaining the inner mitochondrial membrane architecture was identified [10]. The role of MINOS in protein biogenesis of the outer mitochondrial was investigated which revealed a connection of the MINOS core subunit Fcj1 to the assembly of β-barrel proteins. Deletion of *FCJ1* leads to morphological alterations of the inner mitochondrial membrane and affects the import of β-barrel proteins, whereas the mitochondrial lipid status was not altered excluding that the observed defects were consequences of an impaired lipid homeostasis.

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2007 - 2005	Studies of Biotechnology, Biochemistry and Food Chemistry at the University of Technology in Graz, Austria (Master Studies)
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2000 - 1996	Grammar School "Oberstufenrealgymnasium" with focus on Biology, Chemistry and Physics at the Schulverein der Grazer Schulschwestern, Graz Eggenberg, Austria
1996 - 1992	Secondary School, Schulverein der Grazer Schulschwestern, Graz Eggenberg, Austria
1992 - 1988	Elementary School, Schulverein der Grazer Schulschwestern, Graz Eggenberg, Austria

Publications

Horvath SE, Böttinger L, Vögtle FN, Wiedemann N, Meisinger C, Becker T, Daum G (2012). Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1. *J Biol Chem* **287**, 36744-36755.

Böttinger L, Horvath SE, Kleinschroth T, Hunte C, Daum G, Pfanner N, Becker T (2012). Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes. *J Mol Biol* **423**, 677-686.

Bohnert M, Wenz LS, Zerbes RM, Horvath SE, Stroud DA, von der Malsburg K, Müller JM, Oeljeklaus S, Perschil I, Warscheid B, Chacinska A, Veenhuis M, van der Klei IJ, Daum G, Wiedemann N, Becker T, Pfanner N, van der Laan M (2012). Role of the mitochondrial inner membrane organizing system in protein biogenesis of the mitochondrial outer membrane. *Mol Biol Cell* **23**, 3948-3956.

Horvath SE, Wagner A, Steyrer E, Daum G (2011). Metabolic link between phosphatidylethanolamine and triacylglycerol metabolism in the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1811**; 1030-1037.

Teaching Experience

SS 2011	Tutor in practical course “Immunologische Methoden“
SS 2010	Tutor in practical course “Immunologische Methoden”
SS 2009	Tutor in practical course “Immunologische Methoden”

Professional Memberships

ÖGMBT	Österreichische Gesellschaft für Molekulare Biowissenschaften und Biotechnologie
ASBMB	The American Society for Biochemistry and Molecular Biology

Conferences and Seminar Attendance

09/2012	International Conferences on the Bioscience of Lipids (ICBL), Banff, Canada (Oral Presentation)
07/2011	DocDay Nawi Graz Doctoral School Molecular Biosciences and Biotechnology, Graz, Austria (Oral Presentation)
05/2011	Yeast Lipid Conference, Oulu, Finland (Oral Presentation)
10/2009	Euro Fed Lipid Congress, Graz, Austria (Poster Presentation)
09/2009	ÖGMBT Annual Meeting, Innsbruck, Austria (Poster Presentation)
07/2009	DocDay Nawi Graz Doctoral School Molecular Biosciences and Biotechnology, Graz, Austria (Poster Presentation)
06/2009	FEBS/EMBO Advanced Lecture Course: “Cellular and Molecular Biology of Membranes”, Corsica, France (Poster Presentation)

Prizes, Funding and Awards

09/2012	JBC H. Tabor Award for the talk: "Characterization of phosphatidylserine decarboxylase 1 from yeast" at the International Conference on the Bioscience of Lipids (ICBL) in Banff, Canada
06/2009	FEBS (Federation of European Biochemical Societies) Youth Travel Fund to assist the FEBS/EMBO Advanced Lecture Course "Cellular and Molecular Biology of Membranes", Corsica, France

Additional Research Experiences

11/2010 - 08/2010	Laboratory of N. Pfanner, Institute of Biochemistry and Molecular Biology, ZBMZ, Freiburg, Germany
05/2010 - 02/2010	Laboratory of N. Pfanner, Institute of Biochemistry and Molecular Biology, ZBMZ, Freiburg, Germany
11/2007	Institute of Biochemistry, University of Technology, Graz, Austria
01/2006	Institute of Food Chemistry, University of Technology, Graz Austria
09/2004	Institute of Organic Chemistry, University of Technology, Graz, Austria
07/2005	Water Laboratory Holding Graz GmbH, Graz, Austria
08/2004	Water Laboratory Holding Graz GmbH, Graz, Austria
07/2003	Water Laboratory Holding Graz GmbH, Graz, Austria