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Lipid traffic to peroxisomes in the yeast

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Zusammenfassung

Die vorliegende Arbeit befasst sich mit Phospholipidtransport und Lipid-Stoffwechselwegen, die zur Versorgung der Peroxisomen in der Hefe *Saccharomyces cerevisiae* unter Peroxisomen induzierenden Bedingungen beitragen. In *Saccharomyces cerevisiae* erlangen Peroxisomen besondere Bedeutung für das Wachstum auf Fettsäuren, da diese Organellen die ß-Oxidation für den Fettsäureabbau enthalten. Ein Ziel dieser Arbeit war es, die Beiträge der zwei Hauptstoffwechselwege und Transportmechanismen von Phosphatidylcholine (PC) zu den Peroxisomen der Hefe zu erforschen. PC wird über zwei Synthese-Routen gebildet, u.z. (i) den Methylierungs-Weg, und (ii) den sog. CDP-Choline-Zweig des Kennedy-Wegs. Mutationen in den jeweiligen Stoffwechselwegen wurden eingeführt, und Peroxisomen, Mitochondrien und Endoplasmatisches Retikulum der Mutanten und des Wildtyps wurden isoliert, analysiert und miteinander verglichen. Diese Analysen zeigten, dass beide Biosynthesewege in unterschiedlichen Maß zur Versorgung der Peroxisomen mit PC beitragen. Der Einfluss der Mutationen in den PC Biosynthesewegen hat aber weiter reichende Konsequenzen, da auch die Biosynthese von Phosphatidylethanolamine (PE) beeinflusst wird. Diese Veränderungen wirkten sich auf Fluidität/Rigidität der Membranen aus, was mit fluoreszenzspektroskopischen Methoden nachgewiesen werden konnte. Diese Parameter der Membranen sind für die Funktionalität der Organellenmembranen und das Zellwachstum mit verantwortlich. Um die Bedingungen für den Transport von Phospholipiden, im Speziellen von PE zu den Peroxisomen zu untersuchen, wurde ein spezifisches *semi-in-vitro* Assay-System basierend auf dem Gebrauch permeabilisierter Hefezellen etabliert. Ein Hybridprotein der wichtigsten-PE-Methyltransferase, Opi3p, mit einer peroxisomalen Targeting-Sequenz wurde konstruiert und in Peroxisomen eines *opi3*Δ Stamm importiert. Diese Strategie erlaubte es, den Import und die Importbedingungen von PE durch das Erscheinen von PC in den Peroxisomen nachzuweisen. Diese Experimente zeigten, dass direkter Membrankontakt zwischen Peroxisomen und anderen Organellen wahrscheinlich zum Transport von PE zu Peroxisomen beiträgt. Außerdem wurde eine Energieabhängigkeit dieses Prozesses festgestellt. Zusammenfassend behandelte diese Dissertation einige grundlegende Aspekte des intrazellulären Phospholipidtransports in Hefe, die sich jedoch auch auf höhere Eukaryoten übertragen lassen sollten.

Summary

The present Thesis deals with phospholipid transport and supply of phospholipids from different biosynthetic pathways to peroxisomes of the yeast *Saccharomyces cerevisiae* under peroxisome inducing conditions. In *Saccharomyces cerevisiae*, peroxisomes are important for growth on fatty acids because ß-oxidation of this microorganism is restricted to this organelle. The first part of this thesis was focused on the main biosynthetic routes and transport mechanism of phosphatidylcholine (PC) to peroxisomes. There are two pathways which may supply PC to the peroxisomes, namely (i) the methylation pathway, and (ii) the CDP-choline branch of the Kennedy pathway. Mutants for each of the mentioned pathways were constructed and isolated organelles (peroxisomes, endoplasmic reticulum, mitochondria) from mutants were analyzed and compared to wild type. The obtained results showed, that both pathways supply PC to peroxisomes, although with different efficiency. Mutations in the PC biosynthetic pathways, however, did not only have an influence on the PC level in the mentioned organelle membranes, but also on the amount of phosphatidylethanolamine (PE). These changes resulted in altered membrane rigidity/fluidity as measured by fluorescence spectroscopy methods. These parameters are important for normal functionality of organelles and cell viability. To test conditions for phospholipid transport, especially the translocation of PE to peroxisomes, a specific *semi-in-vitro* assay using permeabilized yeast cells was designed. A hybrid protein of the main PE-methyltransferase, Opi3p, with a peroxisomal targeting sequence was constructed and introduced into peroxisomes of an *opi3*Δ strain. This strategy allowed measuring the import and investigating conditions of PE import into peroxisomes by appearance of PC in peroxisomal membranes. These assays showed that direct membrane contact between peroxisomes and other organelles is most likely responsible for delivery of PE to peroxisomes. Energy may be required for this process. In summary, this Thesis addressed a specific aspect of intracellular phospholipid traffic in yeast which may, however, also be relevant for higher eukaryotes.

General introduction

The present work deals with phospholipids, their biosynthetic pathways and their transport routes to peroxisomes of the yeast *S. cerevisiae*. Phospholipids are characterized by their structure, especially the glycerol backbone with two acyl chains attached, and the different head groups. These components are important for the biophysical properties of phospholipids and their functions in biological membranes. Phospholipids with large head groups have the tendency to organize in bilayers whereas phospholipids with smaller head groups induce curvature in membranes. The composition of phospholipids and ratio of different species is therefore most important for the integrity and function of biological membranes. On one hand, membrane curvature is required for certain membrane elasticity whereas on the other hand a solid bilayer is the basis of all biological membranes.

A good model organism for studying the phospholipid biosynthetic pathways and their transport routes is the baker's yeast, *S. cerevisiae*. This single-cell organism shares the main metabolic pathways and subcellular organization with higher eukaryotes, but is still simple enough to be easily manipulated by molecular biological and genetic means. Thus, the yeast cell has also become a very good model organism to study peroxisome biogenesis.

Similar as a busy town, the yeast cell is organized in compartments with individual properties and functions. In the endoplasmic reticulum, the major "protein and lipid factory" within the cell, the majority of structural phospholipids, sterols and storage lipids such as triacylglycerols and steryl esters are synthetized. The mitochondria are the energy producers or the "power plants" of the cell, providing most of the energy for all cellular processes in the form of ATP. Moreover, mitochondria also produce some of the specific cellular lipids. Peroxisomes act as "recycling centers" where the toxic waste such as ROS or H_2O_2 is metabolized. However, peroxisomes also provide some essential substrates for energy production by ß-oxidation of fatty acids as well as glycerolipids, cholesterol and bile acids in mammalian cells.

Since most of the cellular organelles cannot synthesize the required phospholipids themselves, communication and lipid traffic between organelles is of high importance. Transport mechanisms which deliver phospholipids to peroxisomes are not well defined, and also pathways which supply phospholipid classes to the peroxisomes have only recently been studied. The aim of the present thesis was to investigate and shed some more light on the

supply of phospholipids to peroxisomes and to study the connection between peroxisomes and other organelles in the yeast cell.

Peroxisomes

Peroxisomes appear to be simple organelles, but in the end turned out to be a compartment difficult to study due to experimental problems. Peroxisomes were discovered in 1954 using electron microscopy (Schrader and Fahimi 2008) and were considered as a "fossil organelle". Only in 1966, biochemical studies led to the discovery of H_2O_2-producing oxidases as well as catalase, an H_2O_2 degrading enzyme in the matrix of the peroxisomes (De Duve and Baudhuin 1966).

Peroxisomes in contrast to mitochondria have no DNA and have only one membrane surrounding a fine granular matrix. However, peroxisomes can remarkably increase in number and size when induced by certain growth conditions and drugs (Erdmann and Blobel 1995; Fahimi et al. 1982; Lalwani et al. 1983). In the yeast, the primary function of this organelle has been associated with ß-oxidation, which resembles the mitochondrial fatty acid ß-oxidation in animal cells regarding break down and energy transformation of long fatty acyl chains (Lazarow 1978; Ishii et al. 1980; Wanders and Waterham 2006). However, in plants (Cooper and Beevers 1969) and eukaryotic microorganisms such as yeast, peroxisomes are the only site of ß-oxidation (Poirier et al., 2006).

Peroxisomes became even more interesting when their function was connected to the so-called Zellweger syndrome (Zellweger 1983, 1965), a genetic neurodegenerative peroxisomal disorder in humans (Goldfischer et al. 1973; Heymans et al. 1983). Since then biogenesis and molecular functions of the peroxisomes were studied in more detail (Schrader and Fahimi 2008; Islinger et al. 2012; Tabak et al. 2003, 2006). The importance of peroxisomes and their specific functions in neuronal cell types becomes also evident when considering that neurons, glia cells and especially astrocytes, harbor considerable numbers of peroxisome (Islinger et al. 2012).

Beside their essential catabolic (Greek *kata* = downward + *ballein* = to throw) and anabolic (Greek *ana*, "upward", and *ballein*, "to throw") functions in lipid metabolism, peroxisomes play a key role in free radical detoxification and differentiation in animal as well as in yeast cells (Schrader and Fahimi 2008; Islinger et al. 2012). In plant cells, lower eukaryotes and

protozoa, peroxisomes are required for a much wider spectrum of activities than in vertebrates, such as penicillin synthesis in fungi (Müller et al. 1991, 1992; Yang and Poovaiah 2002), glyoxylate cycle in plants (Kornberg and Beevers 1957), plant hormone-isoprenoid biosynthesis (Guirimand et al. 2012; Clastre et al. 2011; Simkin et al. 2011; Thabet et al. 2011), biotin synthesis (Tanabe et al. 2011; Maruyama et al. 2012; León 2013) and production of toxins for interaction of pathogen fungi with hosts (Saikia and Scott 2009; Imazaki et al. 2010). In mammalian cells isoprenoid/cholesterol biosynthesis has also been reported to be at least partially localized to peroxisomes (Kovacs et al. 2012, 2007, 2009; Wanders and Waterham 2006), although this localization still remains a matter of debate (Hogenboom et al. 2004). The existence of pathways which are not exclusively peroxisomal in plants and mammals but also co-localize to mitochondria or other organelles, suggests that these pathways may have been peroxisomal in ancient organisms, but were evolutionary moved to other subcellular locations leading to different functions in individual species (Islinger et al. 2012). Recent discoveries showed that peroxisomes and mitochondria have a much closer relationship than assumed. It is evident that in plant and animal cells these organelles not only share metabolic steps in fatty acid ß-oxidation, but also have in common a division machinery (Koch et al. 2003, 2005; Koch and Brocard 2012; Wiese et al. 2007), a redox-sensitive relationship (Fransen et al. 2012) and cooperate in anti-viral signaling and defense (Ivashchenko et al. 2011).

To investigate the origin of peroxisomes sequenced genomes of different organism have been studied. A high degree of similarity among membrane proteins and components of the matrix import machinery with the endoplasmic reticulum machinery has been recognized (Tabak et al. 2003, 2006; Schrader and Fahimi 2008). Most of the Pex proteins (proteins required for peroxisomal formation) are peroxisome specific and eukaryotic innovations with no prokaryotic counterpart. This fact suggested that peroxisomes do not have endosymbiotic origin (Tabak et al. 2006; Schrader and Fahimi 2008). However, the matrix proteins of peroxisomes show distant relation to alphaproteobacteria (Gabaldón et al. 2006). Interestingly, this is the same group of bacteria which were connected to the origin of mitochondria. However, it was also suggested that the proteins were acquired from the mitochondria-endosymbiont (Tabak et al. 2006). Other scientists argued that peroxisomes could have evolved in a defense strategy in an early eukaryote still lacking mitochondria at a time when oxygen levels were increasing (O_2 holocaust). By conversion of oxygen to hydrogen peroxide, the peroxisomes could remove this product of the oxidases through another reducing peroxidation reaction and produce water with certain substrates as hydrogen

donors (de Duve 1983, 2007). Although this theory is interesting, there is presently no experimental evidence for it.

Phospholipids

Phospholipids are the main building blocks of biological membranes. All cellular membranes, from plasma membrane to organelle membranes contain these lipid classes. A shortage or lack in phospholipid supply to organelles may have severe consequences for the cell. Such consequences appear to be even more dramatic for single-cell organisms like the yeast. The importance of phospholipids in the yeast is underlined by the fact that overlapping routes of biosynthesis exist. Some of them are localized to the endoplasmic reticulum where the majority of phospholipid synthesis occurs (Zinser et al. 1991; Zinser and Daum 1995), but also mitochondria (Daum and Vance 1997) and the Golgi apparatus (Trotter and Voelker 1995) contribute to this process. As example, all three compartments form phosphatidylethanolamine (PE). Mitochondria and Golgi contain phosphatidylserine (PS) decarboxylases which convert PS to PE. Otherwise, the CDP-ethanolamine branch of the Kennedy pathway localized to the endoplasmic reticulum forms PE when ethanolamine is available from external sources. This pathway is, with the exception of hepatocytes, the main phospholipid supply pathway in animal cells (Vance 2008).

The two major phospholipid classes in all eukaryotic cells are PE and phosphatidylcholine (PC). Both are essential for cell survival and cells auxotrophic in pathways for either of those phospholipids are not viable. As already mentioned above, PE can be synthesized by different routes, namely (i) by decarboxylation of PS with the aid of the mitochondrial phosphatidylserine decarboxylase Psd1p (Kuchler et al. 1986), (ii) by Psd2p in the Golgi/vacuolar compartment (Trotter and Voelker 1995; Trotter et al. 1995), (iii) by the so-called CDP-ethanolamine pathway (Kennedy and Weiss 1956) which is located to the endoplasmic reticulum (ER); and (iv) by acylation of lyso-PE (Riekhof and Voelker 2006). The other major phospholipid, PC, can be synthesized *via* two different pathways both located to the endoplasmic reticulum. Synthesis of PC can occur by methylation of PE by the methyl transferases Cho2p/Pem1p and Opi3p/Pem2p using methyl groups of S-adenosyl methionine as co-substrates (Kodaki and Yamashita 1987, 1989; McGraw and Henry 1989; Greenberg et al. 1983). Alternatively, similar to the CDP-ethanolamine branch, incorporation of choline *via* the CDP-choline branch of the Kennedy pathway (Kennedy and Weiss 1956) leads to PC

formation. Deletion of both methyl transferases leads to choline auxotrophy. However, yeast *cho2*Δ mutants are able to survive, because Opi3p/Pem2p is able to suppress the *cho2*Δ mutation (Hancock et al. 2006; Preitschopf et al. 1993).

The cooperative phospholipid biosynthesis in the endoplasmic reticulum and mitochondria has been well researched (Daum and Vance 1997; Choi et al. 2005; Gohil and Greenberg 2009; Osman et al. 2011). Only recently, direct membrane contact between these two organelles has been discovered on a molecular level (Kornmann et al. 2009; Michel and Kornmann 2012). In contrast, routes of phospholipid supply to peroxisomes in yeast are still more or less a *tabula rasa*. Only few studies are available dealing with the lipid composition of yeast peroxisomes (Rosenberger et al. 2009; Wriessnegger et al. 2007). Even less information is available about the mechanism of lipid supply to peroxisomes (Raychaudhuri and Prinz 2008; Titorenko et al. 1996).

It is known that different transport mechanism are possible for the supply of lipids to subcellular compartments such as the plasma membrane (Schnabl et al. 2003; Pichler et al. 2001; Stefan et al. 2011). Alternative routes of phospholipid traffic between mitochondria and endoplasmic reticulum have also been discussed (Achleitner et al. 1995; Gaigg et al. 1995; Kornmann et al. 2009; Nguyen et al. 2012; Shiao et al. 1995; Vance and Shiao 1996; Voelker 1985, 1988; Daum and Vance 1997). However, the exact mechanism of phospholipid transport between the ER to mitochondria is still a matter of dispute. Direct membrane contact between organelles as described for the plasma associated membrane (Pichler et al. 2001) or the mitochondria associated membrane (Vance 1990; Achleitner et al. 1999; Voelker 1990) appears to be one of the most likely mechanisms to be involved.

The questions how phospholipids are directed to organelles lacking phospholipid synthesis and which mechanisms are used remain to be answered. The present Thesis is focused on peroxisomal phospholipids with emphasis on PC supply to peroxisomes and transport mechanisms involved in aminoglycerophospholipid (PE, PC) transport to peroxisomal membranes.

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CHAPTER 1

Lipid transport between the endoplasmic reticulum and mitochondria

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Key words: phospholipids, sterols, sphingolipids, mitochondria, endoplasmic reticulum, transport

Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; CL, cardiolipin; PI, phosphatidylinositol; PG, phosphatidylglycerol; DAG, diacylglycerol; ER endoplasmic reticulum; MT, mitochondria; MAM mitochondria-associated-membrane; OMM, outer mitochondrial membrane; IMM inner mitochondrial membrane; ERMES, endoplasmic reticulum mitochondria encountered structures.

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Abstract

Mitochondria are partially autonomous organelles which depend on the import of certain proteins and lipids to maintain cell survival and membrane formation. Although phosphatidylglycerol, cardiolipin and phosphatidylethanolamine are synthesized by mitochondrial enzymes, phosphatidylcholine, phosphatidylinositol, phosphatidylserine and sterols need to be imported from other organelles. The origin of most lipids imported into mitochondria is the endoplasmic reticulum, which requires interaction of these two subcellular compartments. Recently, protein complexes that are involved in membrane contact between endoplasmic reticulum and mitochondria were identified, but their role in lipid transport is still unclear. In the present review, we describe components involved in lipid translocation between the endoplasmic reticulum and mitochondria and discuss functional as well as regulatory aspects that are important for lipid homeostasis.

Introduction

Biological membranes are major structural components of all cell types. They protect the cell from external influences, organize the interior in distinct compartments and allow balanced flux of components. Besides their specific proteome, organelles exhibit unique lipid compositions which influence their shape, physical properties and function. Major lipid classes found in biological membranes are phospholipids, sterols and sphingolipids.

The major "lipid factory" within the cell is the endoplasmic reticulum (ER). It is able to synthesize the bulk of structural phospholipids, sterols and storage lipids such as triacylglycerols and steryl esters (van Meer et al. 2008). Furthermore, initial steps of ceramide synthesis occur in the ER providing precursors for the formation of complex sphingolipids in other organelles (Futerman 2006). Besides the export of ceramides, the ER supplies a large portion of lipids to other organelles which cannot produce their own lipids or have a limited capacity to do so. Organelle interaction and transport of lipids require specific carrier proteins, membrane contact sites, tethering complexes, and/or vesicle flux. These processes are highly important for the maintainance of cell structure and survival but are still a matter of dispute. Most prominent organelle interaction partners are the ER and mitochondria. As subfraction of the ER named mitochondria associated membrane (MAM) (Vance 1990) was described to be involved in lipid translocation to mitochondria. MAM is composed of the outer ER network membranes which are in contact or close proximity with the outer mitochondrial membrane (OMM). Contact sites between MAM and mitochondria were assumed to facilitate exchange of components between the two compartments. Interstingly, MAM harbor a number of lipid synthesizing enyzmes (Gaigg et al. 1994). Recently, molecular components governing membrane contact between the two organelles were identified (Csordás et al. 2006; Friedman et al. 2010; de Brito and Scorrano 2008; Dolman et al. 2005; Kornmann et al. 2009; Lavieu et al. 2010), although the specific role of these components in lipid translocation is not yet clear.

Major phospholipid classes of mitochondria

Cardiolipin (CL) and/or phosphatidylglycerol (PG) are considered as mitochondria specific phospholipids (Zinser et al. 1991). Both lipids are synthesized by mitochondria themselves (Davidson and Stanacev 1971). The importance of CL for ATP-production has been shown with different membrane types, e.g. bacteria, hydrogenosomes and mitochondria (Acehan et al. 2011; Mileykovskaya et al. 2005; Schlame 2008). In mitochondria, the majority of CL is localized to the inner membrane (IMM) (Zinser et al. 1991), but substantial amounts were also detected in the outer mitochondrial membrane (OMM) (Gebert et al. 2009). With its uncommon, dimeric structure (Fig. 1) CL together with phosphatidylethanolamine (PE) interacts with many mitochondrial proteins (Osman et al. 2011) and stabilizes their conformation (Joshi et al. 2012). Mutants lacking CL and PE biosynthesis are synthetic lethal in yeast and bacteria (Gohil et al. 2005). In bacteria both lipids are organized in membrane clusters (Matsumoto et al. 2006). Cardiolipin, localized only to mitochondria, is the third most common mitochondrial phospholipid and is localized primarly to the inner mitochondrial membrane (van der Veen et al. 2012; Zinser and Daum 1995) where it interacts with proteins of ATP production, mitochondrial transport systems (Bogdanov et al. 2008a; Schlame and Ren 2009) and proteins required for mitochondrial structure and fusion (Joshi et al. 2012).

Another prominent mitochondrial phospholipid is PE. Mitochondria are able to synthesize a large portion of PE by decarboxylation of PS (Birner et al. 2001; Emoto et al. 1999; Nerlich et al. 2007; Trotter et al. 1993; Vial et al. 1982). With its small hydrophilic head group and large hydrophobic tail PE is a typical non-bilayer-forming phospholipid (see Fig. 1). This structure appears to be important for some peripheral and integral membrane proteins (van den Brink-van der Laan et al. 2004). Together with CL, PE - the second most common mitochondrial phospholipid, plays a crucial role in maintaining mitochondrial morphology. Loss of both PE and CL is lethal probably due to defects in mitochondrial fusion (Joshi et al. 2012) and/or protein destabilizing (Bogdanov et al. 2008b; Osman et al. 2009b). Lack of PE synthesized in the IMM cannot be compensated by PE imported from extramitochondrial sites (Joshi et al. 2012; Birner et al. 2001; Bürgermeister et al. 2004), however PE derived from lyso-PE by remodeling processes, can restore both intra- and extramitochondrial deficiencies (Riekhof and Voelker 2006). Noteworthy, mitochondria are the major supplier of PE to other organelles (Voelker 1984).

Mitochondrial enyzmes of CL and PG synthesis, as well as the major PS decarboxylase, are synthesized on cytosolic ribosomes, imported into mitochondria, and assembled into the IMM (Minskoff and Greenberg 1997; Jiang et al. 2000; Nowicki et al. 2005; Nebauer et al. 2007; Choi et al. 2012). Protein precursors in general are imported to the mitochondria trough the outer membrane entry gate, TOM-translocase of the outer membrane and trough the inner membrane gate TIM inner membrane translocase (TIM22, TIM23) complexes. Also the newly identified MINOS (mitochondrial inner membrane organizing system) complex was shown to be important for protein precursor import and organisation of the proteins (van der Laan et al. 2012; Zerbes et al. 2012). MINOS interacts with TOM and the sorting and assembly machinery for outer membrane proteins (SAM) (Bohnert et al. 2012). The translocation trough the inner membrane is accomplished by the TIM. Only recently we were able to demonstrate experimentally the involvement of components of the mitochondrial proteases in import and processing of the mitochondrial TOM complex and of mitochondrial PS decarboxylase Psd1p from the yeast (Horvath et al. 2012). Depending on the sorting signal the precursor protein is, after it arrives form the cytosol and imported by TOM, send to the OMM the IMS, the IMM or the matrix.

Most of mitochondrial matrix and inner membrane proteins (70%) have N-terminal presequences wich serve as import signals and are cleaved off after the import into the matrix by mitochondria by peptidases (Oct1p, IMP, MPP, Atp23p, Icp55,Pcp1p) (Desy et al. 2012), (Mossmann et al. 2012). Many of the protein precursors contain a multitude of internal signals that are non-clevable and remain an integral part of the mature protein. Those signals include cysteine motifs (MISS) as targeting signals which form intramolecular disulfide bonds in the inner mitochondrial space and trap the protein there, or activate it once imported inside the matrix (Mossmann et al. 2012).

Phosphatidylcholine (PC) has a big hydrophilic head group and a long hydrophobic tail (see Fig. 1) and is the most common of mitochondrial phospholipids. Its cylindrical shape makes it a perfect component of bilayer membranes and its role as structural component is essential (van Meer et al. 2008). Specific functions of PC in mitochondria are not well defined. Similar to PC, specific functions of phosphatidylinositol (PI) in mitochondria are not known, but its essential role in total cellular metabolism makes it directly or indirectly indispensible for the maintenance of mitochondria. The bulk head group of PI makes this phospholipid a specific membrane component.

Phosphatidylserine (PS) has also a cylindrical shape which allows integration into bilayer membranes (see Fig. 1). Although PS is present in low concentrations in organelles of eukaryotic cells (Zinser and Daum 1995) it is important as precursor for the two major phospholipids PE and PC. Supply of PS to mitochondria is essential because in many cells the majority of PE is formed by decarboxylation of PS in mitochondria. The metabolic conversion of PS to PE upon import of PS into mitochondria provides a convenient biochemical method to study this import process (Achleitner et al. 1995, 1999; Voelker 1988, 1989b, 1991, 1992, 1993).

Typically, the lipid composition of mitochondria shows as major components 40%-44% PC, 27%-34% PE, 1%-3% PS, 5%-15% PI, and 13%-14% CL depending on the cell type (Daum and Vance 1997; Zinser and Daum 1995; van Meer 2008). OMM and IMM are strongly different with respect to their lipid equipment. Whereas the IMM is strongly enriched in proteins and contains only 20 % lipids of total mass, the OMM is a lipid rich membrane (Daum and Vance 1997; Zinser and Daum 1995; Dolis et al. 1996). Accumulation of CL, PG and also PE in the IMM appears to be related to functions mentioned above.

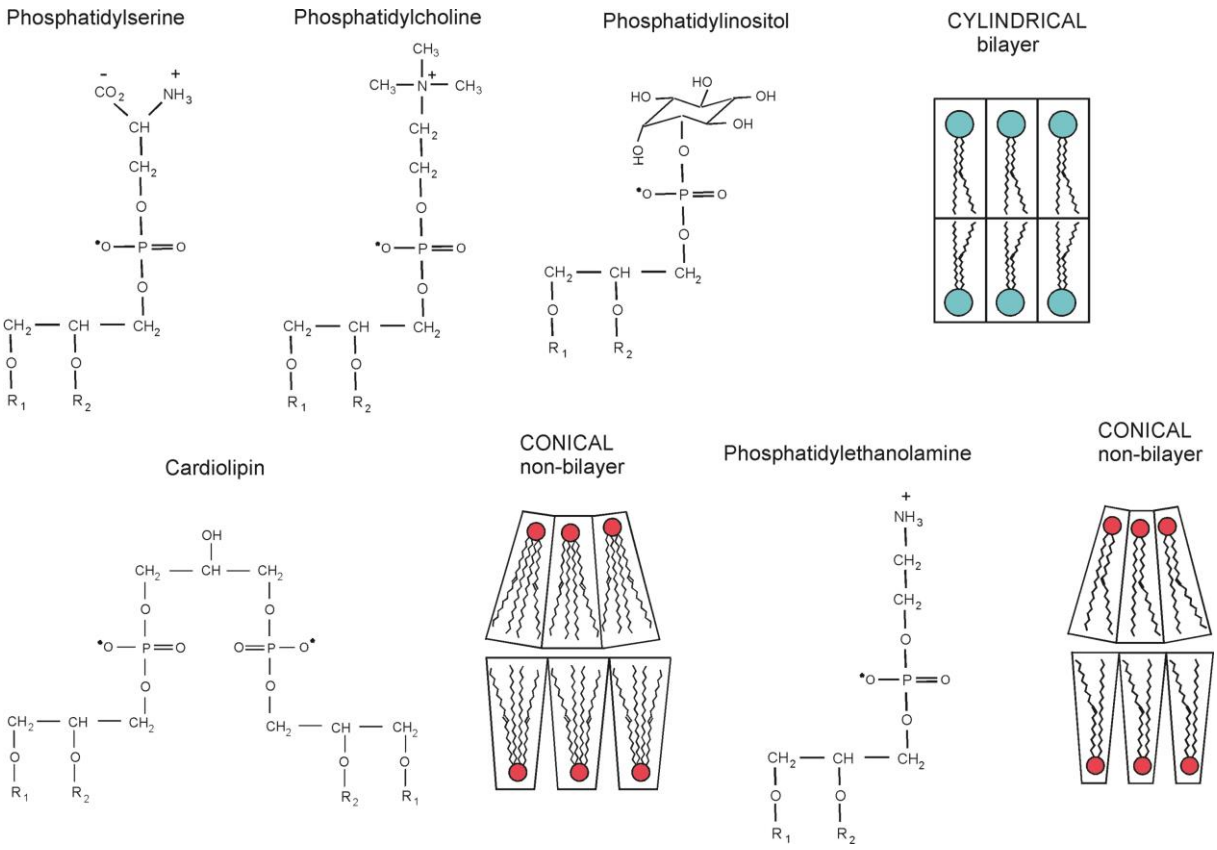

Figure 1: Major phospholipid classes of mitochondria.

Chemical formulas and geometrical forms of the major mitochondrial phospholipids are shown. The shape-structure concept of lipids compares the area of the head group with the area of their acyl chains. If the cross-section area of the head group is similar to the cross section area of the acyl chains, the lipids have an overall cylindrical shape and can self-assemble into bilayer phases similar to phases of a biological membrane. A typical example for such geometry is PC. If the cross-section area of the head group is smaller than that of the acyl chains, lipids have a conical shape and form structures with a negative curvature, such as hexagonal phase. Examples of this type are PE and CL. While PC, PS and PI exhibit cylindrical shape and self-organize into bilayers, PE and CL with their conical shape induce hexagonal phases and disturb bilayer arrangement.

Synthesis of aminoglycerophospholipids involves interaction of organelles

The biosynthetic sequence of aminoglycerophospholipid formation starts with the synthesis of PS in the ER (Fig. 2). The highest concentration of PS synthesizing enzymes has been detected in the MAM (mitochondria associated membrane) (Vance 1990; Kuchler et al. 1986). Interestingly, mammalian cells and yeast have different pathways to produce PS. In mammalian cells, two phosphatidylserine synthases (PSS-1 and PSS-2) produce PS by base exchange at the head group of PE or PC (Vance 2008) in a Ca^{2+} dependent reaction (Fig. 2B). The Ca^{2+} release in the lumen of ER is energy dependent. In yeast, formation of PS is catalyzed by the gene product of *PSS1/CHO1* (Letts et al. 1983) which requires CDP-diacylglycerol and serine (Nikawa and Yamashita 1981) as substrates and depends on Mg^{2+} or Mn^{2+} (Fig. 2A) and ATP for the formation of CDP-DAG and the cleavage of its pyrophosphate bond. In plants, both pathways described above are active for PS production (Delhaize et al. 1999; Manoharan et al. 2000; Rontein et al. 2003). In all types of cells, PS synthesized in the ER is exported to other organelles, including mitochondria where it serves as a substrate for PE synthesis.

PE is the second most abundant lipid of eukaryotic cells. It can be synthesized by four different pathways: (i) the CDP-ethanolamine pathway (also named Kennedy pathway) (Kanfer and Kennedy 1964); (ii) decarboxylation of PS to PE, (iii) base exchange between different phospholipids; and (iv) acylation of lyso-PE (see Fig. 2). Yeast has two PS decarboxylases (PSD) with overlapping functions (Trotter et al. 1993). Psd1p is localized to mitochondria (Trotter et al. 1993), and Psd2p has been found in a Golgi /vacuolar compartment (Trotter and Voelker 1995). Psd1p is a component of the IMM/intermembrane space and produces the majority of total cellular and mitochondrial PE (Birner et al. 2001; Trotter et al. 1993). Yeast can also produce PE through the CDP-ethanolamine branch of the Kennedy pathway (Kennedy and Weiss 1956). The CDP-ethanolamine pathway incorporates externall present ethanolamine by phosphorylation and activation with CTP and attachment to DAG. In the final step, phosphoethanolamine is transferred from CDP-ethanolamine to a diacylglycerol acceptor, forming PE. PE formed by this pathway however can not supply the full complement of mitochondrial PE in *psd*1/2 deficient cells (Trotter and Voelker 1995). Ethanolamine phosphate which is an intermediate in this biosynthetic sequence can also be derived from sphingolipid degradation (Saba et al. 1997; Mao et al. 1997). Finally, yeast

harbors enzymes which catalyze acylation of lyso-PE (Deng et al. 2010; Riekhof et al. 2007b; Riekhof and Voelker 2006). This type of reaction named Land's cycle includes deacylation/reacylation of phospholipids (Lands 1958) and appears to be important for remodeling processes. The reason for the existence of overlapping pathways is still a matter of dispute, although evidence for distinct pools of PE has been presented (Bürgermeister et al. 2004).

Mammalian cells have the capacity to synthesize all their PE through the PSD pathway in mitochondria (Voelker and Frazier 1986), but in the presence of ethanolamine the CDP-ethanolamine pathway can fulfill most of the extramitochondrial requirements for this lipid (Vance 2008). The Land´s cycle is important for remodeling of phospholipids which have been formed by other pathways. As an example formation of PE from lysophosphatidylethanolamine can be accomplished by LPEAT2 (acyl-CoA: lysophosphatidylethanolamine acyltransferase 2) in the brain cells. Ethanolamine phospholipids are major constituents of the myelin sheath increasing the signal transmission speed along the axons (Cao et al. 2008). In plants, remodeling of PE to LPE was reported to be involved in plant growth promotion and leaf senescence (Hong et al. 2009; Cowan 2009). In the parasite *Trypanosoma brucei* and *Plasmodium berghei*, PE is exclusively produced through the CDP-ethanolamine branch of the Kennedy pathway (Serricchio and Bütikofer 2011).

PC is the most abundant phospholipid in eukaryotic cells (van Meer et al. 2008). Since mitochondria lack PC synthesizing enzymes this phospholipid has to be imported from the ER. PC can be produced via three pathways: (i) the CDP-choline branch of the Kennedy pathway (Gibellini and Smith 2010) (ii) methylation of PE (Li et al. 2005; Sundler and Akesson 1975) and (iii) the Land`s cycle, in which LPCAT (lysophosphocholine acyltransferase) produces PC from lyso-PC and fatty acids (Hishikawa et al. 2008; Shindou et al. 2009; Riekhof et al. 2007a) (see Fig. 2). In most mammalian cells the majority of PC is formed through the CDP-choline pathway (Kennedy 1956; Hermansson et al. 2011; Gibellini and Smith 2010), and only in hepatocytes PE methylation is the predominant pathway (Li et al. 2005; Sundler and Akesson 1975). Reactions of the CDP-choline pathway are similar to the CDP-ethanolamine pathway and catalyzed by choline kinase, phosphocholine cytidyltransferase and choline phosphotransferase. In the final step phosphocholine is transferred from CDP-choline to DAG forming PC. In yeast (Boumann et al. 2004) as well as

in mammals (Henneberry and McMaster 1999) enzymes of the both branches of the Kennedy pathway have overlapping substrate specificities.

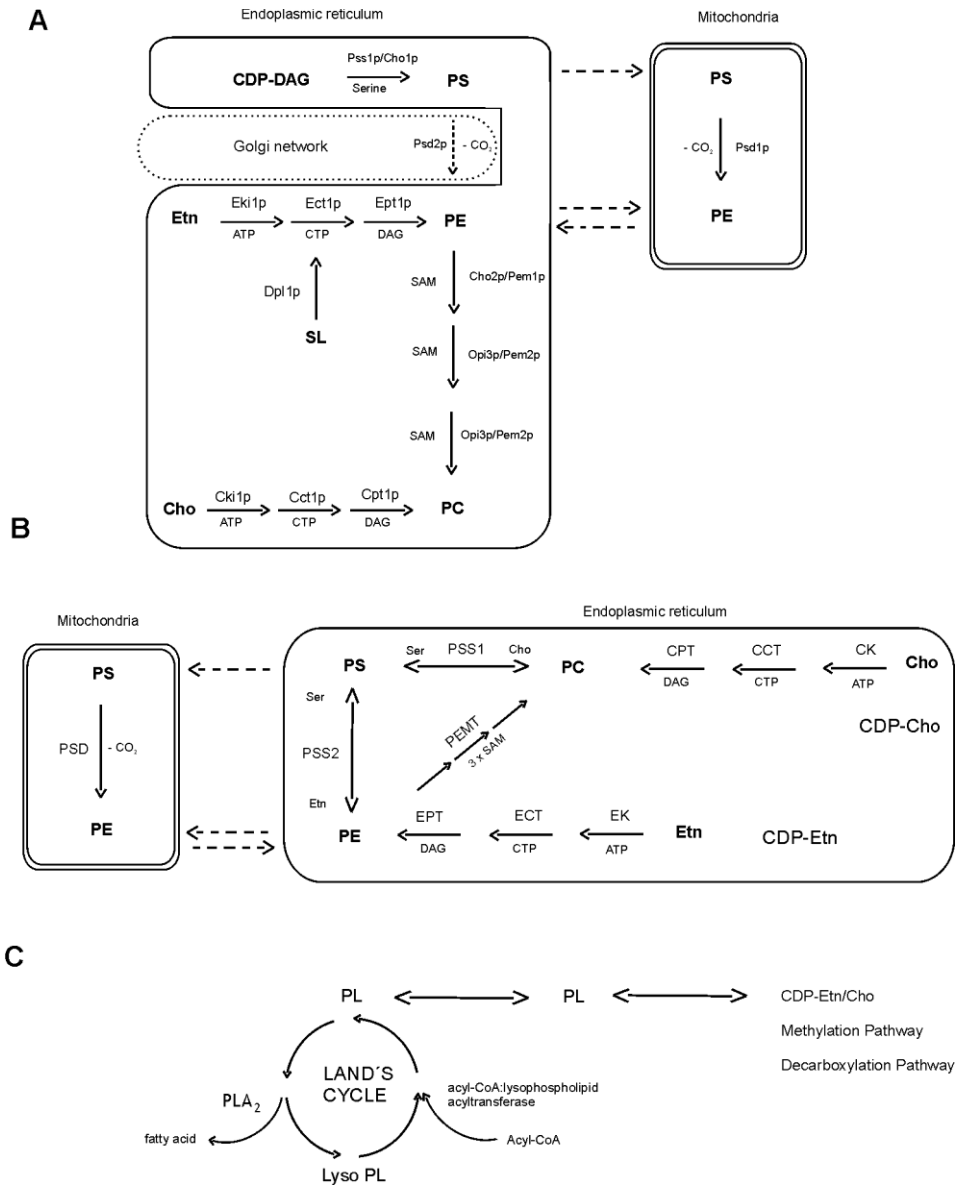

Figure 2: **Biosynthetic pathways of aminoglycerophospholipids**

A: Biosynthesis of aminoglycerophospholipids in yeast. Formation of PS is accomplished in the ER and catalyzed in a CDP-DAG dependent reaction by the PS synthase Pss1p. Decarboxylation of PS yielding PE occurs in mitochondria (via Psd1p) and Golgi/vacuolar Apparatus (via Psd2p). Three step methylation of PE in the ER catalyzed by Cho2p/Pem1p and Opi3p/Pem2p leads to formation of PC with S-adenosine methionine (SAM) as methyl donor. PC and PE can be also formed by the CDP-ethanolamine and CDP-choline branches of the so-called Kennedy pathway making use of exogenous or endogenous choline (Cho) and ethanolamine (Etn), respectively. SL, sphingolipids.

B: Aminoglycerophospholipid biosynthesis in mammalian cells. The major mechanism of PS production in mammals is base exchange with PC (PSS1) and PE (PSS2) as substrates. A major route of PE and PC production are the CDP-ethanolamine and CDP-choline pathways. In mammalian cells, PE can also be produced by decarboxylation of PS. In hepatocytes a single methyltransferase catalyzes methylation of PE to PC.
C: The Land´s cycle describes a sequence of de-acylation and re-acylation. PE and PC are converted to lysoPE and lysoPC and *vice versa*.

The second pathway of PC production in mammalian cells (Van Pilsum and Carlson 1970) and yeast (Kodaki and Yamashita 1987) is PE methylation. In this sequence S-adenosylmethionine (SAM) serves as methyl donor for three steps of methyltransferase reactions, PE is in the first step methylated to monomethyl-PE and to dimethyl-PE and PC in the last two steps. In yeast, the gene products of *CHO2/PEM1* and *OPI3/PEM2* are involved (Kodaki and Yamashita 1987), whereas hepatocytes harbor only one N-methyltransferase which catalyzes all three steps of PE methylation (Ridgway et al. 1989; Sundler and Akesson 1975). The lower eukaryote *Trypanosoma brucei* lacks genes coding for PE N-methyltransferases (Gibellini et al. 2009).

Cardiolipin and phosphatidylglycerol synthesis in mitochondria

Synthesis of PG and CL occurs in mitochondria by a multistep reaction sequence (Schlame et al. 1993). PA is converted to CDP-DAG by CDS1, either in the ER/MAM and is imported to the mitochondria or at the inner mitochondrial membrane directly. The reaction sequence of CL synthesis includes conversion of CDP-DAG to phosphatidylglycerol phosphate (PGP) by PGS1 (Chang et al. 1998a). PGP is further dephosphorylated to PG, the reaction catalyzed by the PGP phosphatase GEP4 (Osman et al. 2010). In the final step a phosphatidate (PA) moiety is transferred from CDP-DAG to the hydroxyl in the head group of PG. The cleavage of the pyrophosphate group is providing the chemical energy for the latter reaction. In yeast (Chang et al. 1998b; Jiang et al. 1997; Tuller et al. 1998), *Arabidopsis* (Katayama et al. 2004; Nowicki et al. 2005) and human cells (Houtkooper et al. 2006; Chen et al. 2006; Lu et al. 2006) CL synthase was localized to mitochondria. Importantly, CL undergoes remodeling processes (Joshi et al. 2009; Schlame and Ren 2009) with so-called tafazzins (Malhotra et al. 2009) involved. Taffazins are phospholipid transaclyases which transfer acyl groups from phospholipids, preferentially PC, to monolysocardiolipin (MLCL). The reverse reaction of

this modification is the transfer of an acyl group from CL to LPC. The reaction does not require activation of fatty acids but occurs in a lysophospholipid-phospholipid complex by deprotonation and nucleophilic attack on the ester bond of the acyl donor (Xu et al. 2006).

PA and CDP-DAG are important intermediates not only for PG/CL synthesis but also for the formation of PI and PS (Athenstaedt and Daum 1999). In the yeast, PA is synthesized from glycerol-3-phosphate and dihydroxyacetone phosphate (DHAP) in the ER and lipid droplets by two steps of acylation. The first acetylation reaction leads to acyl-DHAP, which is reduced by a NADPH-dependent reaction to LPA (lysophosphatidic acid). LPA is in the finaly step converted by a another acetylation step to PA (Athenstaedt et al. 1999a; Sorger and Daum 2003). Alternatively, PA can also be generated by phospholipase D (Osman et al. 2011) in mitochondria. In plants, PA synthesis occurs in plastids, mitochondria and microsomes. Dephosphorylation of PA is catalyzed by mammalian lipin and the yeast ortholog Pah1p (Brindley et al. 2009; Carman and Han 2009, 2006; Harris and Finck 2011) which were recently shown to be pacemakers and switch points in lipid metabolism. Lipins or phosphatidic acid phosphatases (PAH) act in triglyceride synthesis by dephosphorylation of PA to form diacylglycerols (DAG) and are responsible for de novo lipid synthesis and formation of lipid droplets (Adeyo et al. 2011). CDP-DAG synthase activity in yeast as well as in mammals was localized to the ER and mitochondria (Chen et al. 2006; Kelley and Carman 1987; Kuchler et al. 1986).

Phosphatidylinositol synthesis

PI is produced in the ER and has to be imported into mitochondria. PI is synthetized from CDP-diacylglycerol and inositol (Gardocki et al. 2005). In the yeast, deletion of the only phosphatidylinositol synthase gene *PIS1* is lethal (Nikawa et al. 1987). PI and its phosphorylated forms serve as precursors for cell signaling molecules, like phosphatidylinositol phosphates (PIP), bisphosphates (PIP2) and triphosphates (PIP3) and the biosynthesis of GPI anchors (Serricchio and Bütikofer 2011).

Transport of phospholipids between the endoplasmic reticulum and mitochondria

As the biosynthetic sequence of PS-PE-PC synthesis occurs in different subcellular compartments (see above), the amounts of intermediates and products are indication and measure for transport between organelles. Therefore, inter-organelle translocation of aminoglycerophospholipids can be investigated by following the metabolism of serine; formation of PS in the ER, the decarboxylation of PS to PE in the mitochondria and the methylation of PE to PC upon the return of PE to the ER, without isolation of organelles. Making use of this experimental strategy, phospholipid transport studies with intact cells, isolated organelles and permeabilized cells have been performed (Achleitner et al. 1995; Butler and Thompson 1975; Carrasco et al. 2006; Emoto et al. 1999; Kornmann et al. 2009; Kuge et al. 2001; Nguyen et al. 2012; Padilla-López et al. 2012; Riekhof and Voelker 2006; Schumacher et al. 2002; Shiao et al. 1995; Tamura et al. 2012; Vance 1990; Voelker 1985, 1989a,1989b, 1990, 1991; Wu and Voelker 2004). These studies showed that in mammalian cells PS transport from MAM to the OMM requires ATP. Further import of PS to the IMM yields PE (Shiao et al. 1995; Voelker and Frazier 1986; Voelker 1990, 1991).

Experiments with isolated organelles provided more detailed information about the process of phospholipid import into mitochondria. Reconstitution of transport systems using isolated mitochondria and microsomes/MAM (Achleitner et al. 1999; Emoto et al. 1999; Kuchler et al. 1986; Simbeni et al. 1990; Vance 1990) revealed that uptake of PS by mitochondria depended on the PS concentration in the donor membranes (ER, Golgi) (Wu and Voelker 2004). Although ATP was required for transport of PS in intact and permeabilized cells (see below), no such observation was made in the reconstituted *in vitro* system. It was argued that close apposition of donor (ER/MAM) and acceptor (mitochondria) membranes was sufficient for transport, but that ATP was probably required to provide appropriate conditions for transport of this phospholipid in intact cells. Experiments making use of the co-isolation of MAM with mitochondria suggested that the contact of these two cellular fractions is fairly tight (Achleitner et al. 1999; Vance 1990). Altogether, it was concluded that membrane contact between ER and mitochondria is important for lipid translocation between these two organelles.

Experiments performed with permeabilized cells provided a useful alternative to the experiments described above (Achleitner et al. 1995; Kuge et al. 2001; Lim et al. 1986;

Voelker 1992; Wu and Voelker 2001). Permeabilized cells are obtained by mild chemical or mechanical treatment of whole cells, resulting in the internal organelle structures remaining largely intact, while access to the interior is possible for compounds which cannot enter an intact cell. In permeabilized yeast cells (Achleitner et al. 1999) [^{3}H]serine is incorporated into PS in the ER. Transport of [^{3}H]PS to mitochondria yields [^{3}H]PE formed by mitochondrial Psd1p. Use of *psd2*Δ mutants and depletion of ethanolamine from the medium allows attribution of the formed [^{3}H]PE to transport of [^{3}H]PS from the ER. Transport of [^{3}H]PE from mitochondria to the ER results in formation of [^{3}H]PC as a measure for this transport route (Achleitner et al. 1995, 1999; Shiao et al. 1998).

The advantage of permeabilized cells is that reactions can be manipulated by addition of chemicals such as divalent ions, energy blockers or cytoskeleton inhibiting reagents (Eilers et al. 1989) or by removing the cytosol. This feature allowed additional characterization of lipid transport between ER and mitochondria with mammalian (Voelker 1990) and yeast cells (Achleitner et al. 1995). Transport of PS to mitochondria was shown to be dependent on ATP in mammalian cells (Voelker 1989a) but not in yeast (Achleitner et al. 1999). However, a certain amount of ATP must be present in both cell types (Shiao et al. 1995) since upstream reactions of lipid precursor formation are energy consuming. It was also shown that ongoing synthesis of PS is not required for translocation to mitochondria because pre-formed PS was efficiently used as a substrate for mitochondria as acceptors for PS transport. Interestingly, when exogenous mitochondria were added to permeabilized cells, they failed to serve as receptors for PS import (Voelker 1993). This result suggests that pre-existing, stable associations between mitochondria and ER/MAM play an important role in the transport process.

In a yeast genetic screen Voelker and co-workers searched for components that influence the translocation of PS from ER to the sites of decarboxylation in mitochondria (via Psd1p) (Schumacher et al. 2002) and Golgi/vacuoles (via Psd2p) (Trotter et al. 1998; Wu and Voelker 2001, 2004). The first gene product identified was Met30p, which influenced the import of PS into mitochondria. *MET30* encodes a subunit of a multi-component E3 ubiquitin ligase (Aghajan et al. 2010; Ouni et al. 2010) and affects substrate specificity of the ubiquitin ligase (Chandrasekaran et al. 2006; Yan and Xiong 2010). One substrate for the E3 ubiquitin ligase is the transcription factor Met4p, which is inactivated upon ubiquitination. It was suggested that Met30p mediated ubiquination of certain target proteins and may play a role either in ER-mitochondria recognition, in the inhibition of this docking process, or in

regulation of transcription of a factor involved in lipid transport. The second component identified in the screen was the phosphatidylinositol 4-kinase Stt4p, which affects transport of PS to the site of Psd2p-driven conversion to PE (Trotter et al. 1998). The mode of action of Stt4p on organelle interaction and/or PS translocation remains to be elucidated. It was suggested that the C2 domain of Psd2p, which binds Ca^{2+}, interacts with proteins or lipids and recognizes anionic lipids such as PS and polyphosphoinositides (Choi et al. 2005) and may be the bridge for PS translocation. Finally, the gene product of *PDR17*/*SFH4* was found to affect PS transport to Psd2p. Reconstitution assays with permeabilized cells and isolated organelles showed that Pdr17p must be present on the acceptor membrane for transfer of PS to Psd2p (van den Hazel et al. 1999; Wu et al. 2000). The mechanism of action of Pdr17p is still unknown.

Reconstitution of PS synthesis and transport in permeabilized mammalian cells identified two other components which affect PS transport from the ER/MAM to mitochondria (Emoto et al. 1999; Kuge et al. 2001). One of these proteins is S100B (NP_006236), an EF-hand domain-Ca2+ binding protein. It is not known whether this protein participates in transport or promotes stability and/or assembly of interactions of ER/MAM and mitochondria. The second component was only indirectly identified in the mammalian cell line R41, which has a defect in PS transport between the outer and inner mitochondrial membranes.

Studies with isolated organelles treated with protease (Achleitner et al. 1999; Shiao et al. 1998) or dinitrophenol, which alters the distance between the ER and mitochondria (Hovius et al. 1992), suggested the participation of organelle surface proteins in PS transport. Electron microscopy supported this view by demonstrating close contact zones between ER/MAM and mitochondria, which might serve as bridges for lipid translocation (Csordás et al. 2006). Furthermore, the involvement of acetylated microtubules in ER-mitochondria dynamics was demonstrated, a mechanism by which membrane contact can be established and/or maintained (Friedman et al. 2010). Moreover, mitochondrial division sites produced by Dnm1p and Drp1p were found to be physically connected with the ER (Friedman et al. 2011).

To shed more light on the role of ER-mitochondria contact, genetic and synthetic genetic interactions analysis mainly with the yeast *Saccharomyces cerevisiae* were performed (Birner et al. 2003; Kornmann et al. 2009; Osman et al. 2009a). Such screenings were designed in a way that defects in two or more interacting gene products become lethal. Recently, Kornmann et al. (2009) identified components of ER-mitochondria contact in yeast at the molecular

level. A till then unknown protein complex which in WT cells tethers the ER/MAM and the mitochondria was identified. Mutations in this protein complex caused slow growth or were lethal for the cells, but could be suppressed by an artificial tehtering construct called ChiMERA carrying a GFP molecule to visualize the localization of the construct. When staining the mitochondria with a mito-tracker and expressing the ChiMERA the colocalization of both organelles was visualized. ChiMERA acted as a bridge between the two organelles therby supressing the mutations of the protein complex, representing sites of the artificial tethering. The complex was named ERMES (ER mitochondria encountered structures) and contains the OMM proteins Mdm10p, Mdm12p and Mdm34p, which interact with the ER membrane anchored protein Mmm1p. In the absence of ERMES, the CL level and the PS/PC conversion rates were decreased, suggesting the involvement of ERMES in phospholipids transport between ER and mitochondria (Fig. 3). Moreover, genetic interactions between *PSD1*, *GEM1* and the ERMES complex were shown (Kornmann et al. 2009). The Ca^{2+} Miro (mitochondria rho like) GTPase Gem1p is a regulatory component of ERMES (Kornmann et al. 2011; Stroud et al. 2011), maintains mitochondrial morphology and inheritance (Frederick et al. 2004, 2008) and most likely connects ERMES to the actin cytoskeleton (Kornmann et al. 2011; Michel and Kornmann 2012). It was assumed that Gem1p shuttles between a free and ERMES bound form (see Fig. 3) although the molecular mechanism of Gem1p remains unknown. Physical interaction of Psd1p with ERMES or Gem1p was not demonstrated. Nevertheless, it was reported that a *gem1*Δ*psd2*Δ*dpl1*Δ yeast mutant grown on a non-fermentable carbon source was defective in its PC synthesis (Kornmann et al. 2011), most likely by impaired PE and PS transport. A similar growth phenotype was reported before for the *gem1*Δ single deletion strain grown on synthetic glycerol media (Frederick et al. 2008). *GEM1* also shows strong synthetic lethality with *GEP4,* encoding a PGP phosphatase (Kornmann et al. 2011). Gem1p is well conserved in the eukaryotic kingdom, suggesting that Miro GTPases are general components of ER-mitochondria connections. Miro-1, the mammalian homolog of Gem1p, interacts with mitofusin 1 and 2, the human homologs of yeast Fzo1p. Mfn-2 was suggested to tether mitochondria to ER and being involved in mitochondrial movement along axons (de Brito and Scorrano 2008; Misko et al. 2010). Most recently, however, the role of ERMES and Gem1p in the import of PS into mitochondria and its conversion to PE was challenged (Nguyen et al. 2012). It was argued that despite their genetic and physical interaction, ERMES and Gem1p function in distinct pathways, and the absence of ERMES and Gem1p had only little effect on PS import into mitochondria and conversion to PE. The effect on PC formation as described by Kornmann et al. (2009) was

regarded as minor. Nguyen et al. (2012) argued that changes in the lipid profile of cells lacking ERMES were side effects of defects in mitochondrial morphology.

Recently, another mitochondrial complex named MitOS, MICOS or MINOS was identified (Harner et al. 2011; Hoppins et al. 2011; von der Malsburg et al. 2011) . This IMM-associated complex functions in cristae formation and morphology (Rabl et al. 2009). It was proposed (van der Laan et al. 2012) that MINOS forms the central core of a large and complex organizing system named ERMIONE which includes the ERMES complex, the prohibitin ring-like structures, the TOM and TIM complexes and Mdm31/32 proteins required for mtDNA maintenance. MINOS was also found to interact with the protein VDAC and with the fusion protein Ugo1 (van der Laan et al. 2012). Both ERMES and MINOS are genetically linked to the prohibitin ring complexes of the IMM that are integrated into the lipid network metabolism (Psd1, Ups1/Ups2) (Birner et al. 2003; Gohil et al. 2005; Kornmann et al. 2009; Osman et al. 2009a; Potting et al. 2010; Tamura et al. 2009, 2012). However, direct involvement of MINOS in lipid transport and/or assembly into mitochondrial membrane has not been demonstrated.

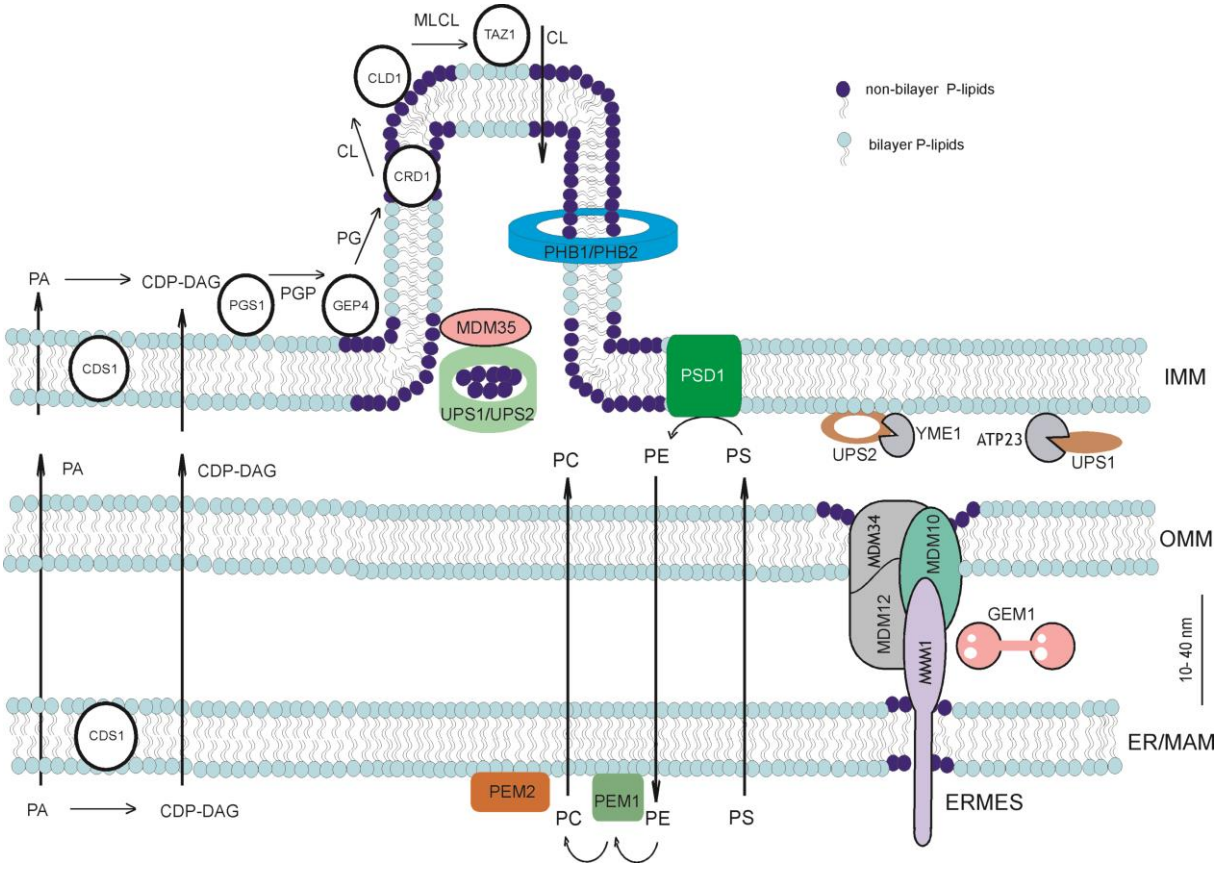

Figure 3: Components involved in translocation of phospholipids to and within mitochondria of yeast. The direction of lipid translocation is indicated by arrows. While PS and PC are imported into mitochondria, a large portion PE is exported. Upon import the majority of PS is converted to PE by Psd1p. Decarboxylation of PS to PE in the mitochondria and methylation of PE to PC in the ER are sufficient for supplying cells with PE and PC even in the absence of external ethanolamine or choline. Similar to PS and PC, also PA and CDP-DAG have to be imported to mitochondria where they are required for CL synthesis in the IMM. The pathway of CL synthesis in the IMM starts with CDP-DAG and is completed during several steps by a multi enzyme cascade. A small portion of CL is exported to the OMM. The ERMES complex containing gene products of *MDM10*, *MDM34*, *MDM12* and *MMM1* tethers ER and mitochondria. In mammalian cells, Mfn1/2 seem to have a similar function. ERMES associates with the GTPase GEM1, cycling between ERMES bound and free form. UPS1/UPS2 interact with Mdm35p and regulate the mitochondrial levels of PE and CL. UPS1 and UPS2 are degraded by ATP23 and YEM1, respectively. Prohibitin ring like structures made from two proteins PHB1/2 contribute to the formation of PE and CL clusters in the IMM. For a detailed description of translocation processes and components involved see text.

Link between cardiolipin formation and intramitochondrial transport of aminoglycerophospholipids

A link between CL and PE assembly in mitochondrial membranes, PE synthesis by Psd1p, and the role of prohibitins was uncovered in synthetic lethal screens with the yeast (Birner et al. 2003; Gohil et al. 2005). It was shown that a *phb1*Δ*phb2*Δ*psd1*Δ triple mutation was lethal due to reduced PE levels and loss of mtDNA. Osman et al. (2009a) demonstrated that ring-like prohibitin complexes organize CL and PE in clusters in the IMM and become essential in strains with low levels of PE and CL. This finding supported the view of physical similarities of CL and PE and their importance for mitochondria morphology (Joshi et al. 2012; Osman et al. 2011). In subsequent work, 35 genetic interactions of prohibitins (GEP) were identified which were required for cell survival in the absence of prohibitins. Among the genes detected *UPS1*, *GEP1/UPS2*, *PSD1*, *MDM35*, *MMM1*, *GEP4*, and *CRD1* were prominent (Osman et al. 2009b) (see Fig. 3). Prohibitins seem to control IMM organization and integrity by acting as lipid scaffolds for PE and CL (Osman et al. 2009a, 2009b). Ups1p and Ups2p antagonistically regulate the CL (Tamura et al. 2009) level, and Ups2p also regulates the PE level (Potting et al. 2010) in the IMM. Most recently, it was shown that Ups1p promotes conversion of PE to PC, whereas Ups2p suppresses this process (Tamura et al. 2012). It was concluded that UPS proteins affect export of PE from the IMM, although the mechanism of this regulation is unknown. The authors showed that loss of Ups1, the ERMES complex and Mdm31p caused similar defects in mitochondria, especially in CL and PE homeostasis in the IMM. Yeast Ups1p and Ups2p are *per se* unstable proteins and degraded by the proteases Yme1p and Atp23p in the IMM (Potting et al. 2010). The newly identified protein Mdm35p protects them from proteolytic degradation (Potting et al. 2010; Tamura et al. 2009, 2012).

Import of phosphatidylcholine and phosphatidylinositol into mitochondria

Although PC and PI are major phospholipids of mitochondrial membranes, little is known about their import into this organelle. Both phospholipids are synthesized in the ER from where they are translocated to mitochondria and assembled into membranes. Lampl et al. (1994) designed an *in vitro* assay to study the import of [^{3}H]-labeled PI and PC from unilamellar donor vesicles to isolated yeast mitochondria. Both phospholipids ended up in the IMM. During import, they were detected in contact sites between OMM and IMM, supporting

the notion that these sites are involved in intramitochondrial phospholipid transport. The uncoupler CCCP, the antibiotic adriamycin and energy depletion did not inhibit this process. Janssen et al. (1999) described transbilayer movement of PC in isolated OMM vesicles from yeast. They showed that this translocation was rapid and bidirectional. Pretreatment of the OMM with proteinase K or sulfhydryl reagents had no effect on PC transport.

Sterols and sphingolipids of mitochondria

Sterols and sphingolipids are minor lipid components of mitochondria. Both lipid classes are synthesized in extramitochondrial compartments and need to be imported and assembled into mitochondrial membranes. Sterol biosynthesis is accomplished by a complex sequence of reactions which are localized to the ER and peroxisomes of mammalian cells (Ikonen 2008; Miller and Auchus 2011; Miller and Bose 2011). In mammalian cells, cholesterol is the end product of this biosynthetic pathway (Brown and Goldstein 2009; Soccio and Breslow 2004), whereas in yeast ergosterol is the major sterol (Jacquier and Schneiter 2012; Wagner and Daum 2005) . In yeast, the ER and lipid droplets contribute to sterol biosynthesis (Athenstaedt et al. 1999b; Leber et al. 1994). Plants produce a variety of sterols such as phytosterols (Suzuki and Muranaka 2007). In mammalian mitochondria the majority of cholesterol is localized to the OMM (Daum 1985) whereas in yeast (Sperka-Gottlieb et al. 1988; Wriessnegger et al. 2009) most of the mitochondrial sterol was found in the IMM.

Biosynthesis of sphingolipids occurs in the ER and in organelles along the protein secretory pathway, mainly the Golgi (Breslow and Weissman 2010; Levy and Futerman 2010; Mencarelli et al. 2010; Perry and Ridgway 2005). However, recent work showed the presence of ceramide metabolic enzymes in mitochondria from mammals and yeast (Kitagaki et al. 2007; Novgorodov et al. 2011). The majority of this lipid class is located to the plasma membrane, whereas amounts in mitochondria are minor (Breslow and Weissman 2010; Stiban et al. 2010; van Meer and Hoetzl 2010). In the OMM, the concentration of ceramide is three-fold higher than in the IMM (Mencarelli et al. 2010).

Import of sterols into mitochondria

Sterols in mitochondria may serve as structural components of membranes and/or as precursors for hormone synthesis. For both purposes, they have to be imported into mitochondria. Transport of sterols from the ER to mitochondria appears to occur by a machinery which is different from that described for phospholipids. A non-vesicular and a vesicular pathway have been proposed for cholesterol translocation to mitochondria (Maxfield and Wüstner 2002).

A number of components appear to contribute to sterol transport to mitochondria (Fig. 4). The best studied protein of this kind is the so-called steroidogenic acute regulatory protein (StAR) (Clark et al. 1994). StAR is almost exclusively expressed in steroid-producing cells and regulated by cAMP (Benmessahel et al. 2002; Strauss et al. 1999). StAR requires ATP and an electrochemical gradient for maximal activity (King and Stocco 1996). Import of StAR into mitochondria results in cleavage of an N-terminal mitochondrial targeting sequence (Arakane et al. 1998). The C-terminus of StAR displays cholesterol transferring capacity (Stocco 2000; Strauss et al. 1999). Ligand binding to StAR changes its secondary structure; the protein itself alters the domain structure of the OMM to facilitate rapid sterol transfer (Petrescu et al. 2001). StAR interacts with the cAMP-dependent protein kinase (PKA) and a peripheral-type benzodiazepine receptor (PBR) (Hauet et al. 2002) which was later named TPSO (translocator protein) (Krueger and Papadopoulos 1990; Mukhin et al. 1989). A protein named PAP7 was identified as a regulator of PBR and PKA. PBR/TSPO (translocator protein) was shown to support import and processing of StAR in the mitochondria (Hauet et al. 2005). VDAC (voltage dependent anion channel) and ANT (adenine nucleotide transporter) were identified as interaction partners of PBR/TSPO (McEnery et al. 1992; Rone et al. 2009). VDAC binds cholesterol and influences its mitochondrial distribution (Campbell and Chan 2007). It appears that TSPO and VDAC together with TOM (translocase of the OMM) function in StAR assembly into mitochondria. Also members of the ACBD family, ACBD1 and ACBD3/ PAP7, seem to participate in the function of the tranduceosome (Midzak et al. 2011). Finally, a protein named MLN64 (metastatic lymph node protein 64) with a C-terminal START domain for cholesterol binding was identified as a mediator for cholesterol transport from late endosomes to the plasma membrane and also to mitochondria (Charman et al. 2010; Liapis et al. 2012; Zhang et al. 2002). It was shown that in Niemann-Pick Type C (NPC) mutated cells MLN64 supplies cholesterol from endosomes to mitochondria. In analogy to StAR, MLN64

needs direct contact with mitochondria to transfer cholesterol, indicating that direct contact between endosomes and mitochondria may occur (Charman et al. 2010).

Once cholesterol has been imported into mitochondria, it is further converted to steroids (Miller and Auchus 2011) (see Fig. 4). The first and rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by a cytochrome P450. Further conversion requires the action of hydroxysteroid dehydrogenases. The steroidogenic cells in humans are localized to a varity of tissues, most prominent are the adrenal glands and gonads. In the adrenal glands mainly in the zona glomerulosa, zona reticularis and zona fasciculate the conversion of cholesterol to aldosterol, dehydroepiandrosterone (DHEA) and cortisol-like is catalyzed. In the gonal glands cholesterol is converted to steroid hormones, estradiol in the ovarian granulose cells and to testosterone by the testicular leyding cells (Miller and Auchus 2011).

Evidence about import of sterols in non-steroidogenic cells is rare. Import of radiolabeled ergosterol and cholesterol from unilamellar vesicles into isolated mitochondria has been studied in *Saccharomyces cerevisiae* (Tuller and Daum 1995). Supply of ergosterol to the mitochondrial surface was enhanced by a cytosolic fraction, whereas no such additive was required for cholesterol transport. Both sterols reached the IMM in an energy-independent process but accumulated to some extent in contact sites between OMM and IMM, supporting the idea that these zones are sites of intramitochondrial lipid translocation. Supply of sterols for membrane requirements is not that well understood. Sterols are very important lipids in the composition of bilayers. Each type of cellular membranes contains a specific sterol as a major component: cholesterol in mammals, ergosterol for fungi, sito-/stigmasterol in plants and hopanoids in primitive bacteria. The fused ring system of the molecule appears to important for increasing the order of fluid phases (Dufourc 2008).

Its biophysical properties enable cholesterol to condense and rigidify bilayers of phospholipids with unsaturated fatty acid chains and fluidize bilayers of di-saturated phospholipids and sphingolipids, being a regulator of membrane dynamics. The same biophysic properties enable easy insertion and extraction into and from bilayers, meaning cholesterol can move rapidly as monomer across membranes compared to other lipids (Maxfield and van Meer 2010). The energy requirements for association of the cholesterol molecules from the hydrophilic environment into a lipid bilayer, with certain phospholipids, like PC (Dai et al. 2010) and with sphingomyelin (Lange and Steck 2008), depend on its insertion depth and orientation within the bilayer. In this process the cholesterols hydrophobic

core is buried within the hydrocarbon region of the bilayer and spans one leaflet of the membrane, with its OH group having contact with the polar phase, therefore avoiding an electrostatic penalty. The transfer is mainly driven by the favourable nonpolar interactions between the hydrocarbon chains (contributions to the solvation free enegy) (Kessel et al. 2001). Cholesterol can be also easily displaced from ordered lipid domains (rafts) by ceramides. Since both lipid groups have small polar headgroups, it is proposed that ceramides and cholesterol compete for association within rafts (Megha and Erwin London, 2004) because of a limited capacity of lipids with bigger head groups to accommodate small headgroup lipids in a manner that prevents unfavourable contact between the hydrocarbon groups of the small headgroup lipids and the surrounding aqueous environment, also called the Umbrella effect hypothesis (Dai et al. 2010).

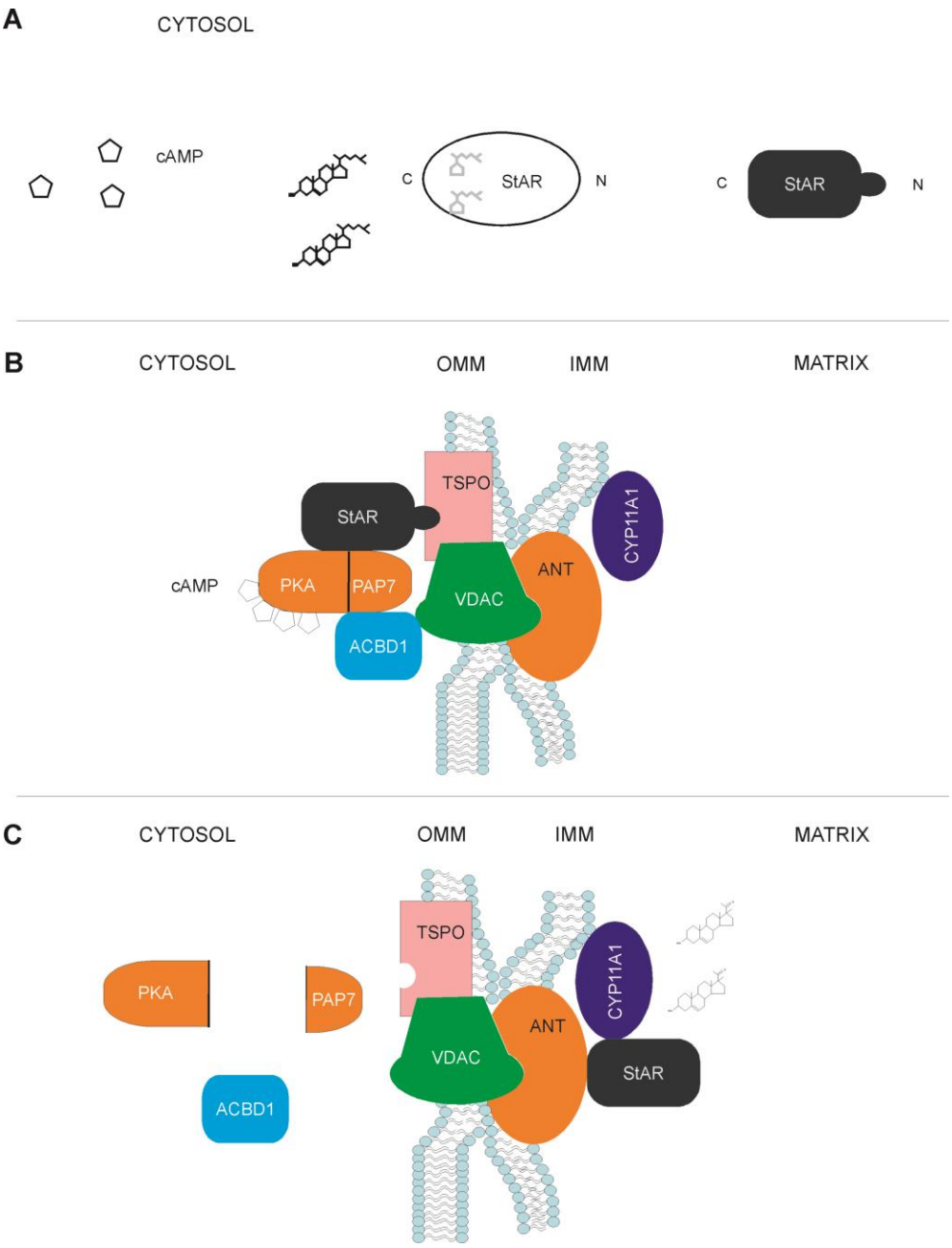

Figure 4: Import of sterols into mitochondria for steroidgenesis

A: Upon binding of cholesterol the StAR precursor protein changes its conformation. StAR expression is regulated by cAMP.

B: StAR gets in contact with PKA, PAP7/ACBD3 and ACBD1 and is imported into the mitochondria with sterols bound. Upon interaction with TPSO, StAR induces changes in the OMM and a transduceosome is formed. This structure which includes TSPO/VDAC of the OMM and ANT in the IMM facilitates imports and maturation of StAR.

C: Upon arrival in the mitochondrial matrix the mature StAR protein delivers cholesterol to CPY11A1 where it is converted to pregnenolone.

Sphingolipid incorporation into mitochondria

Little is known about import and function of sphingolipids in mitochondria. There is evidence that sphingolipid metabolism affects the mitochondrial pathway of apoptosis (Chipuk et al. 2012). Ceramides with their short and long acyl-chains could form large protein-permeable channels in the OMM (Siskind and Colombini 2000), and such channels may contribute to the release of proapoptotic proteins from mitochondria (Futerman 2006). It was proposed that ceramide is produced by hydrolysis of sphingomyelin by a neutral sphingomyelinase in MAM (Chipuk et al. 2012; Mullen et al. 2012) and then transferred to mitochondria where it is converted to sphingosine-1-phosphate and hexadecenal. It has also been proposed that in liver mitochondria, thioesterase and neutral ceramidase produce ceramide from sphingosine and acyl-CoA (Novgorodov et al. 2011; Novgorodov and Gudz 2009). *In vitro*, ceramide synthase or reverse ceramidase activity was detected in mitochondria (Bionda et al. 2004). In yeast Isc1p has been suggested to generate ceramides in mitochondria (Kitagaki et al. 2007, 2009).

Evidence for the existence of two ceramide transporting proteins has been presented: the Goodpasture antigen-binding protein (GPBP) and its splice variant, the ceramide transporter (CERT) (Mencarelli et al. 2010). It was proposed that these proteins might be involved in mitochondrial lipid homeostasis, although this view is still a matter of debate.

Discussion

Among the different subcellular compartments, mitochondria are one of the most unique when considering lipid composition. Presence of PG and CL as well as the large amount of PE are characteristic for mitochondrial membranes. Recent studies have shown that especially these non-bilayer forming lipids are important for mitochondrial function (Acehan et al. 2011; Birner et al. 2003; Gohil et al. 2005; Joshi et al. 2012; Nebauer et al. 2007; Osman et al. 2009a; Tamura et al. 2012). However, low abundant lipids in mitochondria such as sterols or sphingolipids must not be ignored, although at present ascribing specific functions to these components in mitochondria is difficult.

Large amounts of lipids have to be imported into mitochondria. Different mechanisms of intracellular lipid transport have been discussed for several decades (Daum and Vance 1997; Holthuis and Levine 2005; Osman et al. 2011; Schulz and Prinz 2007; Voelker 2005). The role of lipid transfer proteins which were enthusiastically studied in the beginning of these investigations has been scrutinized more recently. Such transfer proteins may be more relevant for lipid sorting or sensing than bulk lipid transport. Vesicle flux was recognized as a more potent mechanism of lipid transport because it allows rapid, robust and efficient translocation to target membranes. It appears that at least significant portions of lipids are co-transported to the cell periphery by the classical pathway of protein secretion (Kohlwein et al. 1996; Salama and Schekman 1995). However, other types of vesicles may also be relevant although experimental evidence for such processes is missing. Last but not least membrane contact has become an attractive alternative for intracellular lipid translocation (de Brito and Scorrano 2010; Elbaz and Schuldiner 2011; Lebiedzinska et al. 2009; Levine and Loewen 2006; Osman et al. 2011; Voelker 2009). Contact of the ER with mitochondria through the MAM fraction, association of the ER with the plasma membrane or nuclear-vacuolar junctions became prominent examples of this kind. Finally, transmembrane movements of lipids by translocases and flippases contribute to appropriate lipid distribution in organelles (Holthuis and Levine 2005).

Close proximity of ER and mitochondria as shown by electron microscopy (Csordás et al. 2006; Friedman et al. 2011) supports the idea of lipid transport by membrane contact. The distance is sufficient for a protein complex to bridge the gap between organelles and to connect membranes without causing fusion (Achleitner et al. 1999). A hemistalk structure caused by high amounts of mitochondrial PE and CL which provide negative membrane

curvature may support attachment and point fusion events (Chernomordik and Kozlov 2008, 2005; Chernomordik et al. 2006). Protein bridges like ERMES stabilize the energy-unfavorable state (Kozlov et al. 2010). Microtubules and actin filaments may help by bridging ER and mitochondria and establishing or maintaining protein contact between membranes (Friedman et al. 2010, 2011). Although ERMES and associated proteins were shown to create or maintain contact between ER and mitochondria (Kornmann et al. 2009; Stroud et al. 2011), direct functions of these components in lipid transport were scrutinized (Nguyen et al. 2012). It was argued that ERMES facilitates membrane contact, but other components may be more directly involved in lipid translocation. MAM-OMM contact sites appear to be further linked to complexes of the IMM (de Brito and Scorrano 2008; Kornmann et al. 2009). Transport of lipids from OMM to IMM may occur either by membrane contact or by translocation through the intermembrane space. Despite its role in IMM organization the cristae forming MINOS complex (Hoppins et al. 2011 Harner et al. 2011; van der Laan et al. 2012) is most likely not involved in this process. The role of Gem1p and Ups-proteins in OMM-IMM lipid translocation has to be taken into account (Tamura et al. 2012). Interaction of prohibitins with the mitochondrial lipid synthesizing machinery also appears to be important for lipid homeostasis in this organelle (Birner et al. 2003; Gohil et al. 2005; Gohil and Greenberg 2009; Osman et al. 2009a, 2009b).

An intriguing although unsolved question is regulation of lipid transport to/from and within mitochondria. Fluidity of the membrane or membrane curvature affected by PE and CL are parameters which may influence biophysical properties of the membrane environment. Also the acyl chain length of specific phospholipid species could have an effect (Dolis et al. 1996; Heikinheimo and Somerharju 1998; Kevala and Kim 2001). A balanced concentration of phospholipids, sterols and sphingolipids appears to be important for membrane fusion or fission. In that respect, the low-abundant lipids in mitochondria such as sterols or sphingolipids may be relevant. Regulation of the mitochondrial lipid assembly by Ups1p and Ups2p, which are in turn subject to proteolytic degradation/protection by Yme1p, Atp23p and Mdm35p (Potting et al. 2010; Tamura et al. 2012) are examples for the wide-spread network of lipid assembly and homeostasis in mitochondrial membranes which we are just beginning to understand in more detail at the molecular level.

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CHAPTER 2

Phosphatidylcholine supply to peroxisomes of the yeast *Saccharomyces cerevisiae*

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Abbreviations: PL, phospholipids; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; CL, cardiolipin; PI, phosphatidylinositol; PG, phosphatidylglycerol; DAG, diacylglycerol; HOMO, homogenate; ER endoplasmic reticulum; MT, mitochondria; PX peroxisomes; SFA, saturated fatty acids; UFA, unsaturated fatty acids; ERG, ergosterol; CDP, cytidine diphosphate; SAM, S-adenosyl-L-methionine; LP, Lipid particles

Abstract

In the yeast *Saccharomyces cerevisiae*, phosphatidylcholine (PC), the major phospholipid of all organelle membranes, is synthesized *via* two different pathways. Methylation of phosphatidylethanolamine (PE) catalyzed by the methyl transferases Cho2p/Pem1p and Opi3p/Pem2p as well as incorporation of choline *via* the CDP-choline branch of the Kennedy pathway leads to PC formation. To determine the contribution of these two pathways to PC supply to peroxisomes, cells were cultivated under peroxisome inducing conditions, i.e. in the presence of oleate in the medium. Mutants bearing defects in the two pathways, respectively, were subjected to biochemical and cell biological analyses. Phenotype studies revealed compromised growth of *cho2*Δ*opi3*Δ (mutations in the methylation pathway) and *eki1*Δ*dpl1*Δ*eki1*Δ (mutations in the Kennedy pathway) strains grown on oleic acid. Analysis of peroxisomes from both mutants revealed that both pathways produce PC for the supply to peroxisomes, although the Kennedy pathway was shown to contribute with higher efficiency. Changes in the peroxisomal lipid pattern of mutants, especially depletion of PC caused changes in membrane properties as shown by anisotropy measurements with fluorescent probes. In summary, our data demonstrate traffic routes of PC from sites of synthesis to peroxisomes and the important role of PC for peroxisome formation and integrity.

Introduction

In the yeast *S. cerevisiae* peroxisomes are important organelles for growth on fatty acids and alkaline media. They are also important for oxidative reactions involving oxygen and hydrogen peroxide (Gurvitz and Rottensteiner 2006). In yeast and in plant cells, the ß-oxidation of fatty acids is localized to the peroxisomes, whereas in mammalian cells also mitochondria are capable of performing fatty acid degradation.

It is believed that peroxisomes originate from the endoplasmic reticulum. How this happens is still a matter of debate. Once the peroxisomes have come to maturation, they can divide autonomously (Fagarasanu et al. 2006; Smith and Aitchison 2009; Motley and Hettema 2007; Tabak et al. 2013; van der Zand et al. 2012). Little is known about the phospholipid composition and especially the supply of phospholipids to peroxisomes. Similar to other biomembranes, peroxisomal membranes contain four major classes of phospholipids: phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI) (Zinser et al. 1991; Rosenberger et al. 2009; Wriessnegger et al. 2007). In mitochondria, cardiolipin (CL) is also a major phospholipid component. In almost all yeast membranes, PC and PE make up to 60-70% of total phospholipids (Schneiter et al. 1999).

Studies from our lab showed, that PE in yeast grown on oleate media can be supplied to peroxisomes through three different pathways (Rosenberger et al. 2009). First, PE can be provided by the mitochondrial PS decarboxylase Psd1p; second, the Golgi/vacuolar Psd2p produce PE for peroxisomes; and finally, the CDP-ethanolamine branch of the so-called Kennedy pathway synthesizes a portion of PE destined for peroxisomes. PC supply to peroxisomes of the yeast *S. cerevisiae* has not been studied in detail. Some information about the supply of phospholipids to peroxisomes from the yeast *Pichia pastoris* has been provided (Wriessnegger et al. 2007).

In the yeast *S. cerevisiae* two main pathways of PC production exist: (i) the methylation pathway and (ii) the CDP-choline branch of the Kennedy pathway (Figure 1). In the methylation pathway, PC is produced from PE that is synthesized from PS. The pathway of aminoglycerophospholipid synthesis starts with the formation of PS in the endoplasmic reticulum by phosphatidylserine synthase Pss1p/Cho1p. PS can then be decarboxylated by two pathways catalyzed by the mitochondrial phosphatidylserine decarboxylase 1 (Psd1p), or

by Psd2p located to the Golgi network. In the methylation pathway, PE is methylated through three steps by the methyl transferases Cho2p/Pem1p (1x) and Opi3p/Pem2p (2x) using the methyl groups from S-adenosyl-methionine (SAM) a co-substrates. The second pathway of PC production in the yeast cell is the CDP-choline branch of the Kennedy pathway. In the CDP-choline pathway, externally added or endogenous choline is stepwise incorporated through phosphorylation by choline kinase and activation with CTP by phosphocholine cytidyltransferase. In the last step, phosphocholine is transferred from CDP-choline to diacyglycerol (DAG) by choline phosphotransferase and converted to PC.

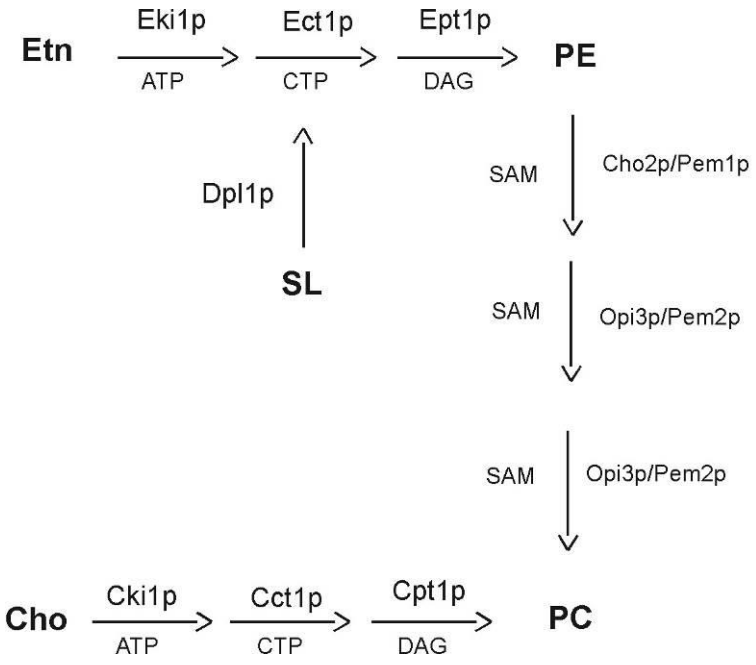

Figure 1: Phosphatidylcholine synthetic pathways in the yeast *S. cerevisiae.* On the right side of the figure the methylation pathway where PE is converted to PC is shown. At the bottom of the figure the CDP-choline branch of the Kennedy pathway is shown. On the top the CDP-ethanolamine branch of the Kennedy pathway is shown which can also provide PE for the methylation pathway.

While PC is the most abundant aminoglycerophospholipid (van Meer et al. 2008) and important for the structure of membranes because of its cylindrical shape (van den Brink-van der Laan et al. 2004), little is known about the distribution of this lipid from the different sites of synthesis to the organelles of the yeast *S. cerevisiae* grown on oleate media, i.e. under peroxisome inducing conditions. Besides the peroxisimes themselves, the supply of lipids to

the endoplasmic reticulum and mitochondria was of interest. To investigate which of the pathways mentioned above delivers PC to the peroxisomes and other organelles, studies were started with a phenotype analysis using mutants compromised in the respective biosynthetic routes. The further aim of the work was to investigate the transport and assembly routes of PC into peroxisomes and other organelle membranes of the yeast *S. cerevisiae*.

Materials and Methods

Strains and culture conditions

Strains used in this study are described in Table 1. Yeast cells were cultivated in YPD medium (1% yeast extract, 2% peptone and 2% glucose) to the stationary phase. For induction of peroxisomes an inoculation to an OD_{600} of 0.1 was performed in YPO media containing 0.3% yeast extract, 0.5% peptone, 0.5% potassium phosphate, pH 6, 0.1% oleic acid (herbal oleic acid pure; Merck, Darmstadt, Germany), 0.2% Tween 80 and 0.1% glucose, and cells were grown to the late logarithmic, early stationary phase. It has to be noted that YPD and YPO media contained low amounts of ethanolamine and choline. It also has to be noted that oleic acid preparations used routinely as carbon source contained impurities of margaric acid, myristic acid, stearic acid, palmitic acid, palmitoleic acid, linoleic acid and linolenic acid. Growth of the different strains on liquid media was followed by measuring the OD_{600}.

Name	Genotype	Origin
BY4742 (wild type)	MAT*α his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0*	Euroscarf, Germany
Kennedy mutant	MATα *his3Δ200, leu2Δ1 trp1Δ63, ura3-52, cki1Δ::HIS3, dpl1Δ::LEU2, eki1Δ::TRP1*	(Birner *et* al., 2001)
Methylation mutant	*MATα his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; cho2::kanMX4*; *opi3::kanMX4*	Kindly provided by Karin Athenstaedt

Table 1: Strains used in this study

Isolation of peroxisomes, mitochondria and microsomes

For cell fractionation and isolation of the organelles, peroxisomes, mitochondria and microsomes (endoplasmic reticulum), late exponential cultures of *S. cerevisiae* grown on YPO media were used. Cells were harvested and spheroplasted as described by Daum et al. (1982). 2 mg Zymolyase 20T were used per 1g wet cell weight. Cells were homogenized on ice with a Dounce homogenizer in the breaking buffer (5 mM MES-pH 6.0-KOH, 1 mM KCl, 0.6 M sorbitol and 0.5 mM EDTA) with 1 mM PMSF added as protease inhibitor. Unbroken cells and nuclei were removed by centrifugation at 5,000 rpm for 5 min. The resulting pellet was collected, resuspended in breaking buffer, re-homogenized and centrifuged again. Altogether, homogenates were collected for lipid analysis from three repeats. The combined supernatants were centrifuged at 15,000 rpm in an SS34 rotor (Sorvall) for 15 min. The supernatant containing the microsomal fraction and the pellet containing peroxisomes and mitochondria were collected. The pellet was gently resuspended in a small Dounce homogenizer in breaking buffer plus 1 mM PMSF and centrifuged at low speed (5,000 rpm) to remove cellular debris. The supernatant was centrifuged at 15,000 rpm for 15 min, the pellet was resuspended in breaking buffer and loaded for further purification on a Nycodenz gradient (17%-24%-35% w/v) in 5 mM MES-KOH, pH 6.0, 1 mM KCl, 0.24 M sucrose. Centrifugation was carried out in a swing out rotor (Sorvall AH-629) at 26,000 rpm for 90 min. A colourless-white band was collected with a syringe at the bottom, diluted in 4 volumes of breaking buffer and sedimented at 15 min 15,000 rpm in an SS34 rotor. The procedure was repeated for mitochondria which formed a separate band at the top of the tube in the density gradient. The supernatant of the previous step containing the microsomal fraction was also centrifuged with an SS34 rotor for 45 min at 18,000 rpm, and the endoplasmic reticulum was sedimented and collected. All organelles were stored at -70 ^{o}C for further analysis.

Protein analysis

Proteins from isolated subcellular fractions were precipitated with trichloracetic acid (TCA) at a final concentration 10% for 1h at 4 ^{o}C. For protein quantification, the pellet was solubilized in 0.1% SDS, 0.1 M NaOH and analyzed by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Proteins were separated by SDS-PAGE (Sodiumdodecylsulfate Polyacrylamide Gel Electrophoresis) as described by Laemmli (1970) using a Biorad mini-protean 3 cell equipment. Separation was performed on 12.5% separation gels, and SDS-

PAGE was carried out at 24 mA for 1.5 h. For studies of protein localization Western blot analysis was performed (Haid and Suissa 1983) with primary rabbit antibodies directed against yeast Fox1p, Por1p, Cytb2p and ER-40kDap. Immunoreactive proteins were visualised by ELISA using a peroxidase-linked secondary antibody (Sigma) and following the manufacturer's instructions (SuperSignal^{TM}, Pierce Chemical Company, Rockford, IL, USA).

Phospholipid analysis

Phospholipids were extracted from homogenate, endoplasmic reticulum (40,000 x g microsomal fraction), mitochondria and peroxisomes by the method of Folch (Folch et al. 1957). Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, Germany) using chloroform/methanol/ 25% NH_3 (65:35:5, per vol.) as first solvent, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol) as second solvent. Lipids bands were stained with iodine vapour, scrapped off the plate and quantified by the method of Broekhuyse and Veerkamp (Broekhuyse and Veerkamp 1968). 1 mg of protein was used for total phospholipid and for individual phospholipid estimations.

Fatty acid analysis

Fatty acids were analyzed by gas liquid chromatography (GLC). Lipid extracts prepared as described above were incubated with 2.5% sulfuric acid in methanol at 85 ^{o}C for 90 min (Morrison and Smith 1964). After incubation and cooling of the samples, water was added, and the fatty acids converted to methyl esters were extracted with light petroleum. Fatty acid methyl esters were separated using a Hewlett-Packard 6890-Gas-Chromatograph equipped with a HP-INNO Wax capillary column (15 m × 0.25 mm i.d. × 0.50 µm film thicknesses) and helium as carrier gas (20 min at 200 ^{o}C, 10 min to 280 ^{o}C, 15 min at 300 ^{o}C). Fatty acids were identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN, USA).

Sterol analysis

Sterols from whole cells or subcellular fractions were identified and quantified by gas liquid chromatography–mass spectrometry (GLC–MS) (Quail and Kelly 1996). Sterol analysis was performed as described in the literature (Quail and Kelly 1996; Tuller et al. 1999). In brief, a mixture of 0.6 ml methanol (Merck), 0.4 ml 0,5% (w/v) pryogallol (Fluka) dissolved in methanol and 0.4 ml 60% (w/v) aqueos KOH solution was placed into 15 ml Pyrex tubes. 5 µl of a cholesterol solution (2 mg/ml) were added as an internal standard. For analyzing the sterol pattern, 0.3 mg of homogenate, and 0.5 mg of organelles were applied respectively. Samples were heated heated in a sand bath for 2 hours at 90 ^{o}C. Then, lipids were extracted three times with 1 ml n-heptane. The upper phase was transferred into a new tube and the lower phase was reextracted. The combined upper phases were dried under a stream of nitrogen and dissolved in 10 µl pyridine. After adding 10 µl N´O´-bis(trimethylsilyl)-trifluoracetamide (Sigma) the samples were diluted with 50 µl ethylacetate and analyzed by GC-MS. GLC–MS was performed on an HP 5890 Gas-Chromatograph equipped with a mass selective detector HP 5972, using an HP5-MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). Aliquots of 1 µl were injected in the splitless mode at 270 °C injection temperature with helium as carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100 °C, 10 °C/min to 250 °C, and 3 °C/min to 310 °C. Mass spectra were acquired in the scan mode (scan range 200–550 amu) with 3.27 scans per second. Sterols were identified based on their mass fragmentation pattern.

Neutral lipids

Lipids from yeast cells were extracted as described above. For quantitation of non-polar lipids, extracts were applied to Silica Gel 60 plates, and chromatograms were developed in an ascending manner by a two-step developing system. Chromatograms were first developed using light petroleum:diethyl ether:acetic acid (70:30:2; per vol.), and then light petroleum:diethyl ether (49:1; v/v) as solvents. Chromatograms for DAG analysis were developed using chloroform:acetone:acetate (45:4:0.5; per vol.). To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g $MnCl_2 \times 4H_2O$, 60 ml water, 60 ml methanol and 4 ml concentrated sulfuric acid, briefly dried and heated at 100 °C for 20 min. Then, lipids were quantified by densitometric scanning at 400–650 nm with diolein and triolein as standards using a Shimadzu dual-wave length chromatoscanner CS-930.

Flourescence anisotropy measurments

Isolated organelles were suspended in breaking buffer, pH 6, at a concentration of 100 µg protein. After addition of diphenylhexatriene (DPH) at a molar ratio of 1:50 (probe to phospholipid), mixtures were incubated for 5 min at 30 °C. Samples were kept in the dark as long as possible. Then, fluorescence measurements were carried out using a Shimadzu RF 540 spectrofluorimeter equipped with polarizers in the excitation and emission light path. Excitation and emission wavelengths for DPH were 350 and 452 nm, respectively (slit width 10 nm). Fluorescence intensities were corrected for background fluorescence and light scattering from the unlabelled sample. The fluorescence anisotropy was calculated according to the eqation $r=(I_{\parallel}-I_{\perp})/(I_{\parallel}+2.I_{\perp})$. $I_{\parallel}$ and $I_{\perp}$ are measured emission intensities parallel and perpendicular to the vertical polarization plane of the excitation light (Lakowicz and Balter 1982).

Electron microscopy

For electron microscopic examination examination, cell precultures were grown under aerobic conditions at 30 ^{o}C on YPD medium containg 2% glucose as the carbon source. The cells were diluted to an OD_{600} 0.1 in fresh YPO medium and grown to in the late exponential phase. Then, cells were harvested by centrifugation and washed twice with 0.5% BSA (fatty acid free) and 3 times with H_2O. Further processing was performed as developed by Perktold et al. (2007). Cells were fixed for 5 min in 1% aqueous solution of $KMnO_4$ for 20 min. Fixed cells were washed three times in distilled water and incubated in 0.5% aqueous uranylacetate for the first three hours with shaking at room temperature and afterwards overnight at 4°C. Samples were dehydrated in a graded series of ethanol (50%, 70%, 90% and 100%) and gradually infiltrated with increasing concentrations of Spurr resin (30, 50, 70, and 100%) mixed with acetone for a minimum of 3 h for each step. Samples were finally embedded in pure, fresh Spurr resin and polymerized at 60 °C for 48 h. Ultrathin sections of 80 nm were stained with lead citrate and viewed with a Philips CM 10 electron microscope.

Results

Growth characteristics of mutants compromised in the two PC biosynthetic pathways

To analyze the influence of mutations in aminoglycerophospholipid synthesis on growth of yeast cells drop tests on YPD and YPO agar plates (Figure 2A) and growth in liquid media (Figure 2B) were performed. The drop test showed that strains affected in PC synthesis grew normally on YPD at 30 ^{o}C. At a temperature of 37 ^{o}C, however, *cho2*Δ, *cho2*Δ*opi3*Δ and the *cki1*Δ*dpl1*Δ*eki1*Δ mutants (Kennedy mutant) showed decreased cell density compared to the wild type. The *cho2*Δ*opi3*Δ mutant showed a strong growth defect whereas the *cki1*Δ*dpl1*Δ*eki1*Δ mutant was less compromised compared to wild type when grown on YPD agar plates.

On minimal media containing oleate as carbon source and supplemented with 5 mM choline and 5 mM ethanolamine, the *cho2*Δ defect was rescued (see Fig. 2A). The *cki1*Δ*dpl1*Δ*eki1*Δ mutant was still growing a little bit worse than wild type under ethanolamine/choline supplementation. Interestingly, the supplementation did not rescue growth of the *cho2*Δ*opi3*Δ mutant. Surprisingly, on YPO rich media the growth defect of all three mentioned mutants was more pronounced than on minimal oleate containing media. This effect may be due to the low amount of ethanolamine and choline present in YPO. Mutant strains did not grow at 37 ^{o}C on minimal oleate media supplemented with 5 mM choline and ethanolamine, each, although the wild type strain showed normal growth (data not shown). Growth of strains on liquid media (YPO) showed similar results as on the agar plates. The *cho2*Δ*opi3*Δ and *cki1*Δ*dpl1*Δ*eki1*Δ mutants showed a clear growth defect. The *cho2*Δ mutant was only slightly affected, and the *opi3*Δ mutant behaved similar to wild type. In minimal oleate media supplemented with 5 mM choline and 5 mM ethanolamine, each, the *cho2*Δ*opi3*Δ mutant did not grow, and the *cho2*Δ mutant had a slight growth delay compared to other strains (6 h delay in reaching of exponential phase; data not shown). The *cki1*Δ*dpl1*Δ*eki1*Δ mutant showed a similar growth phenotype as the wild type when grown on supplemented minimal oleate media.

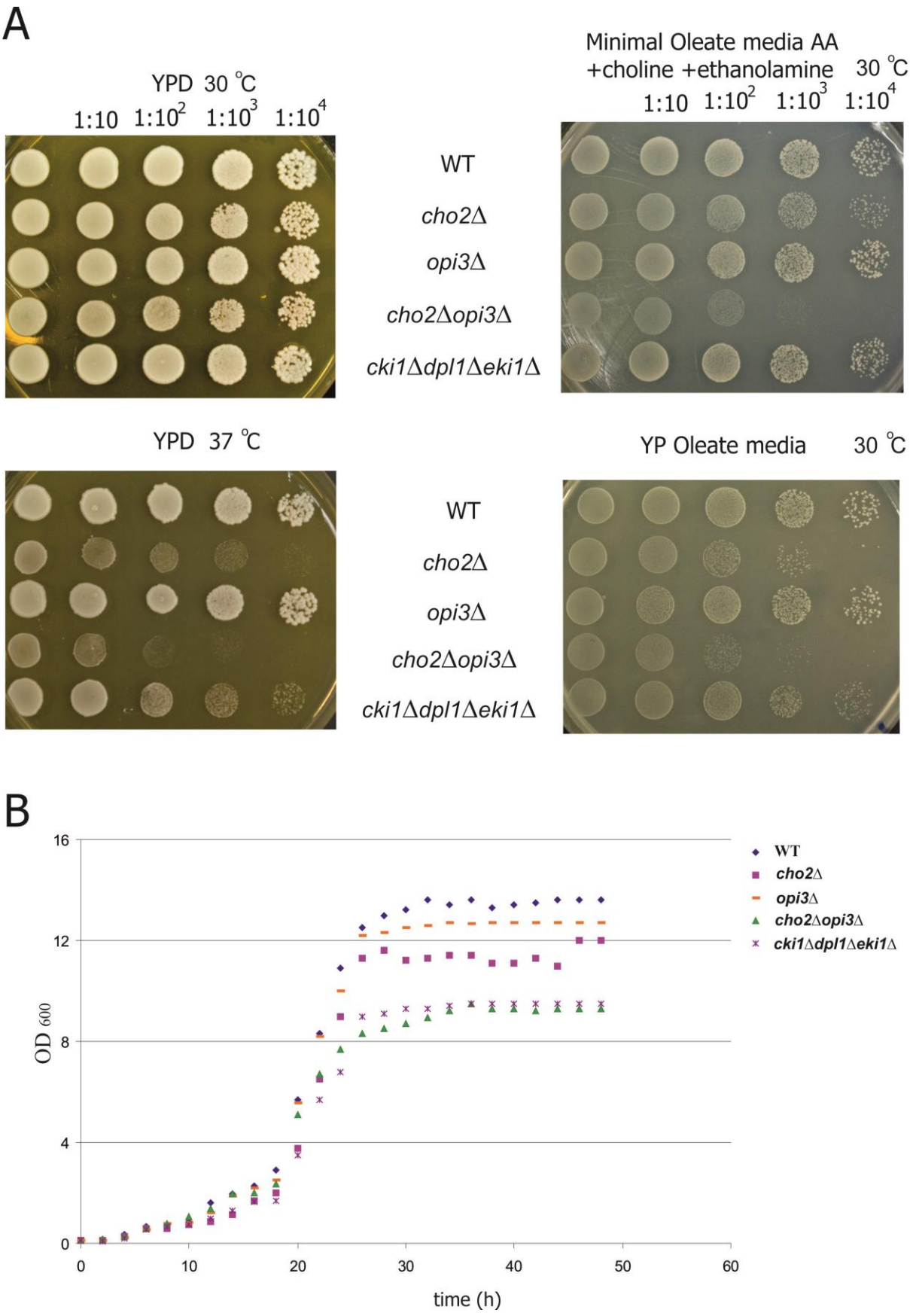

Figure 2 (A, B): Phenotype studies of wild type, *cho2*Δ, *opi3*Δ, *cho2*Δ*opi3*Δ and *cki1*Δ*dpl1*Δ*eki1*Δ strains are shown. Drop test (A); liquid cultures in YPO media (B).

Isolation and characterization of peroxisomes from cells with PC biosynthetic defects

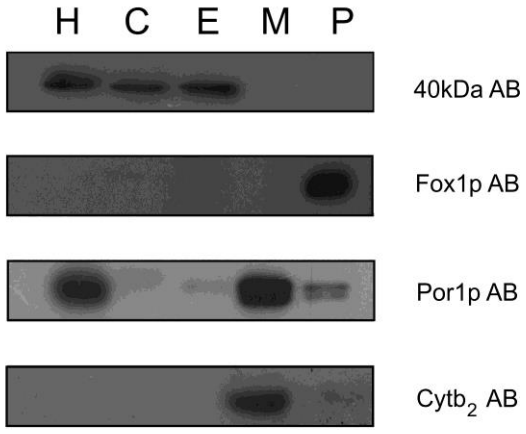

Figure 3: Western blot analysis of subcellular fractions from *Saccharomyces cerevisiae.* WT BY4742 cells were grown on oleic acid. H (homogenate), C (cytosol), E (endoplasmic reticulum), M (mitochondria) and P (peroxisomes).

Peroxisomes were isolated as described previous by Zinser and Daum (1995). The quality of peroxisomal preparation was tested by Western blot analysis to determine the enrichment of peroxisomes and cross contamination with other organelles (Figure 3). Antibodies used for Western blot analysis were: 40kDa AB-antibody directed against 40 kDa protein of the endoplasmic reticulum; Fox1p-antibody directed against the multifunctional β-oxidation protein (Fox1p) for peroxisomal membranes; Por1p-antibody marker for the mitochondrial outer membrane; $Cytb_2$-antibody marker for the mitochondrial intermembrane space. As can be seen from Fig. 3, Fox1p is highly enriched in peroxisomes from wild-type cells. The degree of cross-contamination of peroxisomes with other subcellular fractions was low. Impurities caused by co-isolation of mitochondria were marginal as can be seen from the high enrichment of Por1p and $Cytb_2$ in the mitochondrial fraction and absence from the peroxisomal fraction. Western blot analysis was also performed with subcellular fractions from all PC mutants. The quality of peroxisomes and other subcellular fractions was similar to wild-type (data not shown).

Phospholipid analysis of peroxisomes from cells with PC biosynthetic defects

To find out how the PC levels of peroxisomes changed in the *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ mutants compared to wild type, we analyzed total phospholipids and individual phospholipids of these two mutants and compared them to wild type. These results were compared to phospholipid analyses of the homogenates (HOMO), endoplasmic reticulum (ER), mitochondria (MT) and peroxisomes (PX).

The wild type homogenate exhibited the highest phospholipid to protein ratio (µg PL/mg protein) followed by *cho2*Δ*opi3*Δ and the *cki1*Δ*dpl1*Δ*eki1*Δ mutant (Figure 4). Interestingly, the difference between the *cki1*Δ*dpl1*Δ*eki1*Δ mutant and the wild type strain was small indicating that the total amount of phospholipids formed was not the reason for the observed growth phenotype defect (see Fig. 2). The total phospholipid values in peroxisomes, which proliferate in the presence of oleate in the medium, largely reflected values of the bulk membranes from homogenates. Surprisingly, the phospholipid concentration in peroxisomes is the highest in the *cho2*Δ*opi3*Δ mutant (130 µg PL/mg prot.), followed by the wild type (120 µg PL/mg prot.) and the Kennedy mutant (98 µg PL/mg prot.).

Phospholipid values of the endoplasmic reticulum and mitochondria isolated from yeast cells grown in the presence of oleate reflected values of the homogenates (see Figure 4). The wild type has the highest value, followed by the methylation mutant and the Kennedy mutant. In all strains, mitochondria have the highest phospholipid concentration followed by peroxisomes and endoplasmic reticulum.

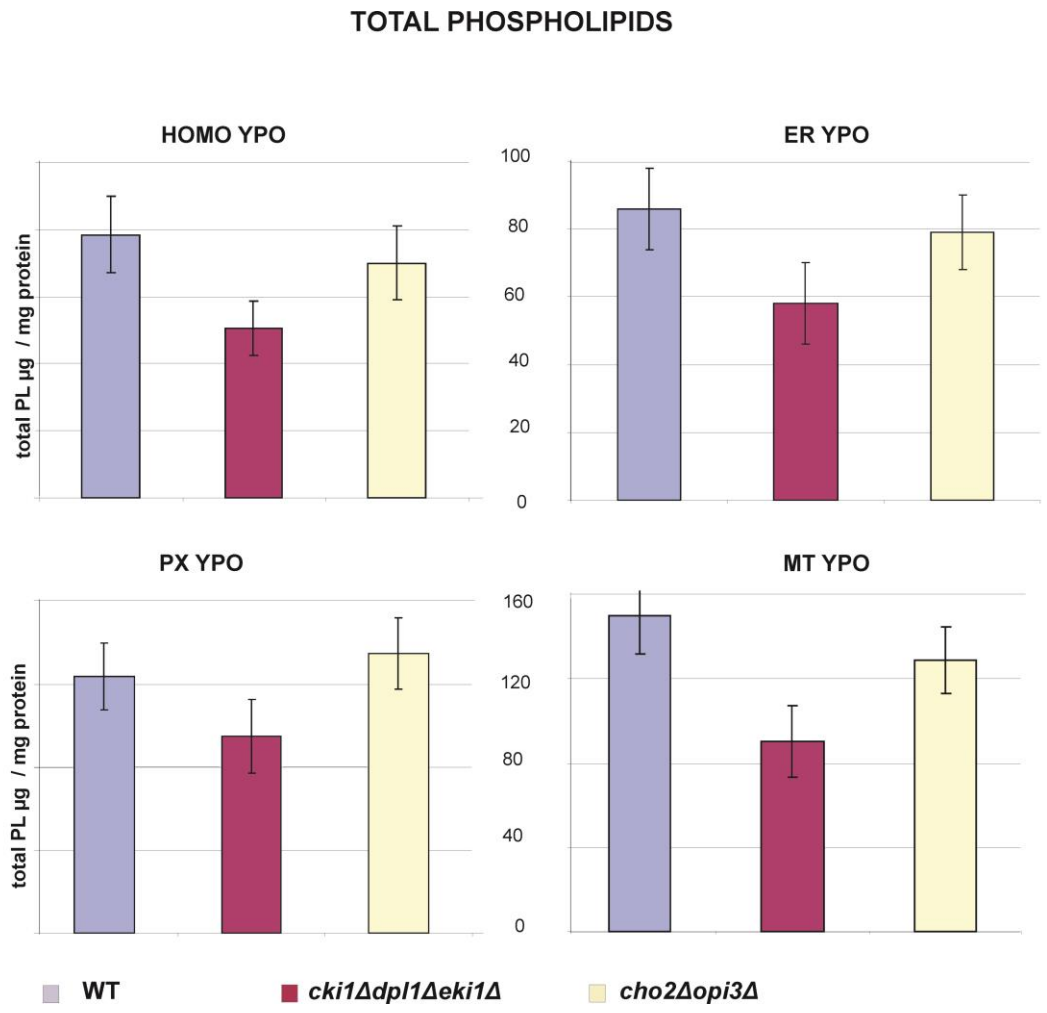

Figure 4: Total phospholipids. Values for the homogenate (HOMO), endoplasmic reticulum (ER), mitochondria (MT) and the peroxisomes (PX) from the wild type and the *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ strains grown in liquid YPO media are shown.

A key experiment for the understanding of PC supply to peroxisomes was the analysis of individual phospholipids in wild type (WT), the Kennedy mutant *(cki1*Δ*dpl1*Δ*eki1*Δ) and the methylation mutant *(cho2*Δ*opi3*Δ) (Fig. 5). LP (lysophospholipids), PI (phosphatidylinositol), PS (phosphatidylserine), PC (phosphatidylcholine), PE (phosphatidylethanolamine), CL (cardiolipin) and PA (phosphatidic acid) were determined in the homogenate, peroxisomes endoplasmic reticulum and mitochondria.

Homogenate samples reflect the overall production of individual lipids in wild type and mutants. In all strains, PC, PE and PI were the major phospholipids followed by PS, LP, CL and PA. While in the wild type and the methylation mutant PC and PE values were similar,

values of these two phospholipids were markedly decreased in the *cki1*Δ*dpl1*Δ*eki1*Δ Kennedy mutant. This result indicated that under the chosen growth conditions with oleate as a carbon source, the Kennedy pathway which is active in the *cho2*Δ*opi3*Δ strain forms the majority of PC. In contrast, the methylation pathway which is active in the *cki1*Δ*dpl1*Δ*eki1*Δ strain produced only a reduced amount of PC. It has to be noted, however, that the PE level is also reduced in this mutant due to deletions of *EKI1, DPL1* and also *CKI1*. The decreased level of PC in this strain cannot be attributed to conversion of PE to PC since methylation enzymes are intact. Thus, direct formation of PE and PC via CDP-ethanolamine and CDP-choline pathways appears to be the reason for the observed defect.

In peroxisomes, deletions of *CHO2* and *OPI3* did not cause a loss of PC supply. Thus, PC produced through the Kennedy pathway appears to be efficiently incorporated into peroxisomal membranes. Interestingly, the PE and PS levels in peroxisomes of the methylation mutant were noticeably higher than in the Kennedy mutant and wild type. Some accumulation of PE and PS due to the lack in further conversion of PC may be the reason for this observation. The peroxisomal level of PC in the *cki1*Δ*dpl1*Δ*eki1*Δ strain is slightly reduced compared to wild type and the methylation mutant. Thus, PC synthesized at a lower amount in this strain by PE methylation appears to reach peroxisomes only at a reduced level. Nevertheless, these data indicate that both the Kennedy pathway and the methylation pathway contribute to PC supply to peroxisomes.

Defects of the PC biosynthetic pathway caused by *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ mutations become more evident in the phospholipid patterns of the endoplasmic reticulum and mitochondria than in peroxisomes. Whereas changes in PC levels of peroxisomes caused by the mutations were moderate, rather dramatically decreased values of PC and also PE were observed with the endoplasmic reticulum and mitochondria. Especially in the *cki1*Δ*dpl1*Δ*eki1*Δ mutant strain where the cell relies on the methylation of PE for PC production a dramatic depletion of PC was observed which was also accompanied by some decrease in PE. Defects were less dramatic although clearly observed in the *cho2*Δ*opi3*Δ strain. These results underline the important role of the Kennedy pathway in PC and also PE production for organelles in cells cultivated on oleate. Minor changes of PI, PS, PA and CL levels were also observed in mutant strains, although these effects were most likely compensations for the missing PC and PE production.

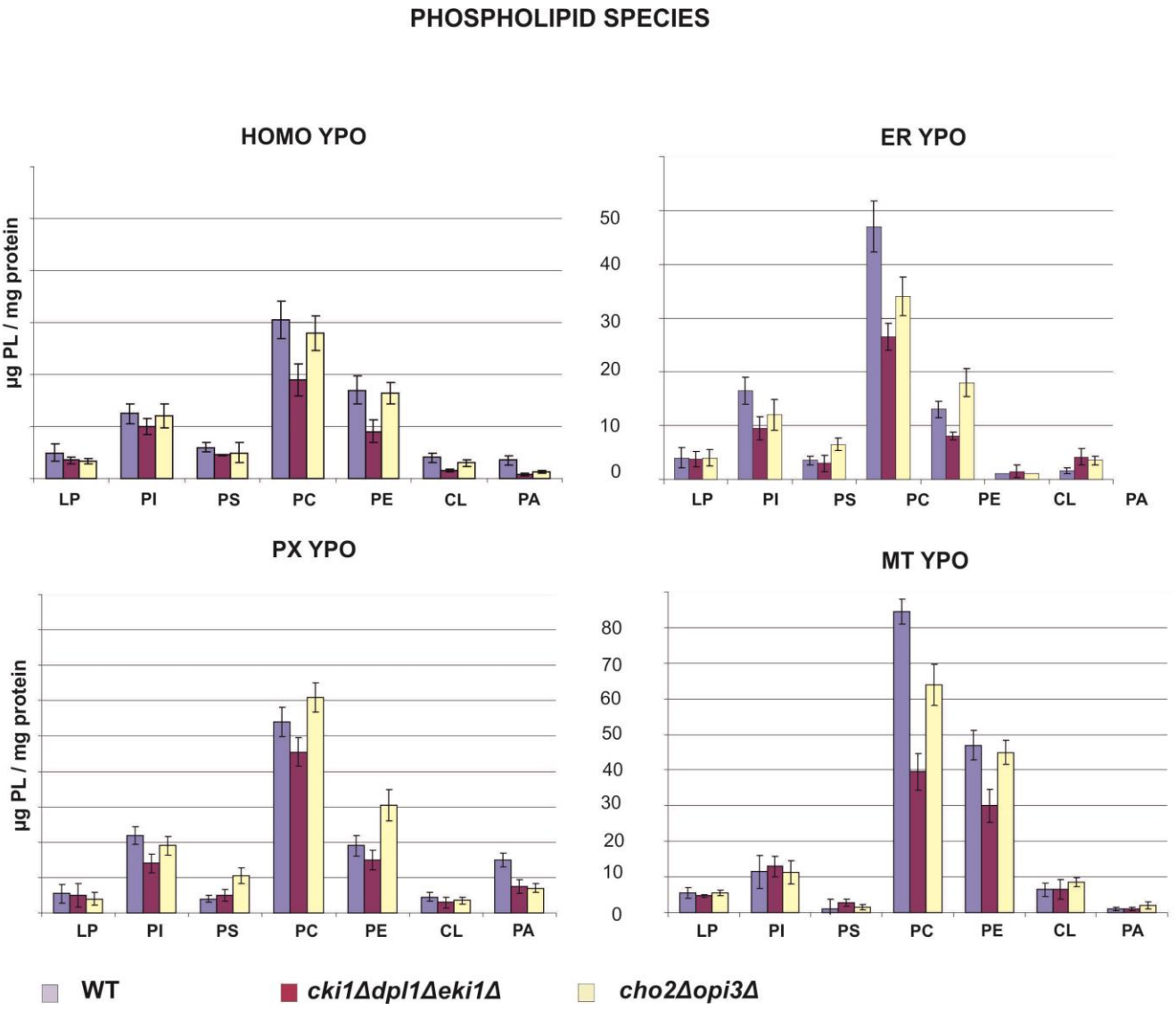

Figure 5: Individual phospholipid species of strains grown on liquid YPO media, which induces peroxisomal proliferation. Changes caused by mutations in the PC pathway are studied for the homogenates (HOMO), endoplasmic reticulum (ER), mitochondria (MT) and peroxisomes (PX).

Fatty acid analysis of peroxisomes from cells with PC biosynthetic defects

Since phospholipid species in the organelle membranes of the three analyzed strains (wild type, methylation and Kennedy mutant) showed a versatile pattern it was also interesting to analyze the fatty acid patterns in the respective organelles (Figure 6). Not surprisingly, oleate (C18:1) is the predominant fatty acid in all samples due to the fact, that this fatty acid present in the medium is not only used as a carbon source but also as a component for the synthesis of membrane phospholipids. Interestingly, the level of C18:1 in peroxisomes was markedly lower than in the endoplasmic reticulum and mitochondria indicating that some selectivity in the supply to organelle membranes may occur.

In all three strains, the C14:1 fatty acid was found only in peroxisomes. C16:0 was present in all three strains and organelles at a level of ~ 10-20%, whereas C16:1 and C18:0 fatty acids were detected only at low concentrations. Presence of C18:2 in all samples is due to impurities of oleic acid samples used as carbon source.

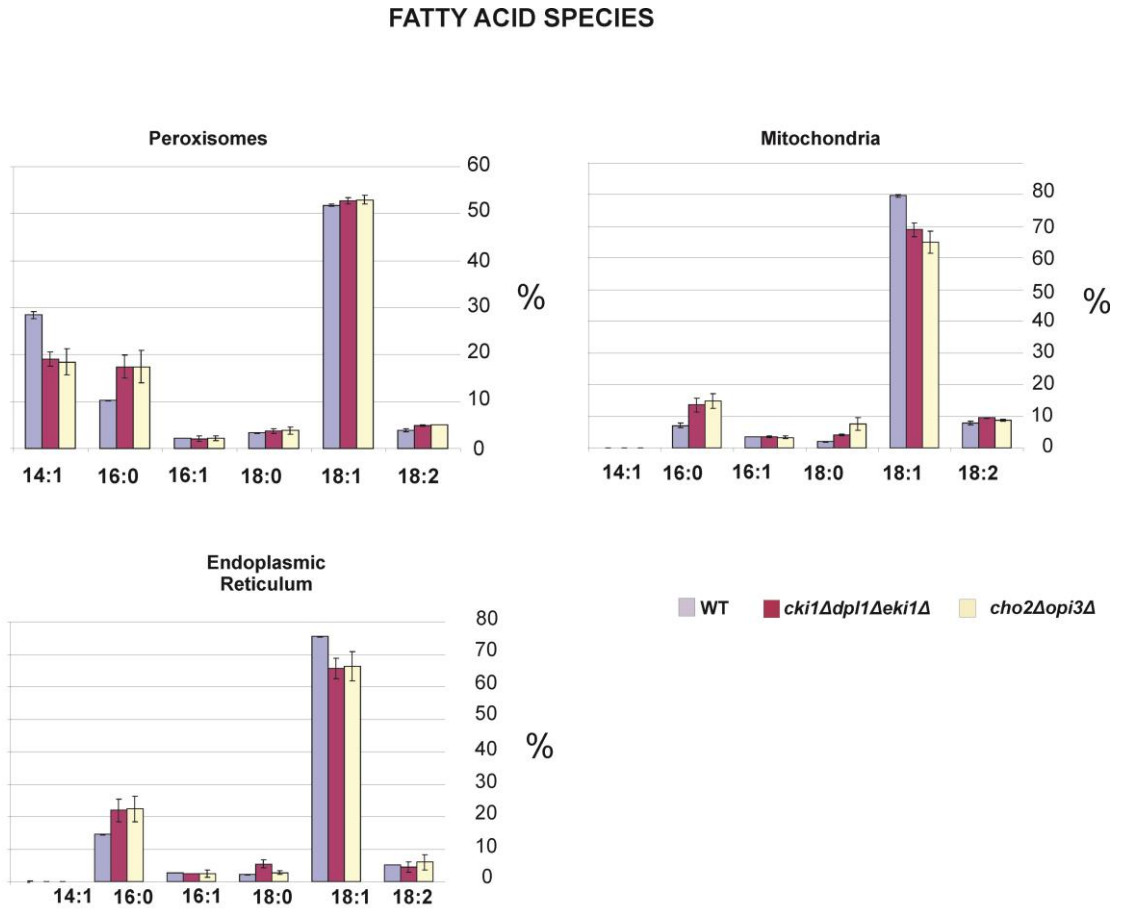

Figure 6: Fatty acid compositions (%) of peroxisomes, mitochondria and endoplasmic reticulum from all three strains.

Electron microscopy of yeast cells with PC biosynthetic defects

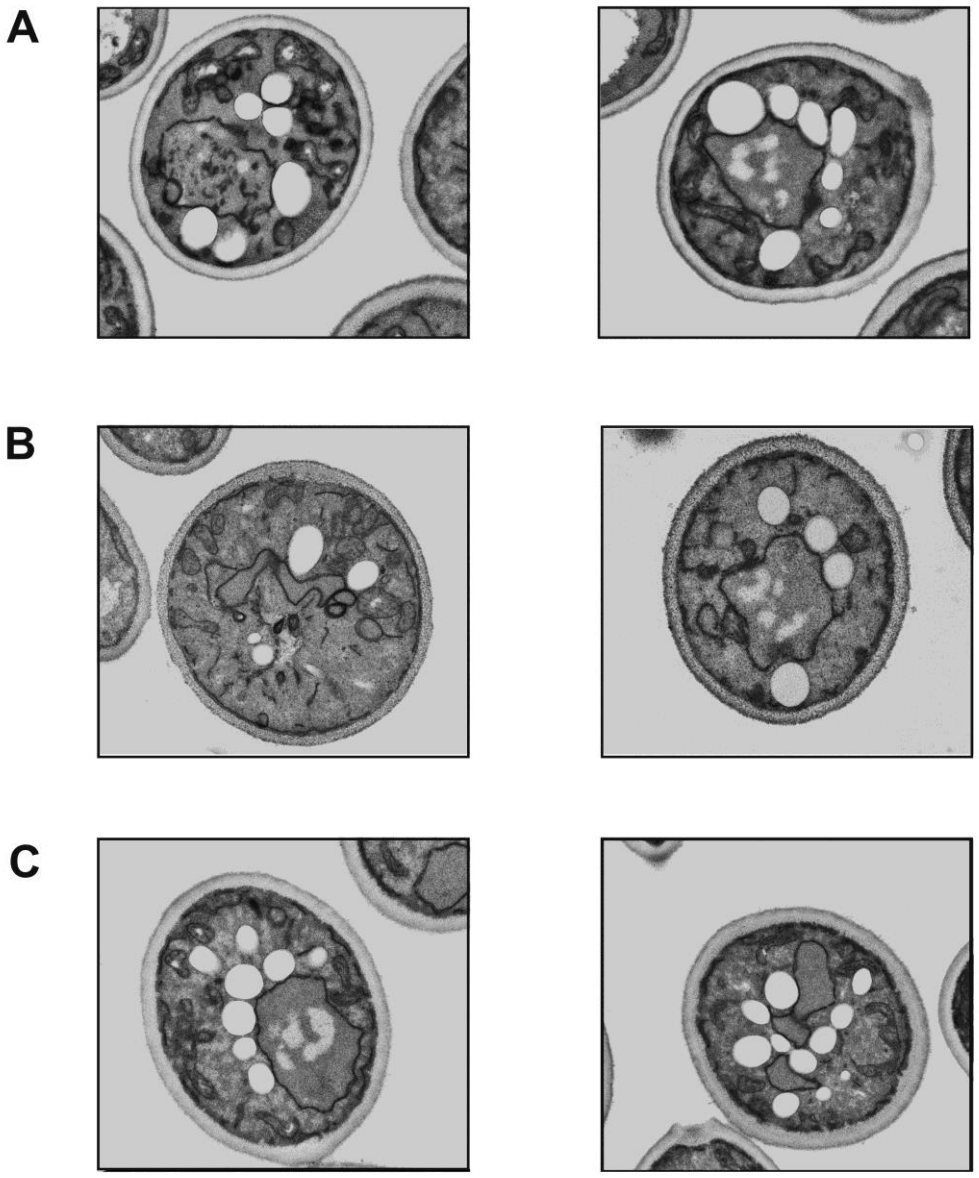

Figure 7: Transmission electron microscopy. Ultra thin sections from the chemically fixed *S. cerevisiae* yeast cells (A, B, C see legend in Figure; bar 1 µm) are shown. The cells were grown on YPO media to induce formation of peroxisomes.

To investigate possible morphological changes caused by mutation in the different PC biosynthetic pathways, wild type and mutant strains grown on oleate were subjected to electron microscopic inspection (Fig. 9). Growth of yeast cells on fatty acids as carbon source not only induces peroxisome proliferation, but also causes enhanced formation of lipid droplets. These droplets mainly consist of non-polar lipids and are a storage compartment for triacylglycerol and steryl esters (Czabany et al. 2008). Data from our group (Rosenberger et al. 2009) but also other studies indicate that peroxisomes have a tendency to associate with other subcellular compartments, especially the endoplasmic reticulum, mitochondria (Schrader et al. 2013; Camões et al. 2009) and lipid bodies (Binns et al. 2006). Especially the contact between peroxisomes and lipid droplets may be relevant for the supply of fatty acids as a substrate for ß-oxidation to peroxisomes, although biochemical evidence for such a route is missing. The association of peroxisomes with mitochondria and endoplasmic reticulum may be more important for the biogenesis of peroxisomes including supply of lipids from the respective sites of synthesis.

As can be seen from electron micrographs (see Fig. 9) dramatic changes of the cellular structure caused by the mutations in the PC biosynthetic pathways were not observed. One obvious difference between the strains analyzed was the decreased number of lipid droplets in the *cki1*Δ*dpl1*Δ*eki1*Δ mutant. These data are in line with previous results from our laboratory (Horvath et al. 2011) showing that a *cki1*Δ*dpl1*Δ*eki1*Δ grown on YPD medium with glucose as a carbon source had a markedly lower level of triacylglycerols than wild type.

Organelle membrane fluidity affected by defects in PC biosynthetic pathways

Membrane properties, especially the rigidity/fluidity of a biomembrane depend on the presence and the amount of various components such as sterols, the saturation degree of fatty acid species and the concentration of polypeptides present in a lipid bilayer. However, also the PC to PE ratio may have an influence on the biophysical properties of organelle membranes. PC is the classical bilayer forming phospholipid, whereas the non-bilayer forming phospholipid PE may induces curvature of the membrane and disturb the order. Therefore, organelles from strains with compromised PC biosynthesis were good candidates for changed membrane properties. To test organelle membrane rigidity/fluidity, anisotropy

measurements using the flourophore diphenylhexatirene (DPH) were performed. High anisotropy values are indicative of membrane rigidity, whereas low anisotropy is typical for more fluid membranes.

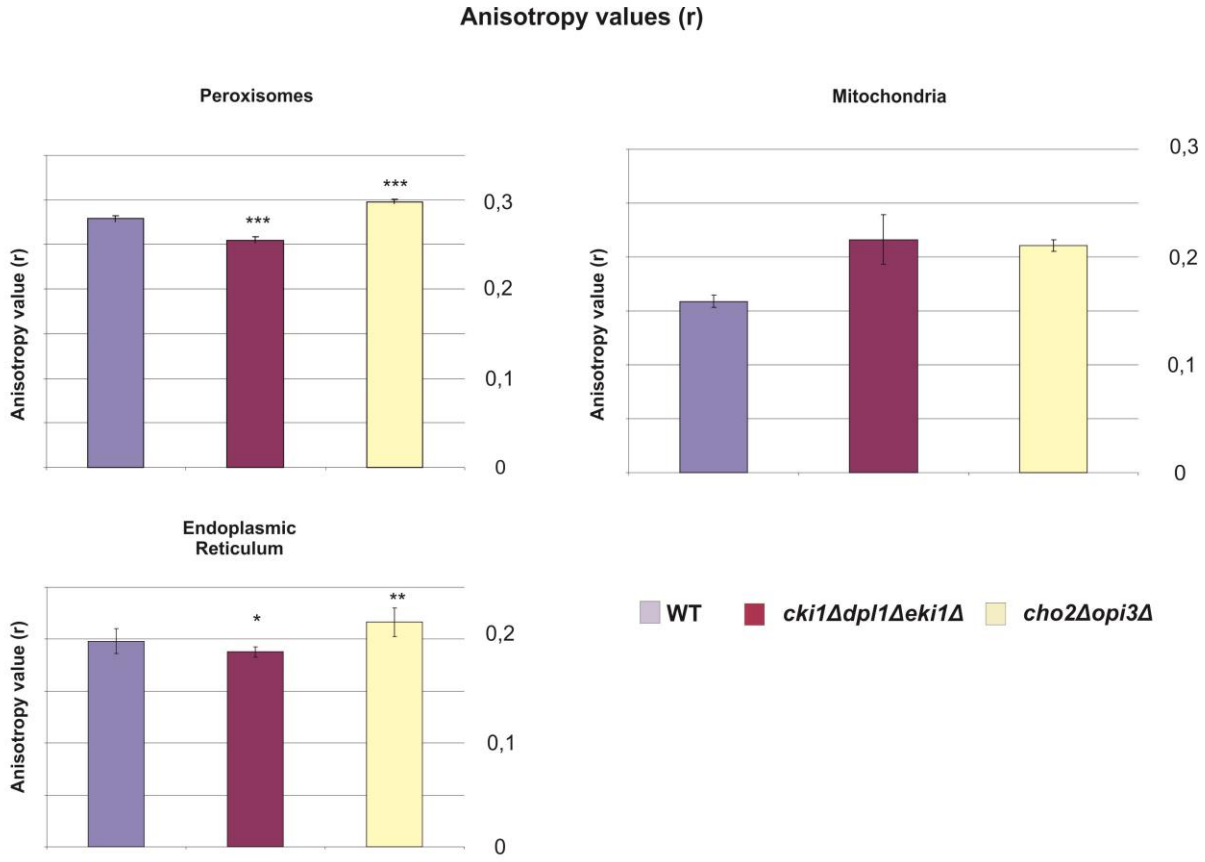

Figure 8: Anisotropy values of peroxisomes, mitochondria and endoplasmic reticulum. An obvious difference of anisotropy values was observed with mitochondria from the different strains. For peroxisomes and endoplasmic reticulum the significance was calculated by a paired two tailed Student's t-test comparing the wild type to each of the mutant strains. P values are as follows: b * ≥ 0,01 b ** ≥ 0,05 b *** ≤ 0,005.

Anisotropy for peroxisomal membranes, endoplasmic reticulum membranes and mitochondria from wild type, *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ mutants are shown in Fig. 10. As can be seen, the fluidity of peroxisomal and endoplasmic reticulum membranes from wild type and mutants are only slightly different. In both organelles, deletion of the Kennedy pathway enzymes caused a slight decrease of the anisotropy, whereas anisotropy values were slightly increased in *cho2*Δ*opi3*Δ. In mitochondrial membranes, both mutations led to higher anisotropy.

To correlate the measured anisotropy values as described above with as many membrane parameters as possible, a comparison of phospholipid patterns, the phospholipid to protein ratio, the PC to PE ratio, the fatty acid patterns, the ratio of saturated (SFA) to unsaturated fatty acids (UFA) and the ergosterol to phospholipid ratio is shown in Fig. 11. High ratios of SFA to UFA, high amounts of ergosterol, low phospholipid to protein ratios and low PC to PE ratios in membranes were considered as parameters that may lead to high anisotropy values indicating high rigidity of membranes. Of course, the overall fluidity/rigidity of a membrane is result of a combination of all parameters mentioned.

A comparison of anisotropy values between peroxisomes from wild type and mutants showed a slight decrease of anisotropy with the *cki1*Δ*dpl1*Δ*eki1*Δ mutant organelles indicating slightly higher membrane fluidity, and a slight increase of anisotropy with peroxisomes from the *cho*Δ*2opi3*Δ mutant. This result was surprising because the ratio SFA/UFA was higher in both mutants than in the wild type, and the sterol content varied. However, anisotropy (fluidity/rigidity) seems to follow the PC to PE ratio in peroxisomal membranes. Although we have to be very cautious with the interpretation of these data, two aspects have to be mentioned. First, sterol measurements are very preliminary and need to be verified. Secondly, the SFA/UFA ratio is very low, because more than 85 % of fatty acids are unsaturated. Thus, small changes in the pattern result in relatively high changes of the ratio. Consequently, we are left with a possible effect of changed amounts of PC and PE in membranes, which might result in the observed fluidity/rigidity changes. This view is supported by results obtained with endoplasmic reticulum and mitochondrial membranes from wild type and mutants. Also in these cases the anisotropy follows the PC to PE ratio. In these samples the low SFA/UFA and ergosterol to phospholipid ratios might indeed be irrelevant for the fluidity status of the membrane.

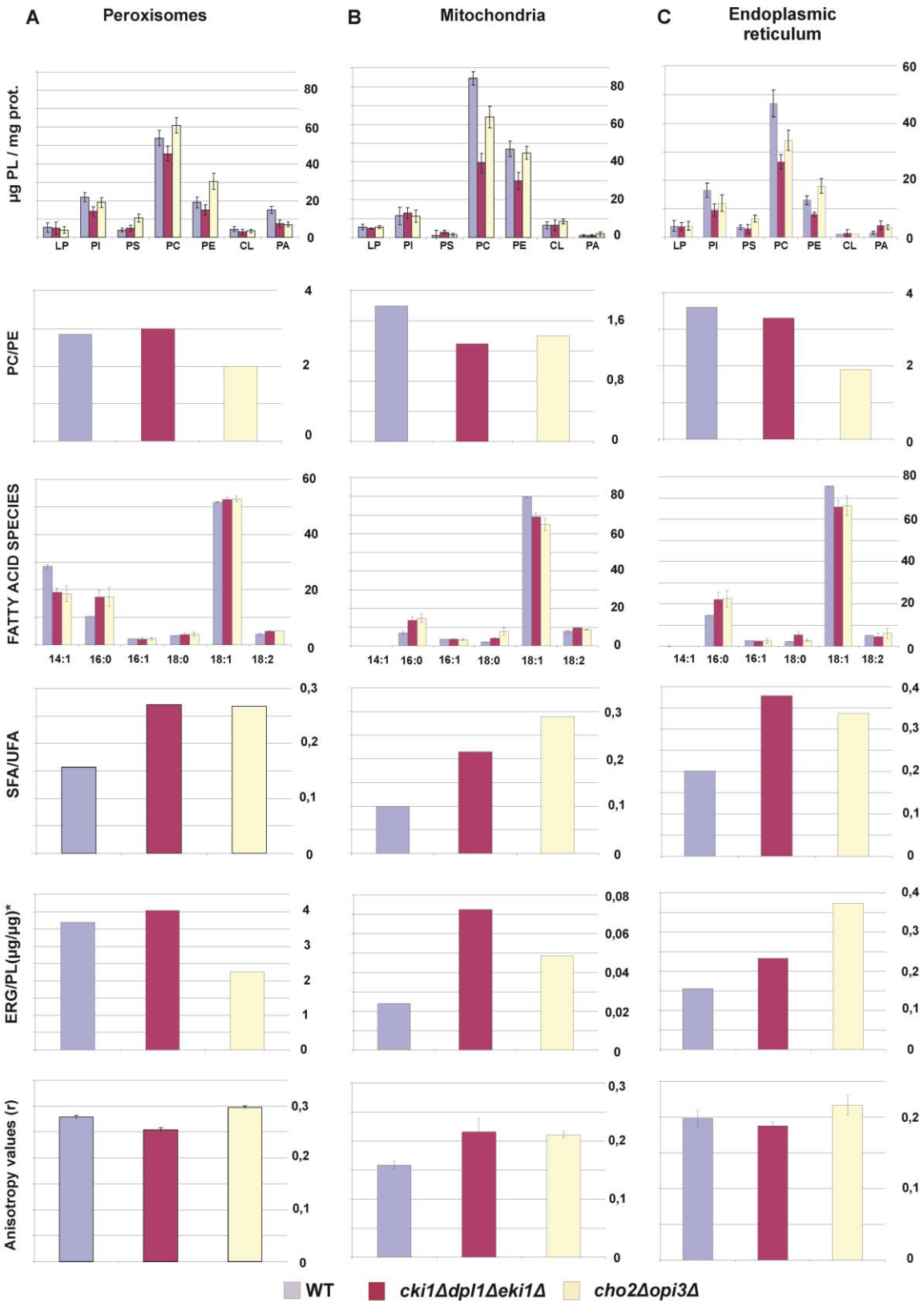

Figure 9: Data which are relevant for the interpretation of organelle membrane anisotropy. SFA, saturated fatty acids; UFA, unsaturated fatty acids; ERG, ergosterol; PL, phospholipid, A peroxisomes B mitochondria C endoplasmic reticulum

Discussion

The supply of PE to peroxisomes in yeast *S. cerevisiae* grown on oleate by three different pathways was presented previous by Rosenberger et al. (2009). The supply pathways of PC to peroxisomes, mitochondria and endoplasmic reticulum and the correlation between PC and PE synthesis in the yeast *S. cerevisiae* are presented in this study. In the yeast proliferation of peroxisomes and expression of peroxisomal proteins is greatly enhanced by growth on oleate media (Dionisi et al. 2012; Brown et al. 2000; Erdmann and Blobel 1995; Eitzen et al. 1997; Ohdate and Inoue 2012; Wu et al. 2012). However, presence of oleate in the media leads to an excess of fatty acids in the yeast cell (Wriessnegger et al. 2007; Rosenberger et al. 2009).

The knowledge about the supply of phospholipids to peroxisomes in yeast and mammalian cells is limited. Here we show that both the methylation pathway and the CDP-choline branch of the Kennedy pathway produce PC destined to peroxisomes, but also to mitochondria and the endoplasmic reticulum. Interestingly, the two pathways exhibit different efficiency in PC formation for peroxisomes, at least under growth conditions chosen for this study. It appears that the CDP-choline pathway is more efficient in the supply of PC to peroxisomes than the methylation pathway. This result also suggests that different pools of PC may exist in the cell. The PC level in peroxisomes of the *cho*Δ*2opi3*Δ mutant is even higher than in wild type. With changing PC levels in the peroxisomes and other organelles also the PE level is altered. In the Kennedy mutant (*cki1*Δ*dpl1*Δ*eki1*Δ) where less PC is present than in wild type, also PE is decreased. This result is not surprising because genes deleted in the mutant used also affect the PE production through the CDP-ethanolamine branch of PE formation. In the methylation mutant (*cho*Δ*2opi3*Δ) where more PC is present the level of PE was largely unchanged.

In peroxisomes of both mutants the PC level reaches nearly wild type levels. This is not the case for other organelles. In mitochondria as well as in the endoplasmic reticulum, the wild type has higher PC levels than mutants, especially when compared to *cki1*Δ*dpl1*Δ*eki1*Δ. It appears that in peroxisomes import and assembly of PC and PE formed by both pathways are more balanced. However, it has to be taken into account that a phospholipid transfer network must exist between phospholipid synthesizing and accepting organelles which may be regulated by individual requirements of the organelles. Our data suggest that PC and PE production and at least their transport to the peroxisomes are linked. Not much is known about the transport mechanism of phospholipids to peroxisomes, but it was shown that non-

vesicular transport to peroxisomes is possible (Raychaudhuri and Prinz 2008). However, evidence has been presented that also vesicle transport from mitochondria to peroxisomes may be possible (Andrade-Navarro et al. 2009; Braschi et al. 2010; Soubannier et al. 2012; Neuspiel et al. 2008). This result could explain why low CL concentrations were found in peroxisomes of all three strains.

The impaired growth of the two mutants on oleate may be explained by the different PC to PE ratio, the ratio of saturated to unsaturated fatty acid and the ergosterol to phospholipid ratio, which have an influence on the fluidity of membranes. According to our data, it seems that the PC/PE ratio has the most influence on the anisotropy. PC and PE differ only in their head groups, PC having a quaternary amine with methyl substituents while PE has a primary amine head group. However, molecular simulations have demonstrated the ability of PE to interact strongly with itself and neighbouring lipids via inner- and intramolecular hydrogen bonding (Leekumjorn and Sum 2006). This biophysical ability generates a close-packed lipid bilayer with hydrogen tails aligned, decreasing the space occupied by each lipid molecule (Kranenburg and Smit 2005). The DMPE and PE molecules which are present in a lipid bilayer, form bridges with each other (Pink et al. 1998; Nussio et al. 2008; Pitman et al. 2005; Kranenburg and Smit 2005; Leekumjorn and Sum 2006). These bridges lead to a higher value of the "main" transition temperature (T_m) from a fluid to a gel state of the membrane or *vice versa* of PE membranes compared to PC bilayers possessing the same hydrocarbon chains (Pink et al. 1998). Furthermore, in model studies insertion of DMPE or PE molecules into a lipid bilayer increased the T_m of the mixed PC:PE bilayer systems substantially (Nussio et al. 2008). Other studies indicate that hydrogen bonds within a cluster remain stable for longer time and that especially PE lipids may diffuse or reorient as groups or clusters, rather than individual lipids (Pitman et al. 2005). The more PE is present compared to PC, the higher the transition temperature of a lipid bilayer will be, i.e. membranes are more rigid as anisotropy values show. Furthermore, the ratio of saturated to unsaturated fatty acid (SFA/UFA) together with the PC/PE ratio seems to have an influence on the anisotropy. The length and saturation degree of fatty acids has an influence on the dynamics of a membrane. Longer fatty acids and more saturated fatty acids make the dynamics of membrane systems slower (Pitman et al. 2005). Since in membrane fractions studied in this work the SFA/UFA ratio is very low, these parameters may be less relevant for membrane properties. Same arguments may hold for the contribution of ergosterol to the properties of membranes studied in this work, although values of sterol analyses have to be scrutinized.

In summary, our study demonstrates routes of PC transport to peroxisomes of the yeast and consequences of PC depletion for properties of peroxisomal membranes. Our data demonstrate the importance of PC for functionality and integrity of peroxisomal membranes.

Acknowledgements

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CHAPTER 3

Phosphatidylethanolamine transport to peroxisomes in permeabilized yeast cells

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Key words: phospholipids, phosphatidylethanolamine, phosphatidylcholine, permeabilized cells, peroxisomes, transport, reporter enzyme, assay, organelle contact

Abbreviations: PL, phospholipids; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; CL, cardiolipin; PI, phosphatidylinositol; ER endoplasmic reticulum; MT, mitochondria; PX peroxisomes; CDP, cytidine diphosphate; SAM, S-adenosyl-L-methionine; LP, Lipid particles; MMPE, monomethyl PE; DMPE, dimethyl PE; p-cells, permeabilized cells; SKL, serine-lysine-leucine; PTS1, peroxisomal targeting sequence; PEX, peroxins, GFP, green fluorescent protein; PEMT, PE methyl transferases

Abstract

In the yeast *Saccharomyces cerevisiae*, phospholipids can be synthesized via different pathways which are partially connected to each other. One of the major yeast phospholipids is phosphatidylethanolamine (PE) which can be synthesized by different routes located to mitochondria, the endoplasmic reticulum and the Golgi. Previous studies from our laboratory suggested that PE formed through all pathways and in different subcellular membranes can be supplied to peroxisomes with comparable efficiency (Rosenberger et al., 2009, Biochim. Biophys. Acta 1791, 379-387). However, mechanisms involved in these translocation processes remained unclear. To address these questions, *in vitro* and *in vivo* assays for studying PE supply to peroxisomal membranes were established. A strain which lacks the gene product of *OPI3*, the major PE methyltransferase responsible for the formation of phosphatidylcholine (PC), was constructed. This strain was transformed with an Opi3p-GFP hybrid with an SKL targeting sequence which directed the enzyme from the endoplasmic reticulum to peroxisomes. In this "reporter mutant", peroxisomes were the only site of PC formation via methylation of PE, and the appearance and amount of PC produced became an indicator and measure for PE translocation from the different sites of synthesis to peroxisomes. Localization of the hybrid protein to peroxisomes and functionality were confirmed by lipid and growth phenotype analyses, subcellular fractionation and enzyme activity assays. Experiments with permeabilized yeast cells revealed that transport of PE to peroxisomes was partially affected by ATP depletion, but not negatively influenced by the absence of cytosol or the presence of the cytoskeleton inhibitor nocodazole. Our data suggest that membrane contact between organelles may contribute to PE transport to peroxisomes.

Introduction

The synthesis of phosphatidylcholine (PC) is a well-studied process in different experimental systems from yeast to mammals. PC is also the most abundant phospholipid in the yeast. Two different pathways, both being located in the endoplasmic reticulum (ER) (Zinser et al. 1991a) are involved in the synthesis of PC in yeast cells (Fig. 1A). The first pathway of PC synthesis involves three methylation steps of phosphatidylethanolamine (PE) using S-adenosyl-L-methionine (SAM) as methyl donor to form monomethyl PE (MMPE) and dimethyl PE (DMPE) as intermediates (Waechter et al. 1969; Waechter and Lester 1971, 1973; Carman and Henry 1999). Enzymes catalyzing this process are the two PE methyltransferases (PEMT) Pem1p/Cho2p and Pem2p/Opi3p with the latter catalyzing the last two methyl transferase steps (Kodaki and Yamashita 1987; McGraw and Henry 1989).

Synthesis of precursor phospholipids for PC synthesis is also spread over various organelles. Whereas phosphatidylserine (PS) is synthesized in the ER, the next intermediate in the biosynthetic pathway of aminoglycerophospholipids, PE, can be formed through four different routes in different organelles. PE can be synthesized by (i) decarboxylation of phosphatidylserine (PS) with the aid of the mitochondrial phosphatidylserine decarboxylase Psd1p; (ii) by Psd2p in a Golgi/vacuolar compartment; (iii) by the so-called CDP-ethanolamine branch of the Kennedy pathway which is located to the ER (ER); and (iv) by lyso-PE acyltransferases such as Ale1p or Tgl3p (for recent review see Flis and Daum 2013). Although PC synthesis occurs in the ER, and PE formation in subcellular fractions mentioned above (mitochondria, ER, Golgi) both lipids are required in all subcellular membranes. Thus, lipid transport becomes a most important issue for the biogenesis of organelles. Different mechanisms of lipid transport between organelles have been described and discussed, such as diffusion, protein mediated translocation, vesicle transport, or membrane contact (Levine and Loewen 2006; Holthuis and Levine 2005; Osman et al. 2011). Most recent investigations favor vesicle flux and membrane contact as the most likely processes to deliver lipids to organelles.

The aim of the present work was to investigate transport of PE to peroxisomes. For this purpose we designed a transport assay based on metabolic conversion of PE to PC. We have chosen Opi3p as a reporter enzyme, which we directed from the ER to peroxisomes by genetic manipulation (Fig. 1B and C). Opi3p catalyzes the second and third step of PE methylation, but can also partially perform the first methylation step, forming MMPE from

PE (Kodaki and Yamashita 1989). Additionally, PC can be formed via the so-called Kennedy pathway using exogenous choline via CDP-choline (Kennedy and Weiss 1956; McMaster and Bell 1994), which in yeast is only active when external choline sources are present.

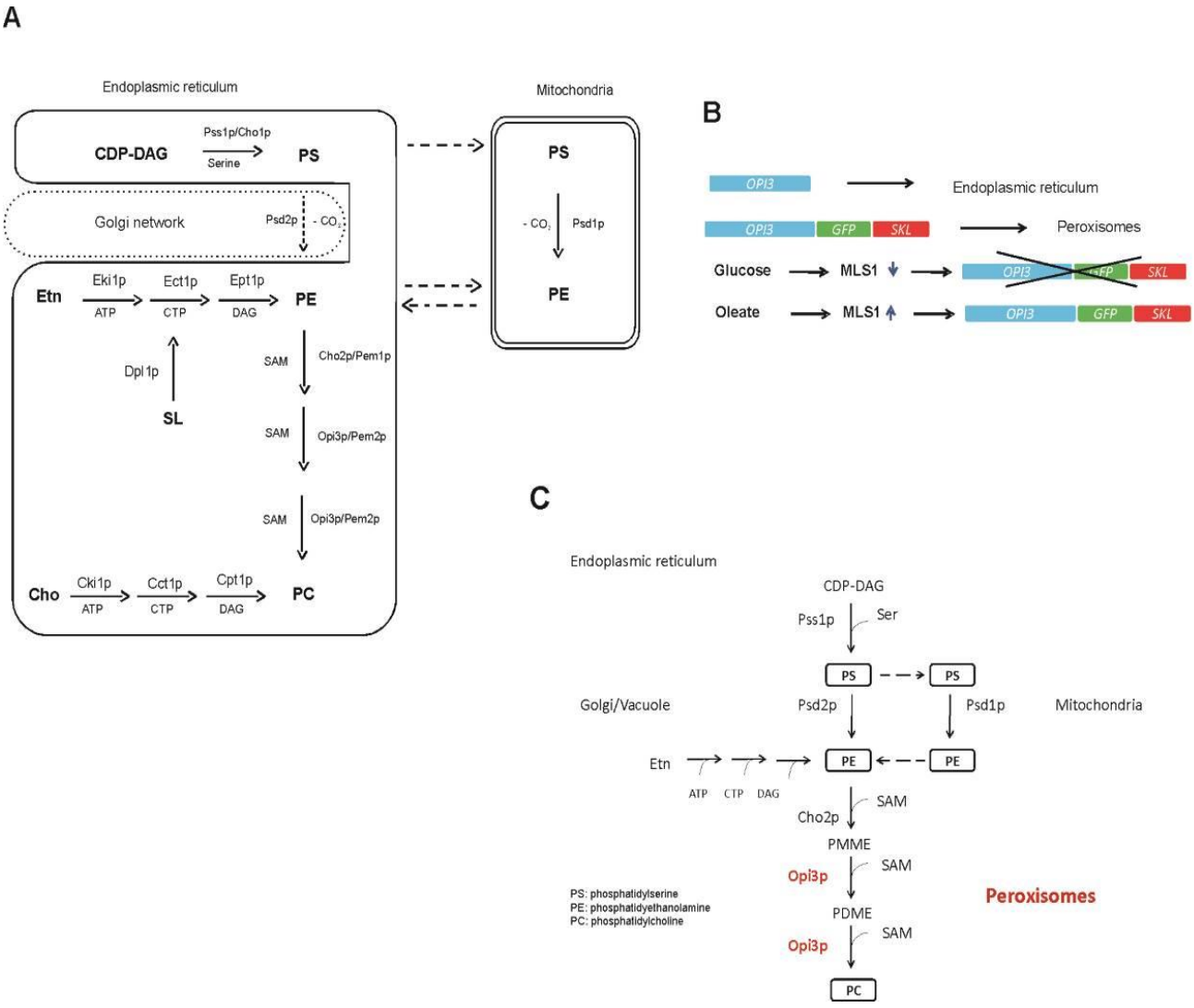

Fig. 1: Phospholipid traffic to peroxisomes

(A) Overview of phospholipid biosynthetic pathways in the yeast with emphasis on localization of enzymes involved. (B) Schematic view of the *OPI3-GFP-SKL* construct. The GFP-tag was fused to the C-terminus of the protein followed by the three amino acid peroxisomal targeting sequence 1 (PTS1) SKL, which directs the protein from the ER to peroxisomes. The hybrid protein was set under the control of the MLS1 promoter, which is repressed in the presence of glucose. (C) Overview of PC synthesis in the "reporter strain". The OPI3-GFP-SKL hybrid protein localized to peroxisomes enables monitoring of PE traffic from its site of synthesis (ER, Golgi and mitochondria) to peroxisomes.

Yeast peroxisomes are incapable of phospholipid biosynthesis (Bishop and Bell 1988; Zinser et al. 1991a). In contrast, mammalian peroxisomes have a certain capacity to synthesize lipids such as plasmalogens and sterols (Kovacs et al. 2007, 2012; da Silva et al. 2012; Wanders 2013; Braverman and Moser 2012; Islinger et al. 2012). In the past years this organelle has gained much interest due to cell biological studies of the Zellweger syndrome and other neuropatholigical metabolic peroxisomal diseases which are due to peroxisomal dysfunction (Krause et al. 2013; Barry and O'Keeffe 2013; Fujiki et al. 2012; Krysko et al. 2007). The biogenesis and proliferation of peroxisomes, however, is still a matter of dispute (Smith and Aitchison 2009; Schrader and Fahimi 2008; Islinger et al. 2012). In plant and yeast cells, peroxisomes are the only organelle harboring enzymes of ß-oxidation (Poirier et al. 2006). Thus, peroxisomes of the yeast are essential for growth on fatty acids as the only carbon source (Erdmann et al. 1989). This ability made yeast a perfect model organism for studying peroxisome proliferation and biogenesis. Proteins essential for peroxisomal formation are the so-called peroxines (PEX). Many of them are conserved from yeast to mammals. Yeast cells deficient in one of the essential peroxines *PEX3, PEX16* or *PEX19* are not able to produce functional peroxisomes (Schrader and Fahimi 2008). Re-introduction of this genes into the mutants however allowed the formation of fully functional peroxisomes in mammals and yeast (South and Gould 1999; Kal et al. 2000). In addition, fission of peroxisomes was observed in yeast (Erdmann and Blobel 1995; Hoepfner et al. 2001; Motley and Hettema 2007; Motley et al. 2008; Nagotu et al. 2008a, 2008b) and mammals (Li and Gould 2003; Koch et al. 2003; Koch and Brocard 2012; Kobayashi et al. 2007; Fujiki et al. 2012). *De novo* synthesis and fission are nowadays widely accepted as processes contributing to peroxisome biogenesis. The so-called growth and division model describes a budding process of pre-peroxisomal membranes from the ER followed by introduction of peroxisomes forming a machinery for import of matrix proteins and thus proliferation to mature peroxisomes (Nashiro et al. 2011; Fujiki et al. 2012; Titorenko and Mullen 2006). Mature peroxisomes are then able to grow and divide. It has been shown for the yeast (Tam et al. 2005; Hoepfner et al. 2005; Haan et al. 2006; Yan et al. 2008) as well as for mammalian cells (Geuze et al. 2003; Tabak et al. 2013, 2003; Toro et al. 2009) that peroxisomes derive from the ER. The role of the essential genes for peroxisomes formation has been largely investigated.

Mechanism(s) of lipid traffic to peroxisomes are still enigmatic. The possibilities of vesicular transport, non-vesicular transport (Raychaudhuri and Prinz 2008) and transporter proteins (Wirtz 1991) have been discussed. None of these mechanisms appeared to be essential for peroxisomal biogenesis. Also the possibility of direct membrane contact, which has been discussed for yeast organelles such as mitochondria and ER (Ardail et al. 1993; Shiao et al. 1998; Achleitner et al. 1999; Kornmann et al. 2009), appears to be a possible mechanism for the supply of lipids to peroxisomes although it has so far not been investigated in detail.

In recent studies investigating the supply of PE and PC to peroxisomes, we found that all pathways of PE and PC biosynthesis contribute to the supply of the two major phospholipid species to peroxisomes (Rosenberger et al. 2009, Flis et al. 2013, manuscript in preparation). We suggest direct membrane contact as possible mechanism for this process tempted by recent evidence employing electron microscopy (Perktold et al. 2007) and assays with permeabilized cells (this study). The present study is aimed at the investigation of PE traffic to peroxisomes in more detail. A "metabolic transfer assay" based on the expression of Opi3p fused to a GFP-SKL-tag in peroxisomes of an *opi3*Δ deletion strain was employed. This experimental setup allowed us to test qualitatively and quantitatively the translocation of PE to peroxisomes by appearance of PC in a *semi in vivo* system under various conditions.

Materials and Methods

Strains used and growth conditions

Strains used in this study are listed in Table 1. Yeast cells were cultivated in minimal medium (SD) containing 2% glucose, or minimal medium (SO) containing 0.1% oleic acid and 0.1% glucose as carbon source and 0.67% Yeast Nitrogen Base. For selection of auxotrophy markers a -ura mix was used. Yeast cells were precultured in minimal SD media for 48h till the late stationary phase was reached, inoculated at OD_{600} of 0.1 in SO media and grown till early stationary phase.

Name	Genotype	Origin
BY4741 (wt)	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
*opi3*Δ	*opi3*Δ: BY4741 opi3Δ::KanMX4	Euroscarf
yMC2	BY4741 opi3Δ::KanMX4 pJR233	This study
yMC3	BY4741 opi3Δ::KanMX4 pJR233 OPI3 GFP SKL	This study

Table 1: Strains used in this study

Cloning of *OPI3*

The open reading frame (ORF) of *OPI3* was used as template for PCR amplification using the following forward and reverse primers: MC42_fw: 5´ CGG CGG GGT ACC ATG AAG GAG TCA GTC CAA GAG 3´ and MC43rev: 5´ GCG CGC GGA TCC CAT ATT CTT TTT GGC CTT ATC ACG 3´. The resulting PCR product was flanked by *Kpn*I and *Bam*HI restriction sites and missing the stop codon. It was cloned into plasmid pJR233 (Brocard et al. 1997) containing a GFP-SKL expression construct, under the control of an MLS-promoter. Sequencing verified correct construction of the plasmid pJR233 encoding for *OPI3-GFP-SKL* (pMC13). This plasmid was transformed into the yeast strain BY4741 *opi3*Δ::KanMX6 and set under the control of the MLS1 promoter, which is repressed in the presence of glucose (Fig. 1 B).

Organelle isolation

Subcellular fractionation was performed with cells grown in liquid SO medium to the early stationary phase according to Rosenberger (Rosenberger et al. 2009). In brief, cells were harvested by centrifugation at 5,000 rpm for 5 min in a Sorvall SLC-3000 rotor. The cell pellet was washed with distilled water and resuspended in twice the volume of buffer A (0.1 M Tris/SO_4, pH 9.4). After addition of 0.66 mg dithiothreitol (DTT) per ml buffer A, the cell suspension was incubated at 30 ^oC with shaking for at least 10 min. Cells were washed and resuspended in 7 volumes per g cells pre-warmed buffer B (30 ^oC) (1.2 M sorbitol, 20 mM KH_2PO_4, pH 7.4). Preparation of spheroplasts was performed using 2 mg Zymolyase 20T per g wet weight in buffer B for approximately 1 h at 30 ^oC with gentle shaking (Daum et al. 1982; Zinser et al. 1991b; Zinser and Daum 1995). Spheroplasts were then washed in buffer B and resuspended in twice the cell volume in breaking buffer containing 5 mM-MES-KOH, pH 6.0, 0.6 M sorbitol, 1 mM KCl, 0.5 mM EDTA and 1 mM PMSF. Spheroplasts were homogenized on ice using a Dounce homogenizer with a tight fitting pestle. Unbroken cells and nuclei were removed by centrifugation at 5,000 rpm for 5 min in a Sorvall SLC3000 rotor. In order to enhance the yield of peroxisomes, the resulting pellet was resuspended twice in breakage buffer, re-homogenized and centrifuged as described above. Combined supernatants were centrifuged at 15,000 rpm in a Sorvall SS34 rotor for 15 min. The crude organelle pellet consisting of mitochondria and peroxisomes was gently suspended in breakage buffer plus 1 mM PMSF, and centrifuged at 5,000 rpm to remove larger aggregates. Then, the supernatant was centrifuged again at 15,000 rpm, and the pellet was resuspended in breakage buffer as described above and loaded for further purification on a Nycodenz gradient (17-24-35%; w/v) in 5 mM-MES-KOH, pH 6.0, 1 mM KCl, 0.24 M sucrose. Centrifugation was carried out in a swing-out rotor (Sovall AH-629) at 26,000 rpm for 90 min. The white peroxisomal layer was withdrawn using a syringe, diluted with 4 volumes of breaking buffer and sedimented at 15,000 rpm in SS34 rotor for 15 min at 4 ^oC. Mitochondria formed a separate band near the top of the density gradient which was also collected and sedimented by centrifugation. For quality control, proteins of the organelle samples were precipitated in 10% (final concentration) trichloroacetic acid (TCA) for at least 1 h on ice and washed in ice cold water. Protein determination was performed using the method of Lowry (Lowry et al. 1951). SDS-PAGE was carried out by the method of Laemmli (Laemmli 1970). Proteins were analyzed by Western blot analysis according to the method of Haid and Suissa (Haid and Suissa 1983). A set of antibodies representing typical marker proteins of various cellular organelles was used to check the quality of the isolated organelles as will be indicated in the

results section. Peroxidase conjugated secondary antibody (Sigma) and enhanced chemiluminescent signal detection reagents (Supersignal™, Pierce Chemical Company, Rockford, IL, USA) were used to visualize immunoreactive bands.

Permeabilization of yeast cells

The method for yeast cell permeabilization was adapted from Achleitner et al. (1995). Cells were grown in SO medium for 24 h and harvested at 5,000 rpm for 5 min in a table top centrifuge. Cells were washed once with 0.1% fatty acid free BSA and twice with distilled water. Then, they were taken up in two volumes per g cell wet weight in Buffer A (0.1 M TrisSO_4, pH 9.6) and incubated with dithiothreitol (DTT) at a final concentration of 154 mg per 100 ml buffer. Cells were sedimented and resuspended in seven volumes per g cell wet weight 1.2 M Sorbitol containing 2 mg Zymolyase/g cell wet weight and incubated at 30 ^{o}C shaking for 1 h. Spheroplasts were regenerated in an aliquot of YPD (2% glucose, 1% yeast extract, 2% peptone) and 1.2 M sorbitol (1:1) for 20 min at 30 ^{o}C with shaking. After harvesting at 5,000 rpm for 2 min spheroplasts were carefully resuspended in Breaking Buffer to a final concentration of 0.5 g cell wet weight per ml. Permeabilized cells were prepared in a permeabilization box by gently freezing aliquots of 200 µl in Eppendorf tubes in a steam of liquid nitrogen for 15 min and stored at -70 °C (Fig. 2). Permeabilization was verified by microscopy after slowly thawing cells on ice for ~30 min.

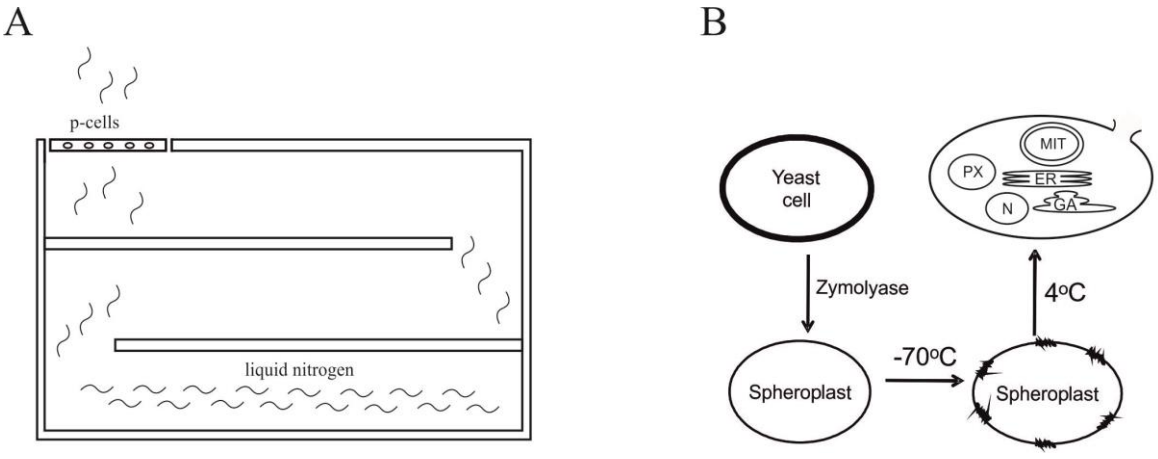

Fig. 2: Overview of yeast cell permeabilization methodology: (A) Permeabilization preparation box for spheroplasted and regenerated yeast cells. (B) A schematic overview of steps which lead to preparation of permeabilized cells. For details see Materials and Methods.

To deplete permeabilized cells of ATP, apyrase (10 U per mg of p-cell protein) and oligomycin (final concentration of 0.07 mM) were added. Samples were preincubated at 30 °C for 30 min prior to the start of the assay. To deplete permeabilized cells of the cytosol, they were centrifuged at 5,000 rpm in a table top centrifuge for 5 min at 4 °C. The two fractions resulting from this centrifugation were the pellet containing permeabilized cells without cytosol, and the supernatant containing cytosolic components. After careful removal of the cytosol, pre-warmed assay mix was added to permeabilized cells and the reaction was started. This procedure led to almost complete removal of the cytosol from permeabilized cells as judged from Western blot analysis. In this analysis GAPDH used as the marker for the cytosol was more or less exclusively found in the supernatant, but not in the pellet. Por1p (outer mitochondrial marker) was enriched in the pellet although a minor amount escaped to the supernatant which, however, did not affect the quality of the pellet fraction (data not shown).

Protein protection assay

Since isolated peroxisomes were too fragile for standard protein protection assays employing mild Proteinase K treatment, we designed and adopted an assay to analyze the topology of Opi3p-GFP-SKL. For this purpose, cells grown to the early stationary phase were harvested, washed twice in 0.1% BSA and three times in distilled water. Spheroplasts were prepared as described above and 200 µl (~ 4 mg protein) of the spheroplasts suspension were used for further assays. To this suspension, 1.4 ml Breaking buffer (5 mM MES, 1 mM KCl, 0.5 mM EDTA, 0.6 M sorbitol, pH 6.0) were added, and a homogenate was prepared by careful pipetting up and down 10 times. The resulting cell homogenate was incubated on ice for 5 min. Cell debris were removed by a low speed spin at 500 rpm for 5 min in a table top centrifuge. The supernatant containing the protein rich lysate was used for Proteinase K treatment. In a typical assay, aliquots of 125 µl lysate were used for Proteinase K protection assays. If not stated otherwise, 1.25 µl of 20 mg/ml Proteinase K were used and 5 µl 20% Triton X-100 as indicated. One aliquot of the lysate was centrifuged at 13,000 rpm (16,000 x g) for 10 min to separate all cellular membranes from the cytosol (supernatant). The resulting membrane pellet was washed once and resuspended in the same amount (125 µl) of Breaking Buffer. Proteins of all fractions were precipitated with 50% TCA (final conc. 10%) and analyzed by SDS-PAGE and Western blotting as described above.

Lipid analysis

Total cell extracts were prepared from cells grown to the stationary phase in minimal SO or SD medium. Cells were harvested and washed twice in 0.1 % BSA and three times in distilled water. Then, cells were frozen at -20 ^{o}C and thawn on ice. The cell pellet was resuspended in 3-4 ml breaking buffer (5 mM MES, 1 mM KCl, 0.5 mM EDTA, 0.6 M sorbitol, pH 6.0). Two volumes of glass beads were added and cells were disintegrated on a Vortex mixer at about 1,200 rpm for at least 1 h at 4 ^{o}C. The cell homogenate was transferred to a fresh tube and the protein amount was determined by the method of Lowry (Lowry et al. 1951). Then, 2 mg equivalents of protein were used for lipid extraction employing the method of Folch (Folch et al. 1957). In brief, protein samples were added to 3 ml $CHCl_3$:MeOH (2:1; v/v) in a Pyrex glass tube. Lipids were extracted to the organic phase by vortexing at room temperature for 1 h. Proteins were removed by consecutive washing steps with 0.2 volumes 0.034% $MgCl_2$, 1 ml 2 N KCl/MeOH (4:1; v/v) and 1 ml of an artificial upper phase ($CHCl_3$:MeOH:H_2O; 3:48:47; per vol.). Washing solutions were added to the extracts and incubated with shaking for 5 min. After centrifugation for 3 min at 3,000 rpm in a table-top centrifuge the aqueous phase was removed by aspiration. Finally, lipids were dried under a stream of nitrogen and stored at -20 ^{o}C.

Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25% NH_3 (65:35:5; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second developing solvent. Lipids were stained with iodine vapor, bands were scraped off the plate, and lipid phosphorus was quantified by the method of Broekhuyse (1968).

Phosphatidylethanolamine methyltransferase enzyme assay *in vitro*

A PE methyltransferase *in vitro* assay with a strain bearing the Opi3p-GFP-SKL hybrid was adapted from Boumann et al. (2004). Yeast subcellular fractions were prepared as described above, and localization and activity of the reporter enzyme were determined. 10 µl (~10 µg protein) of an organelle suspension were used for a 100 µl assay mixture containing 5 µl 1 M TrisCl, pH 8.0, 49.8 µl 1 mM SAM, 0.2 µl [^{3}H]S-adenosyl methionine (SAM) (10 Ci/mol), 20 µl 1 mM Epicuron (carrier) and 15 µl distilled water. The reaction was started by addition of the enzyme source. At time points indicated, aliquots were withdrawn, and lipids were

extracted using 300 µl $CHCl_3$:MeOH (2:1, v:v) and washed twice with 300 µl $CHCl_3$:MeOH:H_2O (3:48:47; per vol.) to remove remaining protein. Lipids were dried under a stream of nitrogen, separated by TLC in a solvent system containing $CHCl_3$:MeOH:NH_3 (65:25:4; per vol.) and visualized by iodine vapor staining. Spots containing PC were scraped off the TLC and subjected to scintillation counting using Ultima gold high flash point luminescence scintillation cocktail (Perkin Elmer) containing 5% distilled water as cocktail.

Aminoglycerophospholipid synthesis in permeabilized cells

For the *semi in vivo* enzyme measurement a modification of the assay described by Achleitner *et al.* (1995) was employed. Reactions were carried out at 30°C in an assay buffer mix of 90 µl 6 mM $MnCl_2$, 90 µl dist. water, 195 µl 1.2 M sorbitol, 400 µl assay buffer (0.6 M mannitol, 50 mM TrisCl, pH 8.0), 45 µl 2 mM serine, 30 µl [^{3}H]serine and 50 µl permeabilized cells (~ 0.5 mg protein, enzyme source, starter of reaction). Aliquots of 100 µl were taken at 0, 10, 15, 25, 30, 50, 50 min. At the time point of 15 min, 5.6 µl 0.5 M EDTA, pH 8.0, and at the time point of 30 min 4 µl 1 M $MgCl_2$ and 1.2 µl SAM were added. The reaction was stopped by removing a 100 µl aliquot which was pipetted into an Eppendorf tube with 300 µl $CHCl_3$:MeOH (2:1; v/v). The extraction was carried out in the Eppendorf tubes for 1 h at room temperature after addition of 100 µl 0.034% $MgCl_2$ for removal of the proteins. Lipids were isolated and samples were loaded on TLC plates as described above. Epicuron was added as lipid carrier to all samples.

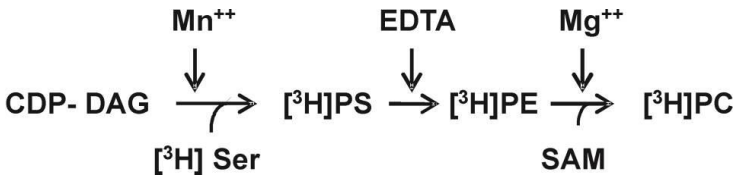

Fig. 3: Overview of the three step enzymatic assay of phosphatidylcholine synthesis in permeabilized cells. In the last two PE methylation steps, Opi3p-GFP-SKL localized to the peroxisomes is used as a reporter enzyme. The amount of PC which is produced in the peroxisomes is an indication for PE having been transferred to peroxisomes.

During the assay described above, radiolabeled PC is formed from CDP-DAG and [^{3}H]serine. In the first step of the assay, mainly PS is produced by PS synthase which requires divalent

ions. In the second step synthesis of PE occurs in the absence of divalent ions which inhibit PS decarboxylases. The depletion of ions is achieved by addition of EDTA to the assay medium. Finally, PE is converted to PC in presence of divalent ions (Fig. 3).

Results

Design of a reporter enzyme assay for monitoring PE traffic to yeast peroxisomes

The present study was focused on the investigation of PE supply and transport to peroxisomal membranes. A reporter enzyme assay was designed and permeabilized yeast cells were employed (see Figs 1-3) to address this question. An Opi3p-GFP-SKL hybrid was targeted to peroxisomal membranes in a *opi3*Δ strain, which lacks the original PE methyltransferase Opi3p in the ER. The reporter enzyme was cloned in the vector system pJR233 under the control of the MLS-promoter together with a GFP-SKL tag (see Material and Methods). Since induction of yeast peroxisomes requires growth of yeast cells on oleic acid, we had to use an expression system inducing hybrid protein expression and peroxisomes proliferation at the same time. The MLS promoter is largely suppressed in the presence of glucose but active in the presence of oleate as carbon source (Brocard et al. 1997). To target the reporter Opi3p to the desired destination, the peroxisomes, we fused it to GFP followed by the SKL-peroxisomal targeting signal 1 (PTS 1) which should result in peroxisome localization of the protein originally located to the ER (see Fig. 1B). This construct was transformed into a mutant deleted of endogenous *OPI3*. As a control, the empty plasmid containing *GFP-SKL* was introduced into a *opi3*Δ strain (see Table 1).

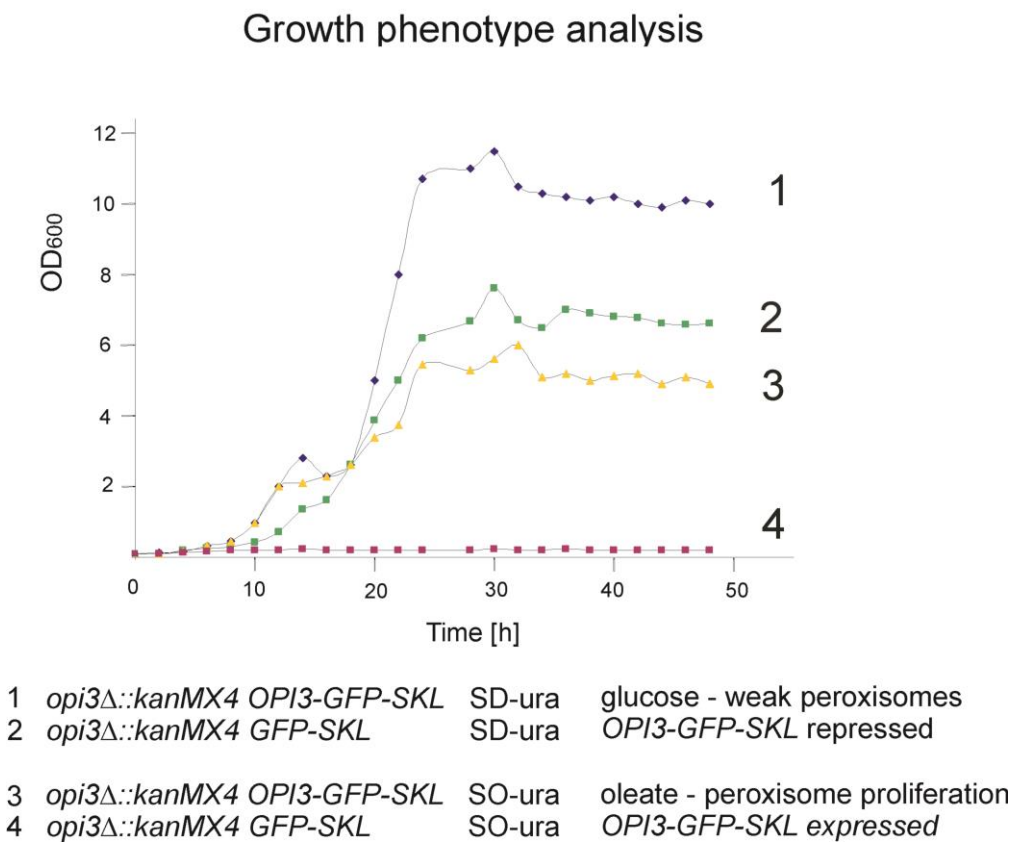

Fig. 4: Growth phenotype analysis. *Opi3*Δ bearing either Opi3p-GFP-SKL or GFP-SKL (control) were grown at 30 °C on SD or SO media. Cells were washed with 0.1% BSA and water and optical density OD_{600} was determined. Data shown are representative for at least three independent experiments. Strains numbered 1, 2 were grown on minimal media supplemented with choline, strains numbered 3, 4 were grown on minimal oleate media.

Opi3p catalyzes the last two steps in the methylation of MMPE to DMPE and PC (see Figs 1A and C). However, it was reported that strains deficient in *OPI3* grown on glucose need not to be supplied with exogenous choline (Greenberg et al. 1983), whereas a *cho2*Δ*opi3*Δ double mutant was auxotrophic for choline (Kodaki and Yamashita 1989). In our experimental set-up, growth retardation of *opi3*Δ on oleic acid indicated impairment of functional peroxisomes. Thus, a restored growth defect of the *opi3*Δ mutant was assumed to reflect expression of a functional Opi3p in peroxisomes. Growth analysis of the respective strains (Fig. 4) revealed that on glucose (non-inducing) the strain carrying the Opi3p-GFP-SKL grew better than *opi3*Δ although the latter mutant also survived in the absence of choline. On oleic acid (peroxisome proliferation induced, hybrid protein expressed), the strain bearing the Opi3p-GFP-SKL hybrid grew reasonably well, whereas the *opi3*Δ deletion mutant was heavily

impaired or practically not viable on oleic acid media. These data indicate that peroxisomal proliferation was paralleled by expression of functional Opi3p.

Opi3p-GFP-SKL is localized to peroxisomes

Subcellular localization of Opi3p-GFP-SKL was analyzed by cell fractionation and by Western blot analysis (Fig. 5). Opi3p-GFP-SKL was mainly localized to peroxisomes, and only a small amount of the protein was found in mitochondria. It has to be mentioned that a small portion of the hybrid protein was also detected in vacuoles (data not shown), most likely due to overexpression of the protein under the MLS-promoter and degradation in the vacuole. No GFP signal was detected in ER membranes indicating that Opi3p-GFP-SKL was successfully targeted to peroxisomes.

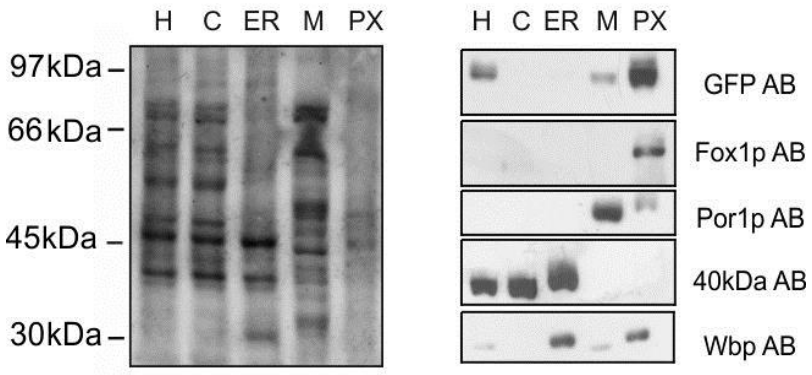

Fig. 5: Subcellular localization of Opi3p-GFP-SKL in yeast cells. Yeast cells were grown for 24 h at 30 °C (SO –ura) with shaking. Cells were harvested and cell fractionation was performed as described in Materials and Methods. Same amounts of protein (10 µg protein per lane) were precipitated with TCA and subjected to SDS-PAGE and Western blotting. Marker antibodies were used as indicated. H homogenate; C cytosol; ER endoplasmic reticulum; M mitochondria; PX peroxisomes. Antibodies used were directed against GFP for Opi3p-GFP-SKL, Por1p (mitochondrial porin), Fox1p (peroxisomal matrix protein), 40 kDa (ER membrane protein), Wbp (ER)

According to hydropathy blots (Kodaki and Yamashita, 1987) and protein membrane prediction programs (Jones et al. 1994, Tusnády and Simon 1998, 2001) Opi3p has been suggested to be an integral membrane protein (Fig. 6A). Therefore, we investigated the topology of Opi3p-GFP-SKL in the peroxisomal host membrane system.

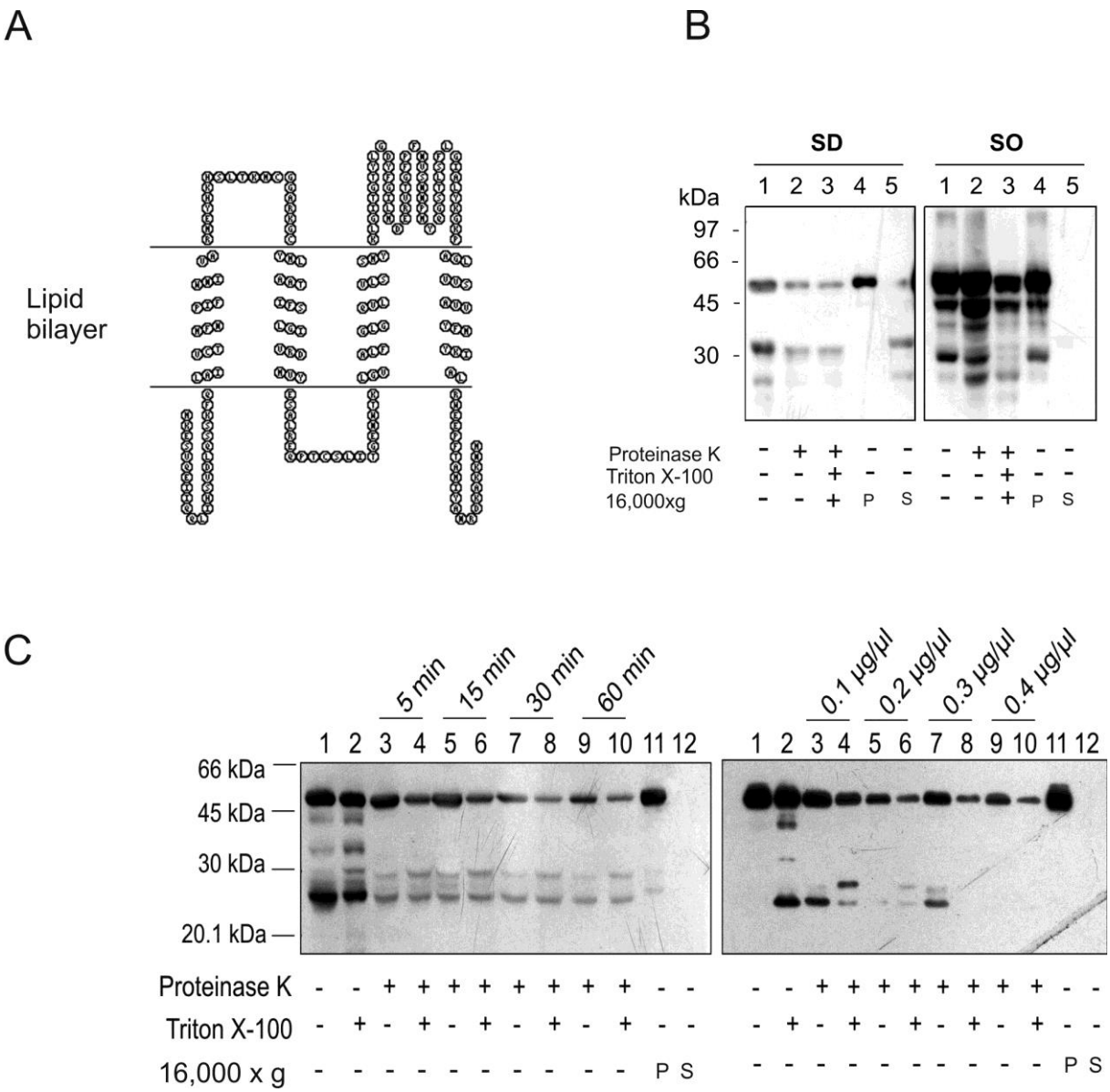

Fig. 6: Topology of Opi3p-GFP-SKL. Proteinase K protection assays with cell lysates were performed as described in Materials and Methods. (A) Topology predictions suggest that Opi3p is an intergral membrane protein (http://www.sacs.ucsf.edu/cgi-bin/memsat.py). (B, C) Cells grown either in SD-ura or SO-ura were used for lysate preparation, and Proteinase K protection assays were carried out as described in the Methods section. When indicated, 5 µl 20% Triton X-100 were added for solubilization of the membranes. Anti GFP-antibody was used to detect the protein. (C) Samples were incubated for different time periods (left panel) or with different amounts of Proteinase K (right panel). P are membranes, and S supernatant fractions from 16,000 x g centrifugations.

Proteins bearing the SKL sequence are usually targeted to the peroxisomal matrix. Protease protection assays as described in the Materials and Methods section were performed to clarify the localization of the hybrid protein within peroxisomes. As isolated peroxisomes are very fragile, a Protease K protection assay had to be adopted in a way that freshly prepared total cell lysates were used, where the organelles remained laregly intact (see Materials and Methods). As can be seen from Fig. 6B, Opi3p-GFP-SKL was also expressed under non-inductive conditions (SD) at a low amount indicating leakiness of the MLS promoter. Under these conditions, i.e. in the absence of peroxisomes, the protein was not protected by any membrane and degraded in the presence or absence of the detergent. However, under conditions where peroxisomes were induced, i.e. in cells cultivated on SO medium, the hybrid protein was not digested by the protease in the absence of Triton X-100 indicating insertion of the protein into peroxisomes. It has to be noted that the protein was not completely digested even after addition of the detergent in standard assays. Therefore, we performed time dependent digestion as well as incubations with increasing amounts of Proteinase K (Fig 6C). These analyses revealed that much of the protein was digested within 60 min even in the absence of the detergent indicating that over longer time incubations peroxisomes became leaky and Proteinase K was able to penetrate peroxisomal membranes. Assays with increasing amounts of Proteinase K revealed that most of the protein seemed to be protected. These data suggests that Opi3p-GFP-SKL was imported into peroxisomal matrix or at least protected in the membrane. 16,000 x g membrane pellets contained all Opi3p-GFP-SKL (Fig. 6C; sample 11), whereas no signal was found in the cytosol (Fig. 6C; sample 12). These results confirmed organelle association of Opi3p-GFP-SKL.

Opi3p-GFP-SKL produces PC *in vitro* and *in vivo*

After verification of the hybrid protein localization to peroxisomes, the ability of the host organelle to produce PC was investigated. For this purpose, yeast cells grown on SD and SO medium were analyzed for the formation of PC *in vivo* (Fig. 7).

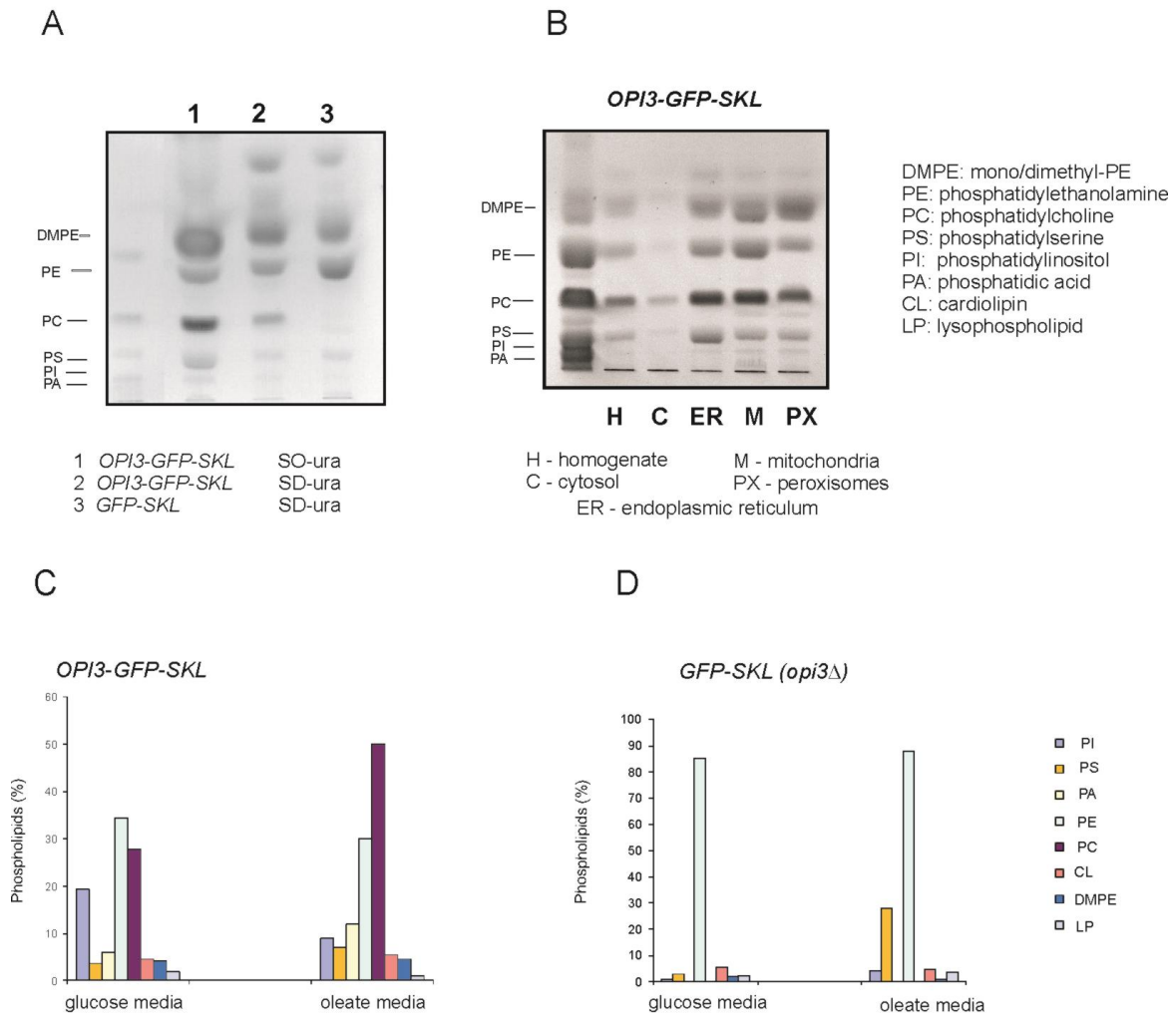

Fig. 7: Phospholipid analysis. (A) Total cell extracts were prepared from *opi3*Δ cells bearing either Opi3p-GFP-SKL or GFP-SKL (control) grown on SO (1) and SD (2, 3) media for 24 h. (B) Subcellular fractions from cells bearing the Opi3p-GFP-SKL hybrid grown on SO media for 24 h were used for lipid extracts. (C, D) Phospholipid were quantified (see Materials and Methods) from *OPI3-GFP-SKL* and *GFP-SKL* expressing strains, respectively, grown on oleate (SO) and glucose (SD) containing media. Individual phospholipid species (%) in the homogenate are shown.

Only trace amounts of PC were detected in *opi3Δ* cells harboring the empty *GFP-SKL* plasmid (Fig. 7A and D). Slightly higher PC amounts were detected in non-induced cells grown on SD bearing the Opi3p-GFP-SKL hybrid in the *opi3Δ* background (Fig. 7A and C) indicating again that the promoter was not completely inactive. As expected, growth of this strain on SO resulted in formation of high quantities of PC, indicating that under these conditions Opi3p-GFP-SKL was active *in vivo*. We also investigated the PC distribution in cell organelles under inducing conditions (Fig. 7B). Amounts of PC were comparable in peroxisomes, mitochondria and microsomes, indicating that PC produced in peroxisomes was distributed to all organelles. To confirm PC production in peroxisomes, we also performed *in vitro* enzyme assays with isolated organelles. The major activity was detected in peroxisomes and only minor activities were ascribed to mitochondria and ER (Fig. 8A). Contamination of mitochondria and ER with the peroxisomal fraction accounted for the remaining Opi3p-GFP-SKL activity in these fractions (Fig. 8B).

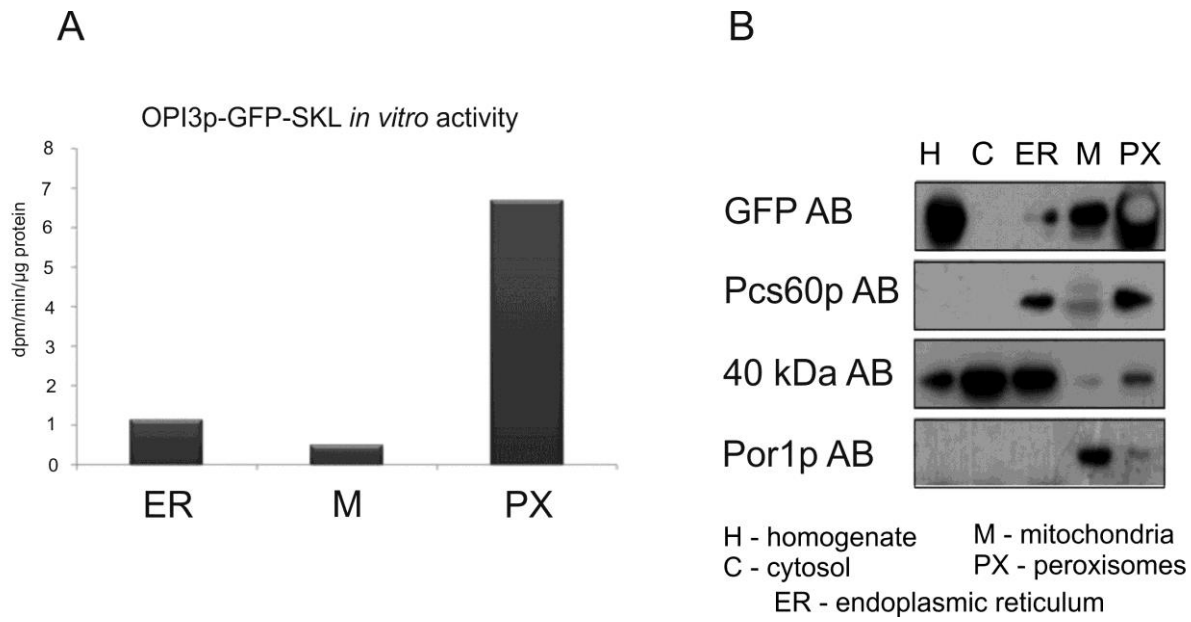

Fig. 8: Phospholipid methyltransferase activity *in vitro*. (A) The strain bearing the Opi3p-GFP-SKL hybrid grown on minimal oleate media was used for organelle isolation. Freshly isolated organelles were used for *in vitro* PE methylation assays as described in Material and Methods. (B) Quality control of the organelle preparation by Western blot analysis. Same amounts of protein (10 µg) were loaded and SDS-PAGE and analyzed by Western blotting. Incubation with primary antibodies against GFP, Pcs60p (peroxisomal AMP-binding protein), 40 kDa (endoplasmic reticulum) and Por1p (mitochondrial porin) is shown.

Phosphatidylethanolamine traffic to peroxisomes

A semi *in vivo* phospholipid assay was adopted from Achleitner *et* al. (1995) and used to investigate PE traffic to peroxisomes hosting the reporter enzyme Opi3p-GFP-SKL. For this purpose, we prepared permeabilized cells from wild type, the strain bearing the Opi3p-GFP-SKL in *opi3*Δ background, and a *opi3*Δ strain to compare efficiency of PE traffic to peroxisomes. The amount of PC formed was an indication how much PE has been transferred to peroxisomes and converted to PC. PC synthesis via PE was monitored by using [^{3}H]serine as substrate for the synthesis of PS which was subsequently used for PE and PC biosynthesis as described in the Materials and Methods section. The required co-substrate, CDP-DAG, for PS formation via [^{3}H]serine incorporation, was found to be present at sufficient levels in permeabilized cells (Achleitner et al. 1995).

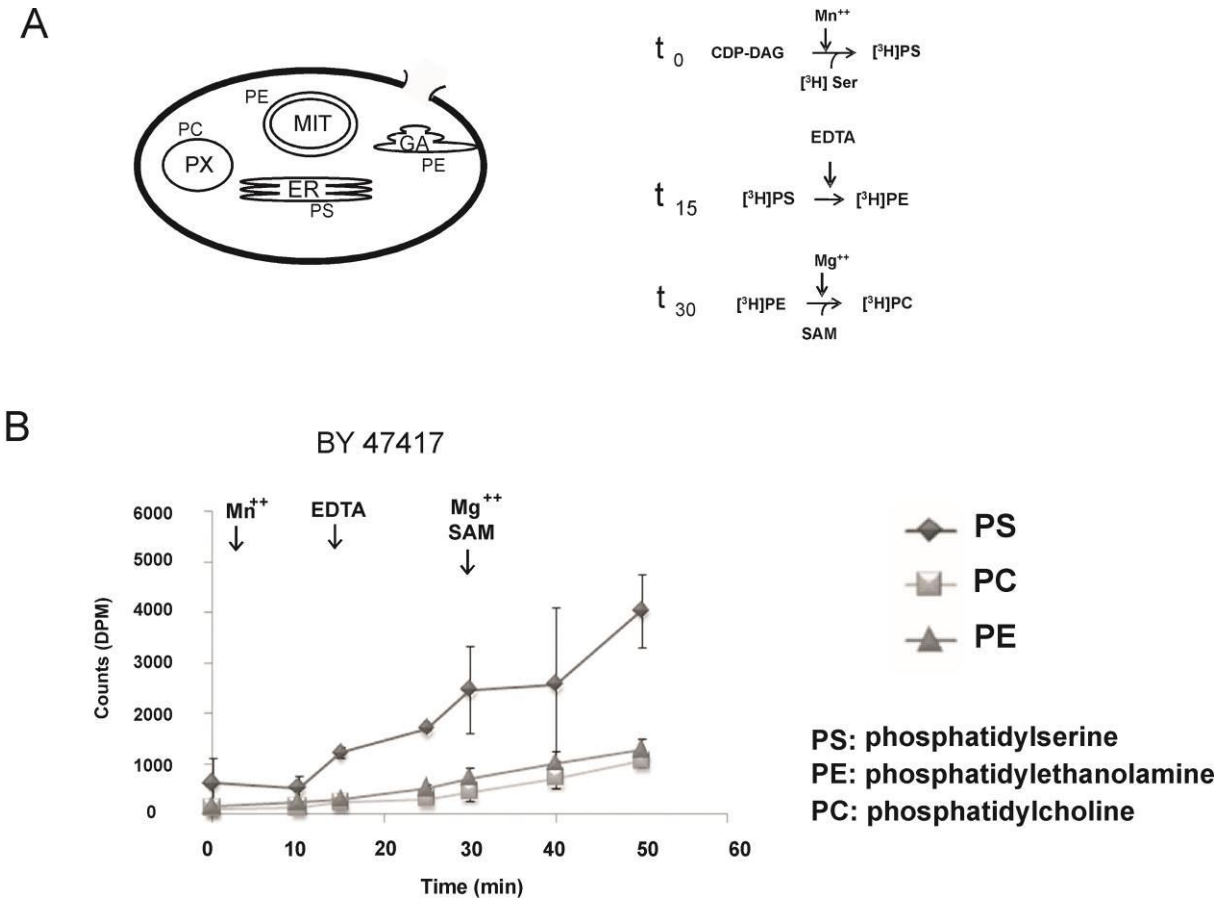

Fig. 9: *Semi in vivo* phospholipid synthesis in permeabilized cells. (A) The assay in permeabilized cells was performed in three steps with additives as indicated. (B) Wild type permeabilized cells served as a positive control for the assay conditions.

The ability of peroxisomes containing the Opi3p-GFP-SKL to produce PC from PE was tested in a three step *semi in vivo* assay (Fig. 9A). Synthesis of PS requires the presence of bivalent cations such as Mn^{2+} (Bae-Lee and Carman 1984; Sperka-Gottlieb et al. 1990) which were added to the assay mixture during the first incubation phase. In the following step, decarboxylation of PS by either Psd1p or Psd2p occurred in the absence of divalent cations, i.e. in the presence of EDTA (Lamping et al. 1991). Methylation of PE to PC requires the presence of Mg^{2+} and SAM as cofactors (Kodaki and Yamashita 1989) which were added in the final period of incubation.

In wild type permeabilized cells where Opi3p is localized to ER (Zinser et al. 1991a), most of the label from [^{3}H]serine was first incorporated into PS (Fig. 9B). Over the incubation period, PE and PC were formed after adding EDTA and SAM/Mg^{2+}, respectively. In *opi3*Δ cells with Opi3p-GFP-SKL directed to peroxisomes, labeled PS, PE and PC were also formed indicating that the biosynthetic sequence of aminoglycerophospholipid biosynthesis was intact, although at some lower efficiency than in wild type (Fig. 10A). Formation of PC in these cells indicated that supply of PE to peroxisomes had occurred. In a strain deleted of *OPI3* (Fig. 10B), high levels of PS and some PE were formed, but synthesis of PC was not detectable.

Permeabilized cells of the *opi3*Δ strain with Opi3p-GFP-SKL present in peroxisomes were also used to test for some conditions which were considered as relevant for the supply of PE to peroxisomes. In one of these assays, ATP was depleted by addition of apyrase and oligomycin to the assay mix (see Methods section). Under these conditions still some PC was synthesized, although at lower amounts than in untreated cells. Thus, presence of ATP might have some influence on the PE transfer to peroxisomes (Fig. 10C). Removal of the cytosol rather stimulated the formation of PC by the peroxisomal Opi3p-GFP-SKL hybrid (Fig. 10D). Closer contact of organelles under these conditions may be the reason for this observed effect. Fig. 10E summarizes the results of the different assays using the PC to PE ratio as a measure for PE transfer efficiency to peroxisomes as described above. In addition to results described above we also tested the influence of nocodazole which interferes with polymerization of microtubules. These experiments, however, did not show an effect of the drug on PE transfer to peroxisomes and PC formation (data not shown).

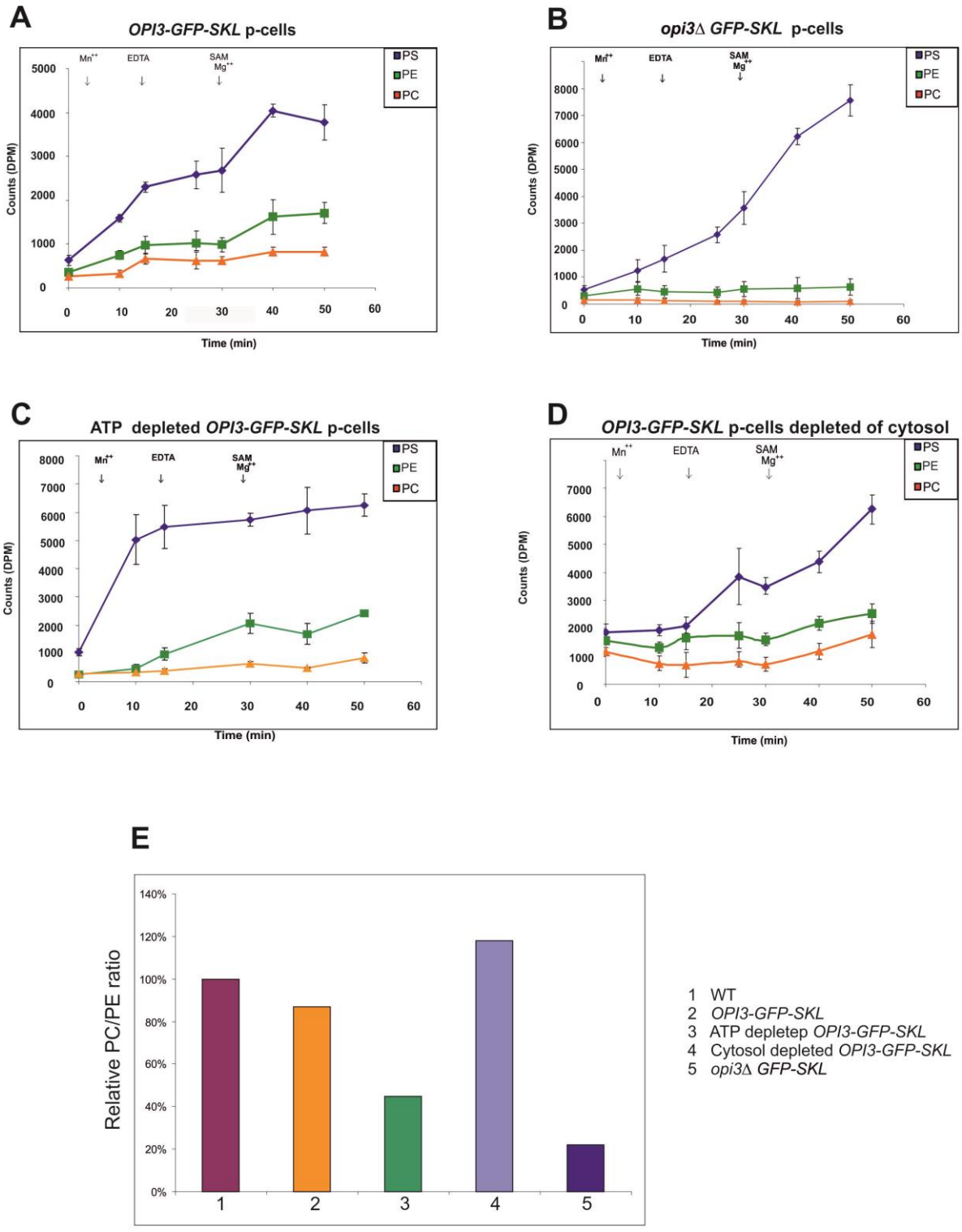

Fig. 10: Semi in vivo translocation of phosphatidylethanolamine to peroxisomes. Permeabilized cells of the *opi3*Δ strain with Opi3p-GFP-SKL present in peroxisomes or with a control strain bearing GFP-SKL were used for assays. Assays with Opi3p-GFP-SKL were performed under standard conditions (A), in the absence of ATP (C) and in the absence of cytosol (D). Assay (B) served as a negative control with *opi3*Δ GFP-SKL. (E) The relative ratios of PC to PE with WT set at 100% are shown.

Discussion

Peroxisome biogenesis has been studied for the past decades and is still a matter of discussion. It is believed and strongly supported by experimental evidence that peroxisomes are non-autonomous organelles derived from the ER, but able to grow and divide. Most studies addressing the biogenesis of peroxisomes were focused on the protein supply. It was found that early and essential peroxines, Pex3p and Pex19p, are localized first to the ER and then shuttled to developing peroxisomes (Hoepfner et al. 2005), thus indicating a connection between the ER and the peroxisomes. Fission of peroxisomes and further growth of daughter organelles makes them *semi*-autonomous (Guo et al. 2007). Since no lipid producing enzymes are localized to the peroxisomes, at least in the yeast, these organelles as several other cell compartments depend on the import of lipids for proliferation.

Mechanisms for lipid import to peroxisomes have not yet been clearly defined. Possible mechanisms of interorganelle lipid transport under discussion are vesicular flux (Raychaudhuri and Prinz 2008; Titorenko et al. 1996), cytosolic lipid transfer proteins (Wirtz 1991; Levine 2011) and direct organelle contact between organelles (Kornmann et al. 2009; Michel and Kornmann 2012; Voss et al. 2012). Only recently, transport proteins for lipid transport within mitochondria have been identified which are regulated by a lipid abundance feedback mechanism (Tamura et al. 2009, 2012; Potting et al. 2010; Connerth et al. 2012). The most favored model of lipid traffic, however, involves direct membrane contact. Indeed, import of PS from the ER to mitochondria has been shown to occur via membrane contact sites (Vance and Shiao 1996; Daum and Vance 1997). Recently, a molecular identification of components from ER-mitochondria contact sites was reported (Kornmann et al. 2009). Membrane contact without contributions of cytosolic proteins was also demonstrated for PS transport to mitochondria of permeabilized yeast cells (Achleitner et al. 1995, 1999). It is tempting to speculate and also supported by data from this study that a similar lipid supply mechanism between ER and peroxisomes may exist. PS transfer to mitochondria from permeabilized mammalian cells is ATP-dependent (Voelker 1989b, 1989a, 1990), whereas the same process in yeast can also occur in the absence of ATP (Achleitner et al. 1995). Requirements for PC import into peroxisomes have not been studied before. Additional evidence has been presented, that contact sites between ER and various other organelles might be important for non-vesicular lipid transfer (Choi et al. 2005; Levine and Loewen 2006). Membrane contact between the ER and mitochondria was also visualized (Csordás et al.

2006; de Brito and Scorrano 2008; Friedman et al. 2010; Lavieu et al. 2010), and acetylated microtubules were shown to be involved in the formation of the contact sites (Friedman et al. 2010, 2011). Our own experiments showed that treatment of permeabilized yeast cells with nocodazole had no influence on PE transfer to peroxisomes. It has to be noted, however, that acetylated microtubules from the ER which are involved in mitochondrial fission are nocodazole resistant (Friedman et al. 2011).

In yeast direct contacts sites of the ER with mitochondria, the so-called MAM-mitochondria contact (Vance 1990; Gaigg et al. 1995; Shiao et al. 1995; Camici and Corazzi 1995), and with the plasma membrane, the so-called PAM-plasma membrane contact (Pichler et al. 2001), were identified. Our own studies aimed at the isolation of peroxisomal associated membranes were unsuccessful so far, although three-dimensional reconstruction of yeast cells from serial sections visualized by electron microscopy showed ER-peroxisome association (Perktold et al. 2007). It has to be noted that purification of peroxisomes often results in co-isolation of ER and mitochondria which may have physiological relevance. Also the peroxisomal fission apparatus is partially shared with those of the mitochondria, pointing to the tight cooperation and cross talk between these two organelles (Schrader and Yoon 2007; Waterham et al. 2007). Recent evidence on mitochondria-peroxisomal cross talk showed that these two organelles have a redox-sensitive relationship (Fransen et al. 2012) and cooperate in mammals in anti-viral signaling and defense (Ivashchenko et al. 2011). A novel vesicular pathway between peroxisomes and mitochondria has also been proposed (Neuspiel et al. 2008). In fungi, peroxisomes and mitochondria cooperate in biotin synthesis (Tanabe et al. 2011). Also contact between peroxisomes and lipid droplet were found to be important (Binns et al. 2006; Rosenberger et al. 2009), indicating that membrane contact may play a role in lipid transport-storage/mobilization of non-polar lipids.

The present study was aimed at establishing an experimental system to test PE transport to peroxisomes on the basis of a combined transport-metabolic assay. For the current study we used an SKL tagged variant of Opi3p, the major yeast phospholipid methyltransferase, to direct the original ER protein to peroxisomes. In an *opi3*Δ background all PC produced through the methylation pathway was assumed to be formed by catalysis of the Opi3p-GFP-SKL hybrid. Such a conversion requires transport of PE to peroxisomes. It has to be mentioned that also minor amounts of monomethyl-PE formed by Cho2p (Kodaki and Yamashita 1987) and present in the cell might be converted to PC. The hybrid protein in peroxisomes rescued the *opi3*Δ defect and formed PC *in vivo* and *in vitro*. Interestingly, PC

synthesized in peroxisomes of this “reporter strain” was also readily transported to other organelles indicating efficient export of this phospholipid from its artificial site of formation to target membranes.

Data obtained in this study indicate that PE formed by decarboxylation of PS in mitochondria (Psd1p) or Golgi (Psd2p) or small amounts of monomethyl-PE eventually formed in the ER (Cho2p) are efficiently translocated to peroxisomes. Assays with permeabilized yeast cells enabled us to test some very basic properties of this translocation system. Depletion of ATP in the permeabilized cell system decreased the translocation/conversion efficiency, but did not stop it completely. Thus, some energy requirement in the form of ATP can be postulated. Interestingly, removal of the cytosol from the permeabilized cell system rather increased the translocation/conversion efficiency of PE to peroxisomes. Consequently, it is very unlikely that soluble proteins present in the cytosol contribute to this process. Finally, treatment of cells with nocodazol, a reagent compromising the cytosol, did not have an effect of PE transport to mitochondria. Although this result has to be interpreted with caution it may be a hint that PE transport governed by vesicle flux is not relevant for this process. These results leave us with membrane contact or other unidentified transport mechanisms as lipid translocation routes to peroxisomes. We have to be aware, however, that more than one mechanism may be relevant for this process.

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Conclusive remarks

The lipid composition of biological membranes is very important for the cell. Lipids of biomembranes are not only a physical barrier, but also play an interactive role with other membrane components, especially proteins. Phospholipids are major components of biological membranes. PC and PE, but also other phospholipids contribute to the bilayer/curvature structure of membranes, interact with proteins or shelter other smaller lipid species such as cholesterol (Nussio et al. 2008; Pink et al. 1998; Pitman et al. 2005).

PC is important for bilayer arrangement and can also protect smaller lipid molecules due its big head group. PE and cardiolipin (CL), a phospholipid specific for mitochondria, interact mainly with proteins (Osman et al. 2011, 2009a; Gohil et al. 2005; Acehan et al. 2011; Schlame 2008) and help to assemble them properly into the membrane. Both lipids induce elasticity of the biomembrane (Chernomordik and Kozlov 2005; Chernomordik et al. 2006; Kozlov et al. 2010). The correct ratio of lipid classes is therefore not only important for membrane stability, but also for interaction with proteins and for contact with other membranes (Ipsen et al. 1987; Róg et al. 2009; Shamitko-Klingensmith et al. 2012; Pan et al. 2008; Wennberg et al. 2012). One phospholipid class alone would not be able to provide the necessary membrane properties, which are required for membrane function and communication. This aspect becomes even more important when membranes from different organelles are studied.

Since many of the organelles are not able to produce phospholipids themselves, or do so only in part (Trotter and Voelker 1995; Birner et al. 2001), transport of lipids and communication between organelles become very important (Kornmann et al. 2009; Osman et al. 2009b; Bohnert et al. 2012; Potting et al. 2010). Lipid transport from sites of synthesis to destinations is an essential process for the existence of the respective target membrane, but in many cases also for the survival of the whole cell. Phospholipids are needed for the expansion of membranes, growth, fission and cellular biogenesis (Schrader et al. 2012; Koch et al. 2005; Waterham et al. 2007; Kornmann et al. 2011).

The exact mechanism how interorganelle lipid transport is achieved is still not known. Many studies about direct membrane contact between organelles have provided new insight into transport of lipids from one organelle to another (Achleitner et al. 1999, 1995; Choi et al. 2005; Voelker 1991; Kornmann et al. 2009; Raychaudhuri and Prinz 2008; Friedman et al. 2010). Recent investigations also addressed intra-organelle lipid transport in mitochondria,

where lipid carrier proteins regulate the concentration of phospholipids (PE, PA, CL) and are regulated themselves by a feedback mechanism (Connerth et al. 2012; Potting et al. 2010; Tamura et al. 2012, 2009).

Little is known about the peroxisomal lipid composition, especially for *S. cerevisiae*. One study from our laboratory addressed PE supply to peroxisomes (Rosenberger et al. 2009). PC supply to the peroxisomes of the yeast is presented in this study. PC and PE supplied to peroxisomes originates at least from two different pathways similar to phospholipid transport to mitochondria (Bürgermeister et al. 2004; Nebauer et al. 2007; Birner et al. 2003; Birner and Daum 2003). PE is supplied to peroxisomes from three different pathways with comparable efficiency (Rosenberger et al. 2009). PC transported to peroxisomes is also derived from two different pathways, namely (i) the methylation pathway catalyzed by Cho2p/Pem1p and Opi3p/Pem2p and (ii) the Kennedy pathway. It has to be noted that transport through these two routes occurs with different efficiency. As shown in this Thesis it seems that in the absence of the methylation pathway the Kennedy pathway of the yeast *S. cerevisiae* is up-regulated. In animal cells, this pathway is the main supplier of PC (Cole et al. 2012; Li and Vance 2008; Lim et al. 1986). The methylation pathway also supplies PC to peroxisomes but at a lower rate than the Kennedy pathway. Although in the literature no evidence supporting our assumption has been presented, we propose that despite PC deficiency in other organelles of the respective mutants under peroxisome inducing conditions much of the available PC is supplied to peroxisomes. It also appears that PC and PE transport in mutants compromised in PC synthesis to peroxisomes are connected, because the PC level is often paralleled by the PE level. Furthermore, disturbance in the PC biosynthetic pathways seems to have an influence not only on the concentration of other phospholipids, but also on the whole lipid network in the baker's yeast. Similar effects have been also observed for disturbance in PE synthesis (Horvath et al. 2011; Gaspar et al. 2011; Choi 2003). It seems that not only DAG levels are affected in the respective PC mutants but also ergosterol levels. These effects demonstrate the connection of lipid biosynthetic pathways within the lipid metabolic network.

Phospholipid biosynthesis and transport in the cell are strictly regulated processes (Athenstaedt and Daum 1999; Carman and Henry 1999; Bae-Lee and Carman 1984). The main sites of phospholipid synthesis are the ER and mitochondria (Zinser et al. 1991; Daum and Vance 1997; Leber et al. 1994), although also the Golgi apparatus seems to produce PE for other organelles especially when mitochondria are unable to provide this lipid class (Trotter and Voelker 1995; Trotter et al. 1995). The exact mechanism how phospholipids

reach their destination and are transported inside the cell is still unknown. The best evidence has been presented for the intra-mitochondrial transport of phospholipids and the mitochondria-ER connection (Connerth et al. 2012; Kornmann et al. 2009; Zerbes et al. 2012; Potting et al. 2010; Nashiro et al. 2011; Tamura et al. 2012). Another strongly investigated transport route is the secretory pathway via vesicle flux from the ER to the Golgi apparatus and to the plasma membrane. This transport route is well established for proteins, but the question as to its contribution to lipid transport, eventually together with proteins, remains open (Kohlwein et al. 1996; Lee et al. 2004; Cole et al. 2012; Kobayashi and Arakawa 1991). Vesicular transport to peroxisomes has been discussed, but supply of phospholipids *via* such a mechanism is still controversial (Titorenko et al. 1996; Raychaudhuri and Prinz 2008; Titorenko et al. 2000). For the primary supply of lipids to peroxisomes a growth and division model is largely accepted (Nagotu et al. 2010) where peroxisomes are formed form the ER. As also fission of peroxisomes is observed (Koch et al. 2003; Koch and Brocard 2012; Koch et al. 2005; Schrader et al. 2012) the question evolved where phospholipids for the peroxisomal membrane come from under these conditions. A mechanism of lipid transport, especially for organelle membranes in close proximity to each other, involves the action of transport proteins (Levine 2011; Holthuis and Levine 2005; Levine and Loewen 2006; Wirtz 1991, 2006; Wirtz et al. 2006). The question remains how the close proximity of 10 to 40 nm between organelles is established since such distances between organelles were visualized before (Friedman et al. 2010, 2011; Csordás et al. 2006). Protein complexes identified recently may contribute to organelle contact (Kornmann et al. 2009, 2011).

The present Thesis supports the idea that peroxisomes like mitochondria (Vance 1990) or the plasma membrane (Pichler et al. 2001) may have associated membranes which supply them with lipids. Attempts to isolate a peroxisomal associated membrane (PXAM) failed so far. However, contamination of peroxisomes during subcellular fractionation with the ER and mitochondria may indicate that such attachments did not occur by chance but by purpose. This finding could be an indication that peroxisomes indeed have close contact with the two other organelles. Recent studies also demonstrated that peroxisomes and mitochondria have more similarities than expected and that some kind of (vesicle) flux between these two organelles may exist (Neuspiel et al. 2008; Braschi et al. 2010; Schumann and Subramani 2008; Rucktäschel et al. 2010; Schrader and Yoon 2007). Also experiments with permeabilized cells and the Opi3p-hybrid protein as an indicator for PE transport to peroxisomes as presented in this Thesis support the view of close contact with other organelles. *Semi in vivo* studies with permeabilized cells showed that the transport of PE from

the sites of synthesis to peroxisomes is independent of cytosolic factors, but seems to require some energy in the form of ATP. PE was successfully converted to PC in the peroxisomes indicating that the hybrid methyltransferase protein artificially directed to peroxisomes was functional. Peroxisomes seem to be a good choice for the use of such hybrid proteins, since they can be strictly targeted and imported in a folded status. For studying peroxisomal contact with other organelles optical and molecular biological methods might be considered in future experiments (Andersson et al. 2007; Kornmann et al. 2009).

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Language skills:
Slovenian (native speaker)
English (fluent spoken and written)
German (fluent spoken and written)
Croatian (excellent)
Serbian (excellent)

Academic degrees

2009	M.Sc. Molecular Microbiology, passed with distinction
2006	B.Sc. Molecular Biology
2000	Matura (high school finishing exam), dux of the school

Education

10/2013 (expected)	Ph.D., Biochemistry, Technical University (TU) of Graz, Austria
08/2009 - 10/2013	DK Molecular Enzymology, NAWI Graz, Austria
2006 - 2009	Master studies, Karl-Franzens University Graz, Austria
2004 - 2006	Bachelor studies, Karl-Franzens University Graz, Austria
2000 - 2004	Diploma studies biology, Karl-Franzens University Graz, Austria
1996-2000	III. Gymnasium (high school) Maribor, Slovenia

Professional experience

General

Biochemistry and Molecular Biology

Proteins - Isolation, Separation, Immunodetection, Assays
Lipids - Extraction and Analysis
Subcellular fractionation
DNA and RNA methodology
Apoptosis and cellular aging
Analysis of blood samples

Other experience

Journalism

Coordination and realisation of projects
Individual- as team-work
Excellent communication skills and social interaction

Chronological

08/2009-04/2013	University project assistant at Institute of Biochemistry TU Graz Research in Cell Biology - Metabolism and transport mechanisms of phospholipids; organelle interaction and association (fractionation of peroxisomes, mitochondria and endoplasmic reticulum) Lipid isolation and analysis of aminoglycerophospholipids and neutral lipids Enzymatic activity assays (semi *in vivo* and *in vitro*) Teaching assistant: Laboratory of Immunological Methods - Laboratory supervision and teaching (SDS-PAGE, Western Blot, ELISA) Supervision of international Ph.D. students
05/2009-06/2009	Junior Researcher, Angewandte Biokatalyse Kompetenzzentrum GmbH (ACIB Graz- Austrian centre of industrial biotechnology) Establishing technologies and methods for expression cassette assembly for *Pichia Pastoris* transformation (primer design, overlap PCR, cloning, sequencing, transfomation)
09/2007-04/2009	Student assistant at Institute for Molecular Biosciences Graz - supervision of molecular biology laboratory exercises during master's degree time (Maturation of the 60S ribosomal subunit): Molecular biology II (01/2009) Infection biology (05/2008) Yeast genetics and molecular biology (11/2007) and other laboratory exercises (12/2008, 11/2008) Isolation and detection of DNA and RNA (Gel electrophoresis, Soutern Blot, Northern Blot) Cloning and expression of yeast and *E. coli* genes (PCR, restriction digestion, ligation ,transformation) Work with proteins; purification, SDS-PAGE, Western Blot, fluorescence microscopy
02/2007-03/2007	Project laboratory: Apoptosis and cellular aging; cloning, expression and observing of mice transgene *S. cerevisiae* mutants
02/2006	Laboratory assistant at Laboratory of Immunology, University Medical Centre Maribor, Slovenia Blood sample analysis for viral marker proteins (Hepatitis Virus, HIV)
2000-2004	Journalist at regional Radio-TV center Maribor, Slovenia, part-time work, coordination and realisation of radio events and radio programme, (RDZ Event, Radijska delavnica znancev) public surveillances, interviews, broadcasts

Computer skills

Windows and Mac OS: MS Office (Word, Power Point, Excel)

Graphic programmes: Adobe Photoshop, Corel Draw

Programmes of molecular biological interest:
pDraw, VectorNTI, NCBI databases,
Saccharomyces Genome Database

Publications and Presentations

Flis V.V. and Daum G.
Lipid Transport between the Endoplasmic Reticulum and Mitochondria
Perspectives on The Endoplasmic Reticulum, *Cold Spring Harb Perspect Biol, 2012, doi: 10.1101/cshperspect.a013235*

09/2012 — 4th ÖGMBT ANNUAL MEETING, Graz, Austria, Poster:
Phosphatidylcholine traffic in the yeast *Saccharomyces cerevisiae*

09/2012 — 10th Euro Fed Lipid Congress, Cracow, Poland, Poster:
Phosphatidylcholine traffic in the yeast *Saccharomyces cerevisiae*

05/2012 — Graduate Seminar 2012 DK Molecular Enzymology,
Graz, Austria, Presentation:
Phosphatidylcholine traffic in the yeast *Saccharomyces cerevisiae*

06/2011 — FEBS Workshop Biomembrane Dynamics:
From Molecule to Cell; Cargèse, Corsica, France
FEBS Fellowship Grant

09/2010 — 51st Inernational Conference on the Bioscience of Lipids (ICBL),
Bilbao, Spain. Poster: *Phospholipid Traffic to Peroxisomal Membranes- OPI3p as Reporter Enzyme*

05/2010 — FEBS Workshop Microbial Lipids, Vienna, Austria, Poster:
Phospholipid Traffic to Peroxisomal Membranes- OPI3p as Reporter Enzyme

04/2010 — Graduate Seminar 2010 DK, Molecular Enzymology,
Loipersdorf, Austria, Presentation:
Phospholipid Traffic to Peroxisomal Membranes-OPI3p as Reporter Enzyme

04/2009 — DK Hearing 2009, Ph.D. DK programme selection, Presentation

08/2008 — RNA 2008 Congress, Berlin, Germany, Poster: *Cytoplasmic Recycling of 60S pre- Ribosomal Factors Depends on the AAA- Protein Drg1*

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Graz, 20.07.2013