Mag. Martina Gsell

Phosphatidylethanolamine, a Key Lipid in Mitochondria

DISSERTATION

zur Erlangung des akademischen Grades

einer Doktorin der Naturwissenschaften

erreicht an der

Technischen Universität Graz

Betreuer: Prof. Dipl. Ing. Dr. Günther Daum

Institut für Biochemie

Technische Universität Graz

March, 2013

Deutsche Fassung:
Beschluss der Curricula-Kommission für Bachelor-, Master- und Diplomstudien vom 10.11.2008
Genehmigung des Senates am 1.12.2008

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am ..... ……..
(Unterschrift)

Englische Fassung:

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

..... ……..
date (signature)

Danksagung

Mein besonderer Dank gilt Günther Daum für die engagierte Betreuung und Unterstützung während meiner Dissertation.

Meinen ArbeitskollegInnen ein großes Danke für die kollegiale und hilfreiche Unterstützung und die vielen lustigen Momente im Labor. Vielen Dank an Gerald, der mir während seiner Diplomarbeit tatkräftig zur Seite stand.

Ganz besonders möchte ich mich auch bei meiner Familie und meinen Freunden bedanken, dass sie mich in dieser Zeit stets unterstützt haben.

Table of Contents

Abstract

The most abundant phospholipids in eukaryotic membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). PE is essential for many types of cells. In the yeast *Saccharomyces cerevisiae* PE synthesis is accomplished by a network of reactions which comprise four different pathways. The enzyme which contributes most to PE formation in the yeast is phosphatidylserine decarboxylase 1 (Psd1p). This enzyme is localized to the inner mitochondrial membrane and catalyzes the decarboxylation of PS to PE. To obtain a general view of the role of PE in the cell and to study the effects of an unbalanced PE level, a Δ*psd1* deletion mutant was subjected to DNA microarray analysis. This approach demonstrated that a number of genes were up-regulated in a Δ*psd1* deletion strain compared to wild type. To study possible physiological functions of these specific gene products, we analyzed the growth phenotype on different carbon sources and the phospholipid profile of the respective mutant strains. This approach identified the products of the three candidates *GPM2*, *GPH1* and *RSB1*, as possible interaction partners with PE formation. *GPH1*, which was investigated in more detail, showed effects in phospholipid as well as in neutral lipid metabolism. Gph1p has originally been identified as a glycogen phosphorylase catalyzing degradation of glycogen to glucose in the stationary growth phase of the yeast. Here, we show that deletion of this gene also causes decreased levels of PC, triacylglycerols and steryl ester. Surprisingly, depletion of the two non-polar lipids in a Δ*gph1* strain leads to a lack of lipid droplets. Moreover, the stability of the plasma membrane appears to be compromised in this mutant. *In vivo* labeling experiments revealed that both pathways of PC biosynthesis, the CDP-choline and the methylation route, are negatively affected by a Δ*gph1* mutation. These findings suggest that Gph1p plays a regulatory role in yeast lipid metabolism, although its specific molecular function is currently not known.

Zusammenfassung

Die am häufigsten vorkommenden Phospholipide in eukaryotischen Membranen sind Phosphatiylcholin (PC), Phosphatidylethanolamin (PE), Phosphatidylinositol (PI) und Phosphatidylserin (PS). PE ist für viele Arten von Zellen essentiell. In der Hefe *Saccharomyces cerevisiae* wird die Synthese von PE durch ein Netzwerk von Reaktionen, welches vier verschiedene Wege umfasst, bewerkstelligt. Das Enzym, das in Hefe am meisten zur Bildung von PE beitragt ist Phosphatidylserin Decarboxylase 1 (Psd1p). Dieses Enzym ist an der inneren Mitochondrienmembran lokalisiert und katalysiert die Decarboxylierung von PS zur Bildung von PE. Um die Rolle von PE in der Zelle und die Auswirkungen eines erniedrigten PE-Levels genauer zu untersuchen, wurde eine Δ*psd1* Deletionsmutante einer DNA-Microarray-Analyse unterzogen. Dieser Ansatz zeigte, dass eine Reihe von Genen in einem Δ*psd1* Deletionsstamm im Vergleich zum Wildtyp hochreguliert waren. Um die physiologischen Funktionen dieser bestimmten Genprodukte zu analysieren, wurden Wachstumsphänotyp auf verschiedenen Kohlenstoffquellen und das Phospholipid Muster der jeweiligen Mutantenstämme untersucht. Dieser Ansatz identifizierte *GPM2, GPH1* und *RSB1* als mögliche Interaktionspartner der Synthese von PE. *GPH1*, welches näher untersucht wurde, zeigte Änderungen im Phospholipidmuster sowie im Stoffwechsel der Neutrallipide. Gph1p ist als Glycogenphosphorylase bekannt und katalysiert den Abbau von Glykogen in Glukose in der stationären Wachstumsphase der Hefe. Hier zeigen wir, dass die Deletion dieses Gens auch eine Reduktion von PC, Triglyceriden und Sterolester verursacht. Überraschenderweise führt diese Reduktion der beiden nicht-polaren Lipide in einer Δ*gph1* Mutante zu einer Reduktion an Lipidpartikel. Darüber hinaus scheint die Stabilität der Plasmamembran in dieser Mutante beeinträchtigt zu sein. *In vivo* Markierungsexperimente

zeigten, dass beide Wege der PC-Biosynthese, der CDP-Cholin und der Methylierungs Pathway negativ durch eine Δ*gph1* Mutation betroffen sind. Diese Erkenntnisse legen nahe, dass Gph1p eine regulatorische Rolle im Lipidmetabolismus der Hefe spielt, obwohl die spezifische, molekulare Funktion bis jetzt nicht geklärt ist.

General Introduction

Saccharomyces cerevisiae is a widely used model organism to study biochemistry, cell biology and molecular biology of eukaryotic cells. Although it is phylogenetically distant from human and mammalian cells, it is an appropriate model because many biosynthetic and regulatory elements are highly conserved between yeast and human. Additionally, its genetic tractability and increasing wealth of accessible data makes *Saccharomyces cerevisiae* the model system of choice for studies in of many biological fields.

In our lab, we use *Saccharomyces cerevisiae* to study lipid metabolism. Lipid metabolism is a complex network in all types of cells. Figure 1 gives an overview of the most important lipid biosynthetic pathways and the contribution of individual enzymes in yeast. Lipids fulfill three major functions. First, they are used as source of energy mostly in the form of triacylglycerols (TG) and steryl esters (SE) which accumulate in lipid droplets under normal conditions [1]. Secondly, the matrix of cellular membranes is formed by polar lipids, especially phospholipids, sphingolipids and sterols. Lipids are specifically distributed among organelles where they contribute to certain cellular processes such as protein sorting or traffic in the secretory pathway. Finally, lipids can act as cellular messengers. The latter function may be accomplished by lipids such as diacylglycerols (DG), polyphosphoinositides and sphingolipids [2].

In Figure 1 the close connection between phospholipid metabolism, neutral lipid biosynthesis and neutral lipid degradation is shown. Additionally to the enzymes, which catalyze the synthesis or degradation of lipids, a sophisticated regulatory machinery is involved in lipid metabolism.

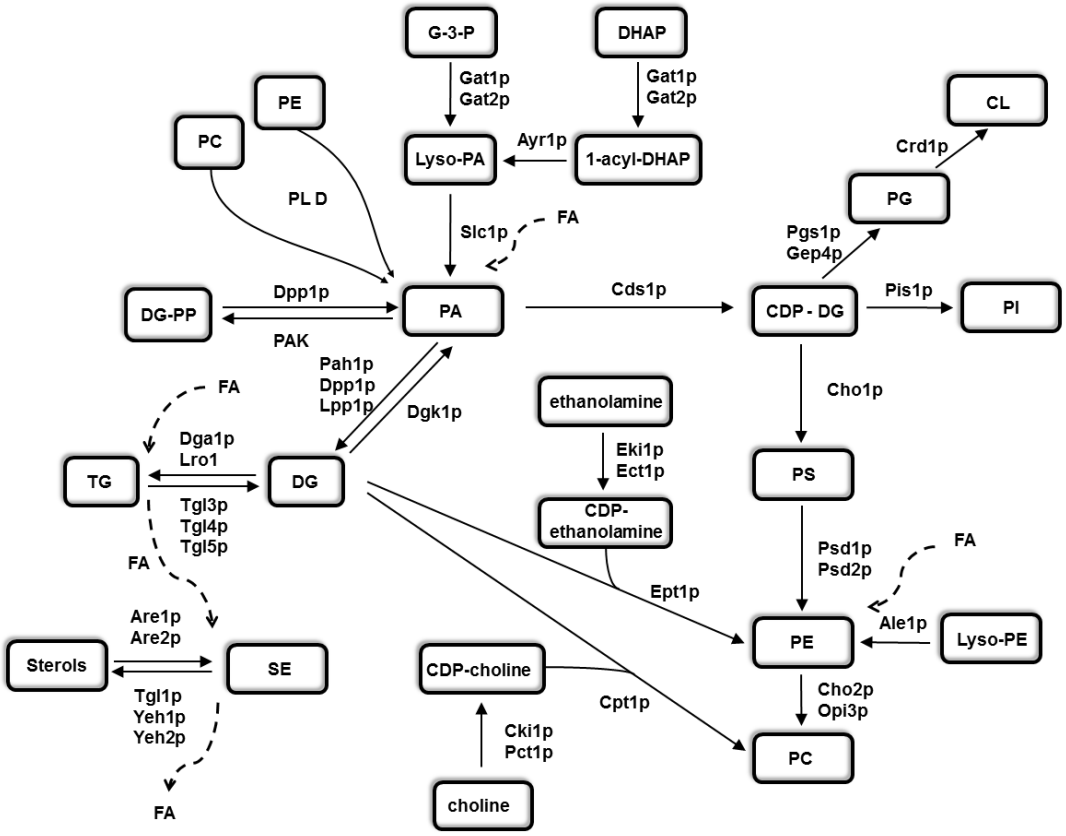

Figure 1: Lipid synthesis in *S. cerevisiae*. The figure shows a simplified overview of lipid synthesis pathways with enzymes involved. Abbreviations: CDP-DG, CDP-diacylglycerol; CL, cardiolipin; DHAP, dihydroxyacetone phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triacylglycerol; DG, diacylglycerol; SE, steryl esters; FA, fatty acid.

De novo, all membrane yeast phospholipids are synthesized from phosphatidic acid (PA), which is also a central metabolite in the synthesis of TG [1]. The *PAH1*-encoded PA phosphatase provides DG, which is acylated to TG by the *DGA1*- and *LRO1*- encoded acyl-CoA-dependent and phospholipid-dependent diacylglycerol acyltransferases, respectively [1,2,3,4].

Enzymes synthesizing and metabolizing lipids and their precursors are present in the cytosol and membranous organelles, such as the endoplasmic reticulum, mitochondria, and

lipid droplets [5]. A complex transport machinery from one to the other organelle is required to keep the lipid composition of each organelle balanced. A prominent example is the plasma membrane which lacks lipid synthesizing enzymes. Thus, all lipids have to be transported to the plasma membrane to maintain its physiological role [5,6].

The expression of lipid enzymes is controlled by a variety of conditions including growth stage, temperature, pH and the availability of nutrients such as carbon, nitrogen, phosphate, zinc, and lipid precursors. Therefore, the lipid composition can be affected by all these factors. Mostly, the regulation occurs at the transcriptional level [7].

The main focus of the present Thesis was on phospholipid metabolism. Phospholipids are the main structural element of biological membranes. They consist of a glycerol backbone which is esterified with fatty acids in the *sn*-1 and *sn*-2 positions. Fatty acids of the diacylglycerol (DG) moiety represent the hydrophobic part of phospholipids [8,9]. The major phospholipids of eukaryotes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) [9], are found at variable amounts in all yeast subcellular membranes. Other lipids such as lyso-phospholipids (LPL), PA, cardiolipin (CL) and phosphatidylglycerol (PG) are only minor components in total cell extracts but may accumulate in certain subcellular compartments or domains [10,11,12,13,14,15].

In this study, we were most interested in PE, which is an abundant membrane phospholipid of many organisms. Similar to other phospholipids, PE contributes to various functions and the integrity of membranes. PE plays a unique role due to its biophysical properties as a non-bilayer (hexagonal phase) forming lipid [16]. Therefore, PE is involved in fusion and fission events of membranes [17]. Furthermore, PE acts as a precursor of glycosylphosphatidylinositol (GPI) anchors [18] and anandamide [*N*-arachidonoylethanolamine] [19]. Moreover, PE is involved in vacuolar protein delivery [20], the activity of various enzymes [21] and possibly in cell signaling [15,22].

The biosynthesis of PE comprises a complex network of reactions distributed among different organelles in the cell. In *Saccharomyces cerevisiae,* four pathways contribute to PE biosynthesis, namely (i) decarboxylation of PS catalyzed by phosphatidylserine decarboxylase 1 (Psd1p) in the inner mitochondrial membrane [23,24,25]; (ii) decarboxylation of PS by Psd2p in a Golgi/vacuolar compartment [26]; (iii) incorporation of ethanolamine through the CDP-ethanolamine branch of the Kennedy pathway [27] in the endoplasmic reticulum [8,28]; and (iv) synthesis of PE through acylation of lyso-PE catalyzed by the acyl-CoA-dependent acyltransferase Ale1p in the mitochondria-associated membrane (MAM) [29,30]. These four pathways form PE with different efficiency [10]. Psd1p is the major supplier of cellular and mitochondrial PE and represents the major cellular *PSD* activity [26]. Inactivation or deletion of the *PSD1* gene leads to a considerable decrease of PE in total cellular and mitochondrial membranes [14,31]. This finding suggests that marked amounts of PE found in all cellular membranes must be derived from mitochondria. However, mechanisms governing PE distribution within the cell are not well understood.

In chapter 1 of this Thesis a general strategy to investigate phospholipid biogenesis pathways by using yeast deletion mutants is described. The use of single and multiple mutants constructed by genetic manipulation which are compromised in individual enzymatic steps or certain metabolic pathways is discussed. We describe selected cases of yeast research on phospholipid metabolism with emphasis on our own work dealing with investigations of PE synthesis. Such studies start with the selection and construction of appropriate mutants and lead to phenotype analysis, lipid profiling, enzymatic analysis and *in vivo* experiments. Comparison of wild type and mutant strains allows us to understand the role of gene products and metabolic processes in some detail. Such studies are valuable contributions to our

knowledge of the complex network of lipid metabolism, but also help us to study effects of lipids on structure and function of cellular membranes.

The second chapter deals with global effects of PE depletion on the gene expression pattern in *Saccharomyces cerevisiae*. To study the genome wide effect of an unbalanced cellular and mitochondrial PE level and in particular the contribution of Psd1p to this depletion we performed a DNA microarray analysis with a Δ*psd1* deletion mutant. This approach revealed that 54 yeast genes were significantly up-regulated in the absence of *PSD1* compared to wild type. Deletion mutants bearing defects in these 54 candidate genes were further analyzed for their growth phenotype and phospholipid profile to investigate a possible link of these mutations to PE deficiency and *PSD1* deletion.

Analysis of deletion mutants compromised in these up-regulated genes identified *GPH1* as a contributor to lipid metabolism. In chapter 3 we focus on Gph1p (glycogen phosphorylase 1), which is known to catalyze the release of glucose 1-phosphate from glycogen in the late stationary growth phase of the yeast to maintain the required energy for cell activity and growth during periods of nutrient starvation. The activity of glycogen phosphorylase is regulated by cyclic AMP-mediated phosphorylation of the enzyme. *GPH1* is not essential in yeast. Δ*gph1* mutants lack phosphorylase activity and attain higher levels of intracellular glycogen [32]. Our findings suggest another role of Gph1p in yeast lipid metabolism, although its specific molecular function remained unclear. This study demonstrates an unexpected link between carbohydrate and lipid metabolism. The dual role of Gph1p may be another example for a player in the complex metabolic network of eukaryotic cells.

References

[1] S.D. Kohlwein, Triacylglycerol homeostasis: insights from yeast, J Biol Chem 285 (2000) 15663-15667.

[2] P. Oelkers, A. Tinkelenberg, N. Erdeniz, D. Cromley, J.T. Billheimer, S.L. Sturley, A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast, J Biol Chem 275 (2000) 15609-15612.

[3] P. Oelkers, D. Cromley, M. Padamsee, J.T. Billheimer, S.L. Sturley, The DGA1 gene determines a second triglyceride synthetic pathway in yeast, J Biol Chem 277 (2002) 8877-8881.

[4] D. Sorger, G. Daum, Synthesis of triacylglycerols by the acyl-coenzyme A:diacyl-glycerol acyltransferase Dga1p in lipid particles of the yeast Saccharomyces cerevisiae, J. Bacteriol 184 (2002) 519-524.

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

[6] H. Pichler, B. Gaigg, C. Hrastnik, G. Achleitner, S.D. Kohlwein, G. Zellnig, A. Perktold, G. Daum, A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids, Eur. J. Biochem. 268 (2001) 2351-2361.

[7] G.M. Carman, G.S. Han, Regulation of phospholipid synthesis in the yeast Saccharomyces cerevisiae, Annu Rev Biochem 80 (2011) 859-883.

[8] G. Daum, N.D. Lees, M. Bard, R. Dickson, Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae,* Yeast 14 (1998) 1471-1510.

[9] G.v. Meer, D.R. Voelker, G.W. Feigensonvan, Membrane lipids: where they are and how they behave, Nat Rev Mol Cell Biol. 9 (2008) 112-124.

[10] I. Schuiki, M. Schnabl, T. Czabany, C. Hrastnik, G. Daum, Phosphatidylethanolamine synthesized by four different pathways is supplied to the plasma membrane of the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1801 (2010) 480-486.

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

[12] M. Bürgermeister, R. Birner-Grünberger, R. Nebauer, G. Daum, Contribution of different pathways to the supply of phosphatidylethanolamine and phosphatidylcholine to mitochondrial membranes of the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1686 (2004) 161-168.

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

[14] R. Nebauer, I. Schuiki, B. Kulterer, Z. Trajanoski, G. Daum, The phosphatidylethanolamine level of yeast mitochondria is affected by the mitochondrial components Oxa1p and Yme1p, FEBS J. 274 (2007) 6180-6190.

[15] M.K. Storey, K.L. Clay, T. Kutateladze, R.C. Murphy, M. Overduin, D.R. Voelker, Phosphatidylethanolamine has an essential role in *Saccharomyces cerevisiae* that is independent of its ability to form hexagonal phase structures, J. Biol. Chem. 276 (2001) 48539-48548.

[16] S.W. Hui, T.P. Stewart, P.L. Yeagle, A.D. Albert, Bilayer to non-bilayer transition in mixtures of phosphatidylethanolamine and phosphatidylcholine: implications for membrane properties, Arch. Biochem. Biophys. 207 (1981) 227-240.

[17] P.R. Cullis, M.J. Hope, C.P. Tilcock, Lipid polymorphism and the roles of lipids in membranes, Chem Phys Lipids 40 (1986) 127-144.

[18] A.K. Menon, V.L. Stevens, Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein, J Biol Chem 267 (1992) 15277-15280.

[19] X.H. Jin, Y. Okamoto, J. Morishita, K. Tsuboi, T. Tonai, N. Ueda, Discovery and characterization of a Ca2+-independent phosphatidylethanolamine N-acyltransferase generating the anandamide precursor and its congeners, J Biol Chem 282 (2007) 3614-3623.

[20] W.P. Huang, D.J. Klionsky, Autophagy in yeast: a review of the molecular machinery, Cell Struct Funct 27 (2002) 409-420.

[21] C. Lange, J.H. Nett, B.L. Trumpower, C. Hunte, Specific roles of protein-phospholipid interactions in the yeast cytochrome bc1 complex structure, EMBO J 20 (2001) 6591-6600.

[22] K.D. Chapman, Emerging physiological roles for N-acylphosphatidylethanolamine metabolism in plants: signal transduction and membrane protection, Chem Phys Lipids 108 (2000) 221-229.

[23] M.A. Carson, M. Emala, P. Hogsten, C.J. Waechter, Coordinate regulation of phosphatidylserine decarboxylase activity and phospholipid N-methylation in yeast, J Biol Chem 259 (1984) 6267-6273.

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

[25] K. Kuchler, G. Daum, F. Paltauf, Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in *Saccharomyces cerevisiae*, J. Bacteriol. 165 (1986) 901-910.

[26] P.J. Trotter, D.R. Voelker, Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (*PSD2*) in the yeast *Saccharomyces cerevisiae*, J. Biol. Chem. 270 (1995) 6062-6070.

[27] E.P. Kennedy, S.B. Weiss, The function of cytidine coenzymes in the biosynthesis of phospholipids, J. Biol. Chem. 222 (1956) 193-214.

[28] R. Birner, G. Daum, Biogenesis and cellular dynamics of aminoglycerophospholipids, Int. Rev. Cytol. 225 (2003) 273-323.

[29] W.R. Riekhof, J. Wu, J.L. Jones, D.R. Voelker, Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in *Saccharomyces cerevisiae*, J. Biol. Chem. 282 (2007) 28344-28352.

[30] W.R. Riekhof, D.R. Voelker, Uptake and utilization of lyso-phosphatidylethanolamine by *Saccharomyces cerevisiae*, J. Biol. Chem. 281 (2006) 36588-36596.

[31] R. Birner, M. Bürgermeister, R. Schneiter, G. Daum, Roles of phosphatidylethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*, Mol. Biol. Cell 12 (2001) 997-1007.

[32] P.K. Hwang, S. Tugendreich, R.J. Fletterick, Molecular analysis of *GPH1*, the gene encoding glycogen phosphorylase in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 9 (1989) 1659-1666.

Chapter 1

Analysis of membrane lipid biogenesis pathways using yeast genetics

Martina Gsell and Günther Daum*

Institute of Biochemistry, Graz University of Technology,

Petersgasse 12/2, 8010 Graz, Austria

*Corresponding author:
Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria
e-mail: guenther.daum@tugraz.at; Tel.: +43 316 873 6462; Fax.: +43 316 873 6952

Key Words: Lipids, phospholipids, mutants, enzyme, yeast

Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine;

PE, phosphatidylethanolamine; DMPE, dimethylethanolamine; TAG, triacylglycerol

Methods in Molecular Biology – Volume on Membrane Biogenesis

2013 IN PRESS

Abstract

The yeast *Saccharomyces cerevisiae* has become a valuable eukaryotic model organism to study biochemical and cellular processes at a molecular basis. A common strategy for such studies is the use of single and multiple mutants constructed by genetic manipulation which are compromised in individual enzymatic steps or certain metabolic pathways. Here, we describe selected examples of yeast research on phospholipid metabolism with emphasis on our own work dealing with investigations of phosphatidylethanolamine synthesis. Such studies start with the selection and construction of appropriate mutants and lead to phenotype analysis, lipid profiling, enzymatic analysis and *in vivo* experiments. Comparing results obtained with wild type and mutant strains allows us to understand the role of gene products and metabolic processes in more detail. Such studies are valuable not only for contributing to our knowledge of the complex network of lipid metabolism, but also of effects of lipids on structure and function of cellular membranes.

1. Introduction

Lipids are a versatile class of biomolecules. Variations in aliphatic chain composition but also in head groups of polar lipids result in the existence of more than 1,000 different lipid species in any eukaryotic cell [1]. This large variation is accomplished by a relatively small set of enzymes because cells use only ~5% of their genes and gene products to synthesize all these lipids [2]. It is well known that lipids fulfill three major functions. First, they are used as stores of energy mostly in the form of triacylglycerols (TAG) which accumulate in lipid droplets under normal conditions [3]. Secondly, the matrix of cellular membranes is formed by polar lipids, especially glycerophospholipids, sphingolipids and sterols. Lipids are specifically distributed among organelles where they contribute to certain cellular processes such as protein sorting or traffic in the secretory pathway. Finally, lipids can act as cellular messengers. The latter function may be accomplished by lipids such as diacylglycerols, polyphosphoinositides and sphingolipids.

In this report, we focus on the biosynthesis of glycerophospholipids, which are major structural lipids in eukaryotic membranes, and on use of the yeast, *S. cerevisiae*, as a valuable experimental system to study this process. The tractable genetics of *S. cerevisiae* has allowed identification and characterization of many structural and regulatory genes involved in synthesis and metabolic conversion of phospholipids [4,5]. Phospholipid synthesis in yeast is governed by a network of reactions which are subject to strict regulation by genetic and biochemical mechanisms. Moreover, distinct spatial organization within the cell plays an important role in the coordinated process of lipid synthesis. Not surprisingly, phospholipid synthesis is also linked to the metabolism of other major lipid classes including fatty acids, triacylglycerols, sterols and sphingolipids [6]. It has also to be noted that phospholipid synthesis in yeast is affected by growth conditions which influence the expression of enzymes

and/or modulate their catalytic activities. As examples, expression of phospholipid biosynthetic genes in yeast is controlled by carbon sources, nutrient availability, growth phase, pH and temperature. Finally, posttranslational modifications of gene products, especially phosphorylation of key proteins involved in phospholipid synthesis, affect metabolism of phospholipids and the balance between certain lipid precursors and final products of lipid biosynthetic pathways [4,7,8,9,10,11,12].

The use of *S. cerevisiae* gene deletion strains has become a standard method for biochemical, cell biological and molecular biological studies. Standard methods of gene deletion based on PCR techniques and yeast cell transformation are well established [13,14], and complete collections of yeast deletion mutants covering non-essential genes are available [15]. Here, we describe the use of such mutants to study yeast phospholipid metabolism with emphasis on the biosynthesis of one of the major yeast phospholipids, phosphatidylethanolamine (PE). All methods presented here are easy to use and can be applied with minor modifications to mutants that affect other aspects of phospholipid metabolism.

Figure 1 gives an overview of phospholipid biosynthetic pathways and the contribution of individual enzymes in yeast. The functions of most of these enzymes have been confirmed by analysis of mutants and/or by biochemical studies. The complex regulatory mechanisms in phospholipid biogenesis has recently been reviewed in Carman and Han [16]. Major phospholipids of the yeast are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI), which are found at variable amounts in all yeast subcellular membranes. Other lipids such as lyso-phospholipids (LPL), phosphatidic acid (PA), cardiolipin (CL) and phosphatidylglycerol (PG) are only minor components in total cell extracts but may accumulate in certain subcellular compartments or domains [17,18,19,20,21,22].

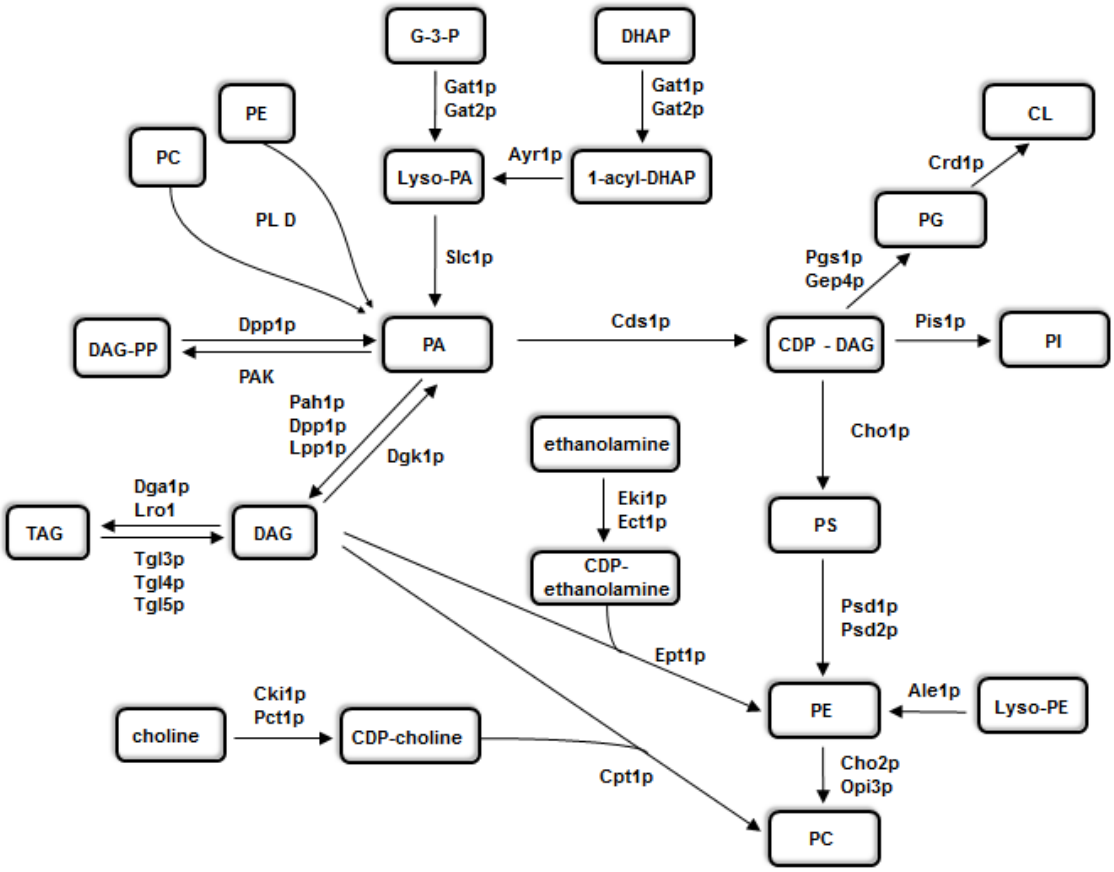

Figure 1. Phospholipid synthesis in *S. cerevisiae*. The figure shows a simplified overview of phospholipid synthesis pathways with enzymes involved. Abbreviations: CDP-DAG, CDP-diacylglycerol; CL, cardiolipin; DHAP, dihydroxyacetone phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol.

Among the phospholipids described above, PE plays specific roles because it is unique regarding its biophysical properties as a non-bilayer (hexagonal phase) forming lipid [23]. Furthermore, a complex network of reactions distributed among different organelles leads to its formation. Four pathways contribute to yeast PE biosynthesis, which are: (i) decarboxylation of PS catalyzed by phosphatidylserine decarboxylase 1 (Psd1p) in the inner mitochondrial membrane [24], (ii) decarboxylation of PS by Psd2p in a Golgi/vacuolar compartment [25], (iii) incorporation of ethanolamine through the CDP-ethanolamine branch of the Kennedy pathway [26] in the endoplasmic reticulum [27,28], and (iv) synthesis of PE

through acylation of lyso-PE catalyzed by the acyl-CoA-dependent acyltransferase Ale1p in the mitochondria-associated membrane (MAM) [29,30]. These four pathways contribute to PE synthesis with different efficiencies [17], but they compensate each other in case of mutations introduced into one or the other route of PE formation.

Here we describe standard methods commonly used for the analysis of mutants affected in PE metabolism. Description of these techniques will start with growth phenotype analysis of mutant strains and lead to lipid profiling, *in vivo* pathway analysis and measurement of enzymatic activities *in vitro*. To study the four pathways of PE synthesis described above (see Figure 1), usage of different mutants is required. Whereas *Δpsd1*, *Δpsd2* and *Δale1* single deletions are sufficient to interrupt the respective pathways, a *Δcki1Δdpl1Δeki1* triple mutant is necessary to silence the CDP-ethanolamine branch of the Kennedy pathway of PE synthesis. Reasons for this requirement are the overlapping substrate specificity of the choline and ethanolamine kinases Cki1p and Eki1p on one hand [31,32] and the fact that ethanolamine phosphate can be provided through sphingolipid degradation by the action of dihydrosphingosine phosphate lyase, Dpl1p [33,34]. Combination of the different mutations allows turning down more than one of the four possible pathways of PE synthesis. It has to be noted, however, that a minimal level of PE is essential for viability, and all PE biosynthetic pathways cannot be interrupted at the same time.

Phenotype analysis is one of the fundamental tools of genetics. In many cases, a particular phenotype or a set of phenotypic features is indicative of the function of a gene product. Usually, phenotypic analysis of a mutant strain starts with assays of growth characteristics, as shown for mutant strains bearing deletions of genes that function in the different PE biosynthesis pathways (Figure 2).

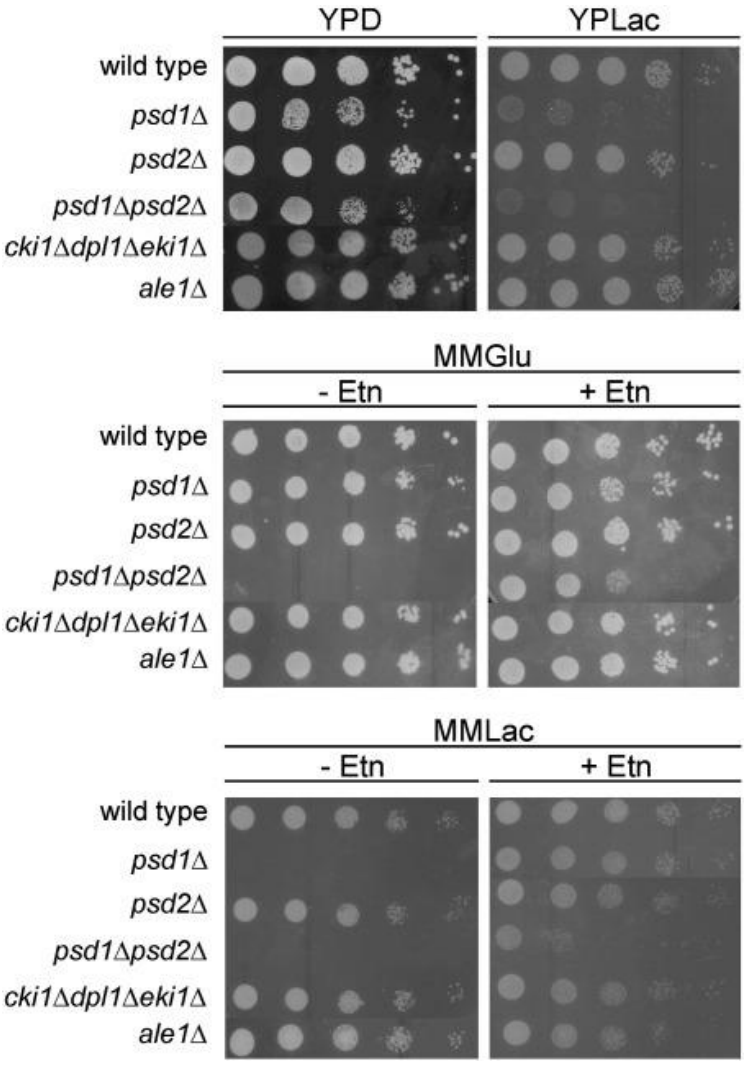

Figure 2. Growth of yeast strains bearing defects in phosphatidylethanolamine biosynthesis depends on the carbon source. Cell suspensions of strains listed in the figure were spotted on YPD, YPLac, MMGlu and MMLac with or without 5 mM ethanolamine. Incubation was carried out at 30°C. YPD, complex glucose media; YPLac, complex lactate media; MMGlu, minimal glucose media; MMLac, minimal lactate media; Etn, ethanolamine. Reproduced from ref. [35] with permission of the publisher.

As mentioned above, a minimal level of PE is essential for yeast cell viability. Mutants lacking one of the PS decarboxylases, Psd1p or Psd2p, respectively, grow like wild type as long as they are cultivated on a fermentable carbon source such as glucose. On a non-fermentable carbon source, e.g. lactate, the requirement for PE increases. Under these conditions, even the single deletion of *PSD1* leads to a growth defect. This result indicates

that Psd1p is the major supplier of PE. Enhanced proliferation of mitochondria in cells grown on non-fermentable carbon sources can be correlated with the importance of PE for cell respiration [36]. A *Δpsd1Δpsd2* double mutant is viable but becomes auxotrophic for ethanolamine or choline on glucose media [37], indicating that the other two pathways of PE formation, especially the CDP-ethanolamine pathway, can compensate for this defect. When cells are grown under conditions that demand more PE with lactate as a carbon source, rescue of *Δpsd1Δpsd2* by ethanolamine supplementation is much less effective [36]. These results also indicate that the efficiency of PE import into mitochondria is limited. Taken together, these simple but well-designed growth phenotype analyses lead to a number of conclusions related to the physiological relevance of PE biosynthetic pathways in yeast [27,35,36].

The next step to understand the role of lipid biosynthetic enzymes in cellular metabolism is lipid profiling. For our routine analysis of yeast phospholipids we use two-dimensional thin-layer chromatography and lipid phosphorus estimation as outlined in detail in Subheading 3.2.3 (Thin-Layer Chromatography and Phospholipid Quantification). In wild type homogenate, the major phospholipids are PC, PE and PI and PS (Table 1) [17]. PC accounts for approximately 50% and PE for approximately 25% of total cellular phospholipids. In *Δpsd1* and *Δpsd2*, and even more pronounced in *Δpsd1Δpsd2* deletion strains, a reduction of the cellular PE content was observed [17,18,19,20,21,22]. In contrast, the phospholipid patterns of *Δcki1Δdpl1Δeki1* (CDP-ethanolamine pathway mutant) and *Δale1* strains largely resembled wild type [17].

Table 1. Lipid composition of cell homogenate from wild type (FY1679; Euroscarf, Frankfurt, Germany) and yeast strains bearing defects in PE formation grown on YPD. It has to be noted that complex YPD medium contains small amounts of ethanolamine and choline which serve a substrates for the CDP-ethanolamine/choline pathway. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPL, lysophospholipids; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid; CL, cardiolipin. Reproduced with adaptations from ref. [17] with permission of the publisher.

	% of total phospholipids							
Strain	PC	PE	PI	PS	LPL	DMPE	PA	CL
FY1679	47.57	24.36	12.19	6.89	1.40	5.49	0.87	1.23
Δpsd1	54.32	18.62	13.24	7.65	1.17	3.58	0.66	0.75
Δpsd2	54.65	16.95	13.60	8.93	1.61	2.77	0.43	1.06
Δpsd1Δpsd2	64.28	7.46	11.74	10.69	1.43	1.31	1.63	1.48
Δcki1Δdpl1Δeki1	48.24	28.91	11.57	4.27	1.20	2.82	1.25	1.74
Δale1	49.85	22.53	11.97	6.75	2.50	3.88	1.00	1.52
Δpsd1Δpsd2Δale1	67.80	5.53	10.91	11.51	1.85	0.58	1.11	0.70

This result confirmed the dominant role of Psd1p and Psd2p in cellular PE formation and suggested that the CDP-ethanolamine pathway and acylation of lyso-PE are only of minor importance in cells harboring functional PS decarboxylases, at least under standard culture conditions (YPD, complex glucose media) [17]. Such data allow estimation of the contributions of different PE biosynthetic routes to total formation of PE in yeast under different physiological conditions.

As mentioned above, growth of yeast cells on non-fermentable carbon sources, e.g. lactate, challenges the cellular requirement of PE mainly through the enhanced proliferation of mitochondria. Under such stringent conditions (minimal medium/lactate with ethanolamine) the cellular PE level of *Δcki1Δdpl1Δeki1* was reduced to 17% of total phospholipids compared to 15% in *Δpsd1Δpsd2* and 25% in wild type. We can conclude from this result that also the CDP-ethanolamine pathway, besides Psd1p, is an important route for

cellular PE formation. The decrease in cellular PE was mainly compensated by elevated levels of PC. Interestingly, an increased level of cellular PE was found in *Δale1* [35]. Ale1p had been identified as a lyso-PE acyltransferase [29,30]. The reason for the increased PE level in the deletion strain may be the broad substrate specificity of Ale1p [38,39,40] and/or compensation of the *Δale1* deletion by other PE forming routes [35].

Whereas lipid profiling of yeast cells provides information about a steady state situation in the cell, *in vivo* labeling experiments reflect the dynamic processes of lipid formation and/or turnover. Formation of PE *in vivo* can be measured by labeling experiments with radioactive serine introduced into the decarboxylation/methylation route of aminoglycerophospholipid biosynthesis; and with radiolabeled ethanolamine incorporated into PE via Kennedy pathway (see Figure 1, and Subheading 3.3, *In vivo* labeling of aminoglycerophospholipids) [19]. When yeast cells are cultivated in the presence of [^{3}H]serine, PS is formed as the first component in the lipid biosynthetic sequence, which is then converted by PS decarboxylases Psd1p and/or Psd2p to PE. Further conversion of PE leads to PC through three-fold methylation. It has to be noted that enzymes involved in the decarboxylation/methylation route of aminoglycerophospholipid biosynthesis are localized to different subcellular compartments, namely the ER and mitochondria or Golgi, respectively. Consequently, translocation of substrates and intermediates between organelles is required. Not unexpectedly, incorporation of radiolabeled serine into PE strongly depends on the presence or absence of Psd1p and Psd2p. Deletion of each of these enzymes, respectively, reduces incorporation of label into PE. The amount of [^{3}H]serine incorporated into PE in the *Δpsd1* strain is decreased to ~30% and in *Δpsd2* mutants to ~70% of wild type [19,21,22,25]. The observation that deletion of *PSD1* has a stronger effect on PE synthesis than deletion of *PSD2* is in line with growth phenotype and lipid analyses described above. In a *Δpsd1Δpsd2* mutant, which completely lacks PS decarboxylase activity, [^{3}H]serine is significantly

accumulated in PS, but nevertheless low levels of radioactivity are incorporated into PE. This observation supports the view that radiolabel in the form of [^{3}H]serine incorporated into sphingolipids through the action of serine palmitoyltransferase ends up in ethanolamine phosphate during sphingolipid turnover [25]. Ethanolamine phosphate can directly serve as a substrate for enzymes of the Kennedy pathway. Double labeling of whole cells with [^{3}H]serine and [^{14}C]ethanolamine in combination with the use of mutants bearing defects in the different PE biosynthetic pathways allows us to estimate the contribution of each pathway to total cellular PE formation.

Labeling of aminoglycerophospholipids *in vivo* using intact, metabolizing yeast as described above can be complemented by experiments using permeabilized yeast cells [36]. These cells do not proliferate but still metabolize. This technique allows dissection of individual steps of the biosynthetic routes of PS, PE and PC formation. The advantage of this system is the possibility to introduce reagents which cannot enter intact cells. Again, the use of different labeled aminoglycerophospholipid precursors and mutants contributes to a better understanding of the complete PE biosynthetic network.

Finally, *in vitro* measurements of enzyme activities can be used to confirm the efficiency of biosynthetic steps and the effects of mutations. It has to be noted that enzyme activities measured in *vitro* do not necessarily reflect the situation *in vivo* due to regulatory effects that may escape detection in the test tube. In the case of PS decarboxylases, however, results obtained *in vitro* largely match *in vivo* data [22,25]. The assay used for these measurements (*see* Subheading 3.4, PS decarboxylase activity assay *in vitro*) is a standard procedure described by Kuchler et al. [18]. PS decarboxylase activity can be measured in total cell extracts as well as in isolated subcellular fractions.

In summary, the use of mutants in yeast lipid research has led to better knowledge about individual steps of lipid formation, and also to a deeper understanding of the links between

pathways in a more global way. As an example, Horvath et al. [35] recently described a novel physiological link between triacylglycerol (TAG) and PE metabolism in yeast. The bridging enzyme between these two pathways is the phospholipid:diacylglycerol acyltransferase Lro1p, which forms TAG from diacylglycerol and a fatty acid derived from a phospholipid, preferentially from PE. Using the set of PE biosynthesis mutants described above, the contribution of the four different PE biosynthetic pathways to TAG formation was analyzed. Interestingly, it was found that the CDP-ethanolamine pathway of PE formation contributes most to the cellular TAG level, whereas mutations in the other pathways for PE synthesis display only minor effects. Such experiments broaden our view of lipid metabolism and set the stage to understand this complex network of reactions including their regulation in more detail.

2. Materials

2.1. Equipment and Supplies

1. Microsyringe (Hamilton, Bonaduz, Switzerland).
2. 12 ml Pyrex glass vials with Teflon liner caps.
3. Table top shaker for test tubes (IKA® Vibrax VXR).
4. Glass tubes (20 ml) with ground neck.
5. Silica gel 60 TLC plates (Merck, Darmstadt, Germany).
6. TLC chamber (Springfield Mill, UK) with saturation paper (e.g., Whatman filter paper).
7. Iodine vapor chamber.
8. Incubator (Heraeus).

9. Table-top centrifuge (Hettich Rotina 46 R, Heraeus Fresco17).
10. Merckenschlager homogenizer (Braun-Melsungen) with fitting glass bottles, and glass beads (0.25-0.30 mm diameter; Sartorius).
11. Scintillation counter Packard 1500 Tri-Carb®.
12. Plastic vials for liquid scintillation counting.

2.2. Reagents

1. Medium for yeast cell cultivation: YPD (2% glucose, 2% peptone and 1% yeast extract).
2. Medium for yeast cell cultivation: YPLac (2.6% lactate, 2% peptone and 1% yeast extract, pH 5.5 with KOH)
3. Medium for yeast cell cultivation: MMGlu (2% glucose, 0.67% yeast nitrogen base and amino acid mixture).
4. Medium for yeast cell cultivation: MMLac (2.6% lactate, 0.67% yeast nitrogen base and amino acid mixture, pH 5.5 with KOH).
5. Solvents: chloroform and methanol, analytical grade
6. Washing solution for lipid extraction: 0.034% $MgCl_2$; 2N KCl/MeOH (4:1; v/v).
7. Washing solution for lipid extraction: artificial upper phase ($CHCl_3$/MeOH/H_2O; 3:48:47; per vol).
8. 0.26% ammonium heptamolybdate tetrahydrate/ANSA (500:22; v/v).
9. ANSA solution: 40.0 g $K_2S_2O_5$, 0.63g 8-anilio-1-naphthalenesulfonic acid, 1.25 g Na_2SO_3 in 250 ml water
10. L-[^{3}H]serine (21.99 Ci mmol^{-1}, Perkin Elmer, Boston, MA).

11. [^{14}C]ethanolamine (2.9 mCi mmol^{-1}, Perkin Elmer, Boston, MA).
12. Scintillation cocktail (Packard Bio-Science, Groningen, The Netherlands) with 5% H_2O.

3. Methods

3.1 Growth-phenotype analysis (*see* **Note 1**)

1. 5 ml YPD are inoculated with 10 µl of an overnight culture and incubated for ~16 hours at 30°C with shaking.
2. The culture is diluted to an OD_{600} 1 with sterile water and dilutions (1, 1/10, 1/100, 1/1000, 1/10000) are prepared in 96-well microtiter plates.
3. 4 µl of the suspensions are spotted on agar plates. Alternatively, a sterile stamp can be used.
4. Agar plates are incubated at 30°C for a few days depending on the medium and the strain.

3.2 Lipid analysis

3.2.1 Preparation of cell homogenate

1. Homogenate is prepared from a minimum of 50 ml culture of full or selective media. Cells are inoculated from a 48 h preculture to an OD_{600} of 0.1 and grown to the early stationary phase at 30°C with shaking (*see* Note 2).
2. After harvesting at 4,500 x g for 5 min, cells are washed with distilled water.

3. Cells are suspended in 10 mM Tris/HCl, pH 7.5, 1 mM EDTA (ml/g CWW) and protease inhibitor phenylmethylsulfonyl fluoride (stock solution 1 M PMSF in DMSO) (2 µl/g cell wet weight)
4. Cells are disintegrated with glass beads in a Merckenschlager homogenizer for 3 min under CO_2 cooling.
5. The cell extract is transferred to a fresh tube and cleared of glass beads, unbroken cells and cell debris by centrifugation at 2,500 x g for 5 min at 4°C. The supernatant fraction represents the homogenate.
6. Proteins are quantified using the method of Lowry et al. [41] or Bradford [42].

3.2.2 Lipid extraction

1. Lipids from the homogenate are extracted using the method of Folch et al [43]. Solvents should be handled with care and work should be performed in a fume hood.
2. Following the procedure of Folch et al. [43] an aliquot of the sample (~3 mg protein) is added to 3 ml of $CHCl_3$:MeOH (2:1; v/v) in a Pyrex glass tube.
3. Lipids are extracted to the polar organic phase by vigorous shaking with a table top rotary shaker (IKA® Vibrax VXR) at room temperature for 30 min.
4. Proteins and polar substances are removed by consecutive washing steps with 1 ml 0.034% $MgCl_2$, 1 ml of 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.). These solutions are added to the extracts and incubated with shaking for 10 min (*see* Note 3)
5. After each washing step samples are centrifuged for 3 min at 2,500 x g in a table-top centrifuge, and the aqueous phase is removed by aspiration (*see* Note 3).
6. Washing steps are repeated until no protein intermediate layer is formed any more.
7. Finally, lipids are dried under a stream of nitrogen and stored at -20°C.

3.2.3 Thin-Layer Chromatography and Phospholipid Quantification

1. Phospholipids are separated by 2D TLC due to different properties of their head groups. Lipids are dissolved in 50 µl $CHCl_3$/MeOH (2:1; v/v) and applied as single spot to a TLC plate (10x10 cm) approximately 1-1.5 cm distant from a corner. TLC plates can be loaded using a Hamilton syringe or a sampler pipette.
2. For the first dimension, $CHCl_3$/MeOH/25% NH_3 (65:25:6; per vol.) is used as a solvent, and for the second dimension $CHCl_3$/acetone/MeOH/acetic acid/H_2O (50:20:10:10:5; per vol.). Separations usually take 50 min/10 cm distance on TLC plates for each dimension.
3. Phospholipids are visualized by staining with iodine vapor in a saturated chamber for some minutes (Figure 3). Spots are marked with a pencil. The iodine vapor develops after putting a spoonful of solid crystals of iodine into the chamber. Staining of TLC plates should be done in a fume hood.

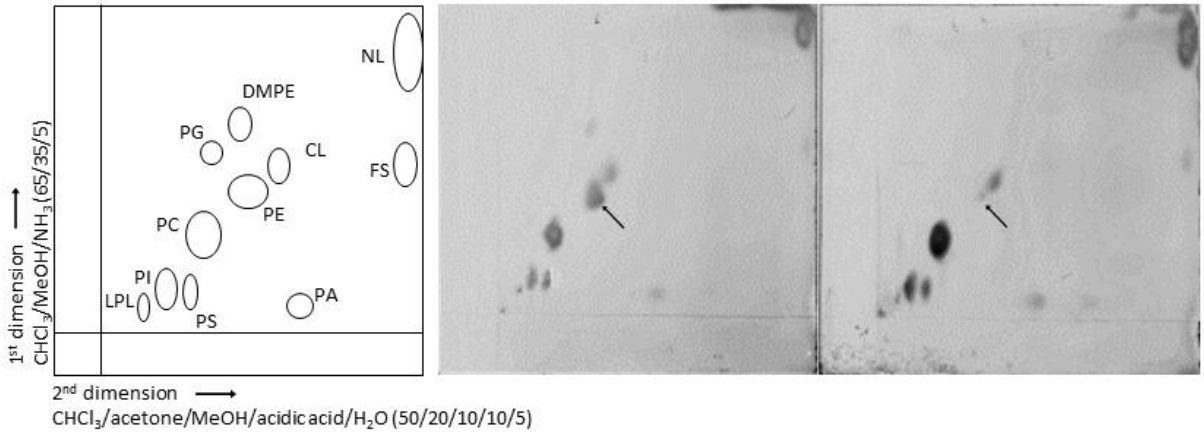

Figure 3. Separation of yeast phospholipids by two dimensional TLC. Left: schematic overview of phospholipid separation by two dimensional TLC. Middle: Two dimensional TLC of phospholipids from wild type yeast. Right: Two dimensional TLC of phospholipids from a Δ*psd1*Δ*psd2* double mutant. The arrows point to the spot of PE. Abbreviations: LPL, lyso-phospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FA, fatty acids; PG, phosphatidylglycerol; DMPE, dimethylethanolamine; CL, cardiolipin.

4. For destaining of spots, TLC plates are incubated in a heating chamber (~50-60°C) for a few minutes.
5. Phospholipids can be quantified from TLC plates after removal of the iodine staining. The plate is moistened with deionized water, phospholipid spots are scrapped off and transferred to a phosphate free glass tube with ground neck (*see* Note 4).
6. The lipid phosphorus of the respective spot can be measured by subjecting the sample to hydrolysis as described by Broekhuyse [44]. In brief, 0.2 ml of conc. H_2SO_4/72% $HClO_4$ (9:1; v/v) are added to each sample. Hydrolysis is performed at 180°C in a heating block for 30 min. Please note that this step has to be performed in a hood due to formation of acidic fumes!
7. Samples are cooled to room temperature, and 4.8 ml freshly prepared 0.26% ammonium heptamolybdate tetrahydrate/ANSA (500:22; v/v) is added. Tubes are closed with phosphate-free glass caps, and after vigorous vortexing, samples are heated to 100°C for 30 min in an oven.
8. Finally, samples are cooled to room temperature and centrifuged briefly in a table-top centrifuge at 1,000 x g to sediment the silica gel. The intensity of the blue color in the supernatant is a measure for lipid phosphorus. Samples are measured spectrophotometrically at a wavelength of 830 nm using a blank spot from the TLC plate without phospholipid as a control. Inorganic phosphate is used as a standard.

3.3 *In vivo* labeling of aminoglycerophospholipids

1. Cells are inoculated from a 48 h preculture to an OD_{600} of 0.1 and grown to stationary phase at 30°C in YPD with shaking.
2. 10 OD_{600} units of cells are divided into three equal portions and put into three sterile Pyrex tubes, harvested by centrifugation and suspended in 500 µl of fresh YPD.

3. Then, cells are incubated for 30 min at 30°C with shaking.
4. 10 µCi L-[^{3}H]serine or 2 µCi [^{14}C]ethanolamine are added to the cultures (*see* Note 5).
5. The cultures are incubated for 10, 20 and 30 min, respectively, at 30°C with shaking and then immediately cooled to 4°C to stop cellular metabolism (*see* Note 6).
6. After centrifugation and washing with ice cold water, cells are deep frozen with liquid nitrogen.
7. To extract lipids, cells are vortexed for 60 min together with 1.5 ml of glass beads and 2 ml $CHCl_3$/MeOH (2:1; v/v).
8. After centrifugation, the supernatant is transferred to a fresh Pyrex tube.
9. Glass beads are washed with 2 ml $CHCl_3$/MeOH (2:1; v/v) and centrifuged; and supernatants are combined.
10. Supernatants are washed as described in Subheading 3.2.2 **(**Lipid extraction).
11. Lipids are dissolved in 50 µl $CHCl_3$/MeOH (2:1; v/v) and spotted on TLC plates.
12. For the analysis of PS, PE and PC, lipids are separated by one-dimensional TLC using $CHCl_3$/MeOH/25%NH_3 (50:25:6; per vol.) as developing solvent (Figure 4).
13. Phospholipids are visualized by staining with iodine vapor in a saturated chamber for some minutes and marked with a pencil. For destaining, TLC plates are incubated in a heating chamber (~50-60°C) for a few minutes.
14. Bands of PS, PE and PC are scraped off the plate and transferred into liquid scintillation vials with LSC SAFETY Cocktail (Packard Bioscience B.V., Meriden, USA) plus 5 vol.% water and incubated for at least 1 hour.
15. Radioactivity is determined by liquid scintillation counting in a Packard 1500 Tri-Carb® liquid scintillation analyzer.

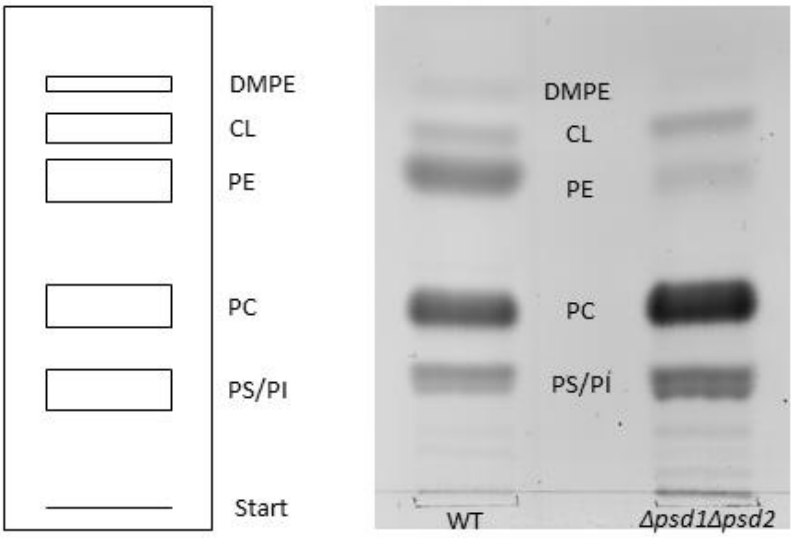

Figure 4. Separation of phospholipids by one dimensional TLC. $CHCl_3$/MeOH/25% NH_3 (65:25:6; per vol.) is used as solvent. Left: schematic overview of phospholipid separation by one dimensional TLC. Right: one dimensional TLC of phospholipids from wild type and Δ*psd1*Δ*psd2* double mutant.

3.4 PS decarboxylase activity assay *in vitro*

3.4.1 Preparation of the substrate

1. [^{3}H]Phosphatidylserine is used as a substrate for PS decarboxylase measurements. It is synthesized *in vitro* in a solution consisting of 200 µl 1M Tris/HCl (pH 8), 200 µl 50 mM NH_2OH (in 2% Triton X-100), 200 µl 6 mM $MnCl_2$, 10 µl 20 mM CDP-DAG, 200 µl 5 mM L-serine and 20 µCi L-[^{3}H]serine.
2. To start the reaction, 1 ml of wild type homogenate containing 10 mg protein (*see* Subheading 3.2.1, Preparation of cell homogenate) is added and the sample is vortexed.
3. Samples are agitated for 60 min in a 30°C water bath using a magnetic stirrer.

4. The reaction is stopped by adding 20 ml $CHCl_3$/MeOH (2:1; v/v) and vigorous vortexing. Then, the sample is incubated for 10 min at room temperature.
5. After centrifugation for 5 min at 2,500 x g in a table-top centrifuge, the aqueous phase is removed by aspiration.
6. The lower polar phase is washed five times with 2N KCl/MeOH (4:1; v/v).
7. Finally, the product is dried under a stream of nitrogen and dissolved in 2 ml $CHCl_3$/MeOH (2:1; v/v).
8. The radioactivity of the sample is determined by liquid scintillation counting in a Packard 1500 Tri-Carb® liquid scintillation analyzer using LSC SAFETY Cocktail (Packard Bioscience B.V., Meriden, USA) plus 5 vol.% water.

3.4.2 Enzyme assay

1. For PS decarboxylase assays, the substrate (displaying radioactivity corresponding to 40,000 dpm) is put into a glass tube with ground neck and dried under a stream of nitrogen. 500 µl 0.2 M Tris/HCl (pH 7.2), 20 mM EDTA is added, and the sample is ultrasonicated for 7 min in a water bath.
2. The glass tube containing the substrate is put into a water bath (30°C) with agitation, and the reaction is started by adding 500 µl of homogenate (2 mg of protein in 10 mM Tris/HCl, pH 7.5, 1 mM EDTA).
3. At different time points (0, 10 and 20 min), 200 µl samples are transferred to a Pyrex-tube containing 4 ml $CHCl_3$/MeOH (2:1; v/v) to stop the reaction.
4. Extraction of lipids is performed as described in Subheading 3.2.2 (Lipid extraction).
5. After drying samples under a stream of nitrogen they are dissolved in 50 µl $CHCl_3$/MeOH (2:1; v/v) and spotted onto a TLC plate.

6. PE formed during incubation is isolated by TLC and analyzed as described above (Subheading 3.3, *In vivo* labeling of aminoglycerophospholipids).

4. Notes

1. A summary of phenotypes which can be easily screened and is associated with primary or suppressor mutations was provided by Hampsey [45].
2. It is very important that all strains tested are in the same growth phase, because the lipid composition varies strongly at different stages of yeast cultivation.
3. For subsequent analysis of lipids by TLC it is very important to remove all protein aggregates by discarding the upper aqueous phase as well as the protein interface layer. Alternatively, the lower polar phase can be transferred to a fresh Pyrex tube by using a glass pipette.
4. Note that all tubes and pipettes have to be free of phosphate contaminations. Such contamination would lead to incorrect measurements. For this reason, phosphate free detergent and ddH_2O has to be used for all steps.
5. As a variation of this assay 0.1 µCi [methyl-^{14}C]choline chloride or 0.5 µCi [1-^{14}C]acetic acid can be used to label lipids. Exogenously added choline incorporates into PC via CDP-choline pathway, and acetate is used as a substrate for fatty acid synthesis.
6. Time points can be varied depending on the problem to be addressed. Early time points mainly represent the synthesis rate of a phospholipid. To obtain steady-state data cells are incubated with labeled precursors for several hours. As an example, 20 ml YPD are inoculated to OD_{600} 0.1 from a 48 hours preculture, and 10 µCi L-[^{3}H]serine, 2 µCi [^{14}C]ethanolamine, 0.1 µCi [methyl-^{14}C]choline chloride or 0.5 µCi

[1-^{14}C]acetic acid, respectively, per ml culture are added. Then, cells can be cultivated to the late logarithmic or even to the stationary growth phase. 10 OD_{600} units are harvested in a Pyrex tube and lipids are extracted as described above.

Acknowledgement

This work was supported by the Austrian Science Fund FWF (project 21429 and DK Molecular Enzymology W901-B05 to GD). The authors are grateful to Edina Harsay for critical reading of this manuscript.

References

[1] M. Sud, E. Fahy, D. Cotter, A. Brown, E.A. Dennis, C.K. Glass, A.H.M. Jr, R.C. Murphy, C.R.H. Raetz, D.W. Russell, S. Subramaniam, LMSD: LIPID MAPS structure database, Nucleic Acids Res, Nucleic Acids Res, 2007, pp. D527-532.

[2] G.v. Meer, D.R. Voelker, G.W. Feigensonvan, Membrane lipids: where they are and how they behave, Nat Rev Mol Cell Biol. 9 (2008) 112-124.

[3] K. Athenstaedt, G. Daum, Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast *Saccharomyces cerevisiae* are localized to lipid particles J. Biol. Chem. 280 (2005) 37301–37309.

[4] M.L. Gaspar, M.A. Aregullin, S.A. Jesch, L.R. Nunez, M. Villa-García, S.A. Henry, The emergence of yeast lipidomics, Biochim Biophys Acta 1771 (2007) 241-254.

[5] G.M. Carman, G.M. Zeimetz, Regulation of phospholipid biosynthesis in the yeast Saccharomyces cerevisiae, J Biol Chem 271 (1996) 13293-13296.

[6] S. Rajakumari, K. Grillitsch, G. Daum, Synthesis and turnover of non-polar lipids in yeast, Prog Lipid Res 47 (2008) 157-171.

[7] G.M. Carman, S.A. Henry, Phospholipid biosynthesis in the yeast *Saccharomyces cerevisiae* and interrelationship with other metabolic processes, Prog Lipid Res 38 (1999) 361-399.

[8] M. Chen, L.C. Hancock, J.M. Lopes, Transcriptional regulation of yeast phospholipid biosynthetic genes, Biochim Biophys Acta 1771 (2007) 310-321.

[9] G.M. Carman, G.S. Han, Regulation of phospholipid synthesis in Saccharomyces cerevisiae by zinc depletion, Biochim Biophys Acta 1771 (2007) 322-330.

[10] J. Patton-Vogt, Transport and metabolism of glycerophosphodiesters produced through phospholipid deacylation, Biochim Biophys Acta 1771 (2007) 337-342.

[11] H. Santos-Rosa, J. Leung, N. Grimsey, S. Peak-Chew, S. Siniossoglou, The yeast lipin Smp2 couples phospholipid biosynthesis to nuclear membrane growth, EMBO J 24 (2005) 1931-1941.

[12] G. Li, S. Chen, M.N. Thompson, M.L. Greenberg, New insights into the regulation of cardiolipin biosynthesis in yeast: implications for Barth syndrome, Biochim Biophys Acta 1771 (2007) 432-441.

[13] A. Hinnen, J.B. Hicks, G.R. Fink, Transformation of yeast, Proc Natl Acad Sci U S A 75 (1978) 1929-1933.

[14] K. Struhl, D.T. Stinchcomb, S. Scherer, R.W. Davis, High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules, Proc Natl Acad Sci U S A 76 (1979) 1035-1039.

[15] E.A. Winzeler, D.D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J.D. Boeke, H. Bussey, Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis, Science 285 (1999) 901-906.

[16] G.M. Carman, G.S. Han, Regulation of phospholipid synthesis in the yeast Saccharomyces cerevisiae, Annu Rev Biochem 80 (2011) 859-883.

[17] I. Schuiki, M. Schnabl, T. Czabany, C. Hrastnik, G. Daum, Phosphatidylethanolamine synthesized by four different pathways is supplied to the plasma membrane of the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1801 (2010) 480-486.

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

[19] M. Bürgermeister, R. Birner-Grünberger, R. Nebauer, G. Daum, Contribution of different pathways to the supply of phosphatidylethanolamine and phosphatidylcholine to mitochondrial membranes of the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1686 (2004) 161-168.

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

[21] R. Nebauer, I. Schuiki, B. Kulterer, Z. Trajanoski, G. Daum, The phosphatidylethanolamine level of yeast mitochondria is affected by the mitochondrial components Oxa1p and Yme1p, FEBS J. 274 (2007) 6180-6190.

[22] M.K. Storey, K.L. Clay, T. Kutateladze, R.C. Murphy, M. Overduin, D.R. Voelker, Phosphatidylethanolamine has an essential role in *Saccharomyces cerevisiae* that is independent of its ability to form hexagonal phase structures, J. Biol. Chem. 276 (2001) 48539-48548.

[23] S.W. Hui, T.P. Stewart, P.L. Yeagle, A.D. Albert, Bilayer to non-bilayer transition in mixtures of phosphatidylethanolamine and phosphatidylcholine: implications for membrane properties, Arch. Biochem. Biophys. 207 (1981) 227-240.

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

[25] P.J. Trotter, D.R. Voelker, Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (*PSD2*) in the yeast *Saccharomyces cerevisiae*, J. Biol. Chem. 270 (1995) 6062-6070.

[26] E.P. Kennedy, S.B. Weiss, The function of cytidine coenzymes in the biosynthesis of phospholipids, J. Biol. Chem. 222 (1956) 193-214.

[27] R. Birner, G. Daum, Biogenesis and cellular dynamics of aminoglycerophospholipids, Int. Rev. Cytol. 225 (2003) 273-323.

[28] G. Daum, N.D. Lees, M. Bard, R. Dickson, Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*, Yeast 14 (1998) 1471-1510.

[29] W.R. Riekhof, D.R. Voelker, Uptake and utilization of lyso-phosphatidylethanolamine by *Saccharomyces cerevisiae*, J. Biol. Chem. 281 (2006) 36588-36596.

[30] W.R. Riekhof, J. Wu, J.L. Jones, D.R. Voelker, Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in *Saccharomyces cerevisiae*, J. Biol. Chem. 282 (2007) 28344-28352.

[31] K. Kim, K.-H. Kim, M.K. Storey, D.R. Voelker, G.M. Carman, Isolation and characterization of the *Saccharomyces cerevisiae EKI1* gene encoding ethanolamine kinase, J Biol Chem 274 (1999) 14857-14866.

[32] K. Hosaka, T. Kodaki, S. Yamashita, Cloning and characterization of the yeast *CKI* gene encoding choline kinase and its expression in *Escherichia coli*, J Biol Chem 264 (1989) 2053-2059.

[33] J.D. Saba, F. Nara, A. Bielawska, S. Garrett, Y.A. Hannun, The *BST1* gene of *Saccharomyces cerevisiae* is the spingosine-1-phosphate lyase, J Biol Chem 272 (1997) 26087-26090.

[34] D. Gottlieb, W. Heideman, J.D. Saba, The *DPL1* gene is involved in mediating the response to nutrient deprivation in *Saccharomyces cerevisiae*, Mol Cell Biol Res Commun 1 (1999) 66-71.

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

[36] R. Birner, M. Bürgermeister, R. Schneiter, G. Daum, Roles of phosphatidylethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*, Mol. Biol. Cell 12 (2001) 997-1007.

[37] P.J. Trotter, J. Pedretti, R. Yates, D.R. Voelker, Phosphatidylserine decarboxylase 2 of *Saccharomyces cerevisiae*. Cloning and mapping of the gene, heterologous expression, and creation of the null allele, J Biol Chem 270 (1995) 6071-6080.

[38] U. Stahl, K. Stalberg, S. Stymne, H. Ronne, A family of eukaryotic lysophospholipid acyltransferases with broad specificity, FEBS Lett. 582 (2008) 305-309.

[39] S. Jain, N. Stanford, N. Bhagwat, B. Seiler, M. Costanzo, C. Boone, P. Oelkers, Identification of a novel lysophospholipid acyltransferase in *Saccharomyces cerevisiae*, J Biol Chem 282 (2007) 30562-30569.

[40] H. Tamaki, A. Shimada, Y. Ito, M. Ohya, J. Takase, M. Miyashita, H. Miyagawa, H. Nozaki, R. Nakayama, H. Kumagai, *LPT1* encodes a membrane-bound *O*-acyltransferase involved in the acylation of lysophospholipids in the yeast *Saccharomyces cerevisiae*, J Biol Chem 282 (2007) 34288-34298.

[41] C.V. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.

[42] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem 72 (1976) 248-254.

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

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

[45] M. Hampsey, A review of phenotypes in *Saccharomyces cerevisiae*, Yeast 13 (1997) 1099-1133.

Chapter 2

Transcriptional response to phosphatidylethanolamine depletion in the yeast *Saccharomyces cerevisiae*

Martina Gsell, Gerald Mascher, Irmgard Schuiki and Günther Daum*

Institute of Biochemistry, Graz University of Technology,
Petersgasse 12/2, 8010 Graz, Austria

*Corresponding author:
Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria
e-mail: guenther.daum@tugraz.at; Tel.: +43 316 873 6462; Fax.: +43 316 873 6952

Key Words: phosphatidylethanolamine, phosphatidylethanolamine decarboxylase, yeast, phospholipids, microarray

Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPE, dimethylethanolamine; TG, triacylglycerol

Manuscript submitted, March 2013

Abstract

In the yeast, *Saccharomyces cerevisiae*, the synthesis of the essential phospholipid phosphatidylethanolamine (PE) is accomplished by a network of reactions which comprises four different pathways. The enzyme contributing most to PE formation is the mitochondrial phosphatidylserine decarboxylase 1 (Psd1p) which catalyzes conversion of phosphatidylserine (PS) to PE. To study the genome wide effect of an unbalanced cellular and mitochondrial PE level and in particular the contribution of Psd1p to this depletion we performed a DNA microarray analysis with a Δ*psd1* deletion mutant. This approach revealed that 54 yeast genes were significantly up-regulated in the absence of *PSD1* compared to wild type. Surprisingly, marked down-regulation of genes was not observed. A number of different cellular processes in different subcellular compartments were affected in a Δ*psd1* mutant. Deletion mutants bearing defects in all 54 candidate genes, respectively, were analyzed for their growth phenotype and their phospholipid profile. Only three mutants, namely Δ*gpm2*, Δ*gph1*, Δ*rsb1*, were affected in one of these parameters. The possible link of these mutations to PE deficiency and *PSD1* deletion is discussed.

Introduction

Phosphatidylethanolamine (PE) is an essential phospholipid in many types of cells from bacteria to man. In the yeast, *Saccharomyces cerevisiae*, depletion of PE which is one of the major cellular and mitochondrial phospholipids causes dysfunction of respiration, defects in the assembly of mitochondrial protein complexes, and loss of mitochondrial DNA [1,2,3]. Moreover, PE plays a specific role due to its unique biophysical properties as a non-bilayer (hexagonal phase) forming lipid [4]. The biosynthesis of PE comprises a complex network of reactions distributed among different organelles in the cell. Four pathways contribute to PE biosynthesis in yeast, namely (i) decarboxylation of phosphatidylserine (PS) catalyzed by phosphatidylserine decarboxylase 1 (Psd1p) in the inner mitochondrial membrane [5,6,7]; (ii) decarboxylation of PS by Psd2p in a Golgi/vacuolar compartment [8]; (iii) incorporation of ethanolamine through the CDP-ethanolamine branch of the Kennedy pathway [9] in the endoplasmic reticulum [10,11]; and (iv) synthesis of PE through acylation of lyso-PE catalyzed by the acyl-CoA-dependent acyltransferase Ale1p in the mitochondria-associated membrane (MAM) [12,13]. These four pathways form PE with different efficiency [14]. Psd1p is the major supplier of cellular and mitochondrial PE and represents the major cellular *PSD* activity [8]. Inactivation or deletion of the *PSD1* gene leads to a considerable decrease of PE in total cellular and mitochondrial membranes [1,15]. This finding suggests that marked amounts of PE found in all cellular membranes must be derived from mitochondria. However, mechanisms governing PE distribution within the cell are not well understood.

To obtain a more global insight into the biosynthesis of PE, its regulation and the role of PE in organelle membranes with special emphasis on the biosynthetic capacity of Psd1p we performed DNA microarray analysis using a Δ*psd1* deletion strain. Here we show that a relatively small number of genes were affected in such a mutant. The corresponding gene

products are involved in various cellular processes including transport, carbohydrate metabolism, stress response and energy metabolism. Moreover, a number of unassigned ORFs were detected during this analysis. In a more detailed analysis, genes and gene products either affecting growth or lipid metabolism were selected from this screening. Three mutants bearing deletions of *GPM2*, *GPH1* and *RSB1* were analyzed in some more detail for their possible link to PE or lipid metabolism.

Materials and Methods

Strains and culture conditions

The wild type yeast strains *Saccharomyces cerevisiae* BY4741 and BY4742 and all deletion mutants in the BY4741 background were obtained from the Euroscarf strain collection (Frankfurt, Germany) (see Table 1). Strains were cultivated on YPD media containing 1% yeast extract, 2% peptone and 2% glucose under aerobic conditions with shaking at 30°C.

Table 1. Yeast strains. All deletion strains used in this study are in the BY4741 background.

Strain	Genotype	Source
BY4741	MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	Euroscarf
BY4742	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0	Euroscarf
BY4742 [pUG35-*GPH1*-GFP]	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 [pUG35-*GPH1*]	This study
BY4742 [pUG35-*RSB1*-GFP]	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 [pUG35-*RSB1*]	This study
BY4742 [pUG35-*GPM2*-GFP]	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 [pUG35-*GPM2*]	This study

Growth tests on fermentable and non-fermentable carbon sources were performed on solid media containing 1% yeast extract, 2% peptone, and 2% agar supplemented with 2% glucose, 2.66% lactate, 2% glycerol or 8 mM sorbitol, respectively. For SDS resistance assays on solid media, 0.05% SDS was added to the media immediately prior to pouring plates. MMLac (minimal medium with lactate) culture plates contain 0.67% yeast nitrogen base without amino acids, 0.073% amino acid mix, 2.66% lactate, adjusted to pH 5.5 with KOH and 2% agar.

The BY4742 wild type strain harboring recombinant plasmids was grown in uracil-free minimal medium (0.67% yeast nitrogen base, 2% glucose and amino acid stock) or in uracil- and methionine-free minimal medium for induction of the MET25-promotor. For growth phenotype analysis cell suspensions of overnight cultures grown in YPD were spotted at dilutions 1, 1/10, 1/100, 1/1000 and 1/10000 on YPD, YPLac, MMLac, YPGlycerol, YPSorbitol and YPD with 0.05% SDS. Incubations were carried out at 30°C.

Strain constructions

GPH1, *GPM2* and *RSB1*, respectively, were cloned into the plasmid pUG35 (provided by J. Hegemann and U. Güldener) with standard genetic methods. pUG35 allows a C-terminal in-frame fusion of the yEGFP3 encoding open reading frame to the gene. The expression of the fusion protein is under control of the MET25-promoter, and under methionine restriction the inserted gene is expressed at high yield. The genes *GPH1*, *GPM2* and *RSB1* were amplified from yeast chromosomal DNA of the strain *S. cerevisiae* BY4741 by PCR under standard conditions using the proof reading *Ex Taq*^{TM}-DNA polymerase (Takara). The sequence specific primers used are listed in Table 2. Primers for *GPH1* amplification include a *BamH*I or a *Cla*I restriction enzyme recognition sequence, respectively. The *GPM2* PCR-product was cut with *BamH*I and *Hind*III and the *RSB1* PCR-product with *BamH*I and *Sal*I to

facilitate the subsequent cloning of the amplified DNA into the specific sites of the pUG35 vector. Genes were sequenced by LGC Genomics (Berlin, Germany). BY4742 was transformed with the cloned vector by the lithium acetate method [16].

Table 2. PCR primers for amplification

Recognition sites for restriction enzymes are underlined

Primer	Sequence
GPH1*BamH*If	5´-CGCGGATCCTGAACAATGCCGCCAGCTAGTAC-3´
GPH1*Cla*Ir	5´-CCCATCGATAGTCACTGGTTCAACGTTCCAAATG-3´
RSB1*BamH*If	5´-CGCGGATCCGGTGGTATGGTACCGAACCTTC-3´
RSB1*Sal*Ir	5´-CCCGTCGACAAGTTTAGCCTTCTTTTTAGAGGAAAC-3´
GPM2*BamH*If	5´-CGCGGATCCATGACTGCAAGCACACCATCCAA-3´
GPM2*Hind*IIIr	5´-CCCAAGCTTAGGATTTTTTATGAAACCCTCATTACGG-3´

Isolation of plasma membrane and mitochondria

Yeast cells were grown aerobically in YPD to the early stationary phase at 30°C. Then, cells were disrupted with glass beads using a Merckenschlager homogenizer under CO_2-cooling. Cell extracts were cleared of glass beads, unbroken cells and cell debris by centrifugation at 2,500 x g for 5 min. The supernatant fraction represented the homogenate. Crude plasma membrane was isolated essentially as described by Serrano [17] and further purified as reported by van den Hazel et al. [18] and Pichler et al. [19].

To isolate mitochondria, spheroplasts were prepared and homogenized in breaking buffer consisting of 0.6 M mannitol, 10 mM Tris, pH 7.4 and 1 mM PMSF by using a Dounce homogenizer as described previously [20]. Unbroken cells and debris were removed by centrifugation at 3,000 x g for 5 min. The resulting supernatant was used to isolate mitochondria by published procedures [21].

Relative enrichment of markers and cross-contamination of subcellular fractions were assessed as described by Zinser and Daum [22]. Protein was quantified by the method of Lowry et al. [23] using bovine serum albumin (BSA) as standard. SDS-PAGE was carried out as published by Laemmli [24]. Western blot analysis of proteins from subcellular fractions was performed as described by Haid and Suissa [25]. Immunoreactive bands were visualized by enzyme-linked immunosorbent assay using a peroxide-linked secondary antibody (Sigma-Aldrich, St Louis, MO) following the manufacturer´s instructions.

RNA preparation

For the preparation of RNA, BY4741 and Δ*psd1* were pre-grown overnight. For main cultures, inoculations to an OD_{600} of 0.1 in fresh medium were made by diluting pre-cultures. Cells were grown to the logarithmic phase and harvested by centrifugation. Cell pellets were shock frozen in liquid nitrogen and stored at -70°C till use. Total RNA was isolated using an RNeasy Kit (Qiagen) including DNase I treatment according to the manufacturer´s instructions. Yeast lysates were prepared by mechanical disruption of cells using a bead mill. Integrity of RNA was tested by agarose gel electrophoresis and determination of the 260 to 280 nm absorbance ratio. The RNA concentration was estimated by measuring the absorbance at 260 nm.

Microarray analysis

For expression profiling, 1 µg of total RNA was linearly amplified and biotinylated using the One-Cycle Target Labeling Kit (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. 15 µg of labeled and fragmented cRNA were hybridized to Affymetrix Yeast 2.0 Gene Chip® arrays (Affymetrix). After hybridization, the arrays were washed and stained in a Fluidics Station 450 (Affymetrix) with the recommended washing

procedure. Biotinylated cRNA bound to target molecules was detected with streptavidin-coupled phycoerithrin, biotinylated anti-streptavidin IgG antibodies and again streptavidin-coupled phycoerithrin according to the manufacturer's protocol. Arrays were scanned using the GCS3000 Gene Chip scanner (Affymetrix) and GCOS 1.4 software. Scanned images were subjected to visual inspection to test for hybridization artifacts and proper grid alignment, and analyzed with Microarray Suite 5.0 (Affymetrix) to generate report files for quality control.

Statistical data analysis was performed using the bioconductor packages "affy" and "limma" [26,27]. Initially, the expression data from all arrays were normalized with RMA [28] to yield log2-transformed signal values. The assay was performed in two independent experiments with two and four samples, respectively. A batch effect was observed (data not shown). A linear model was generated including the factors "batch" and "strain" to correct for this effect. The signal values were then averaged for the individual subgroups and differences in the expression level were calculated as x-fold change. Differences between Δ*psd1* and BY4741 strains were extracted and analyzed with the moderated T-test (empirical Bayes method). Transcripts with an at least two-fold change of expression level and a p-value of less than 0.05 were regarded as differentially expressed. Functions of differentially expressed transcripts were annotated using the latest array annotation in the NetAFFX analysis center (www.affymetrix.com/analysis/index.affx).

Bioinformatic analysis

To retrieve information about ORFs of interest, the *Saccharomyces* Genome Database (SGD, http://www.yeastgenome.org/) was queried. The SGD tool GO Slim Mapper [28] was used to assign the general Gene Ontology (GO) terms to identified ORFs. Genes were categorized by using the SGD GO Slim Mapper (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper) to define the biological processes, molecular functions and cellular

components of gene products, in conjunction with GO annotations for yeast gene products curated by the SGD.

Phospholipid analysis

Phospholipids were extracted from the homogenate containing 3 mg protein or from mitochondria and plasma membrane fractions containing 2 mg protein, respectively, by the procedure of Folch et al. [29] using 3 ml chloroform/methanol (2:1, v/v). 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 (50:25:6; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second developing solvents. Lipids were stained with iodine vapor, scraped off the plate, and quantified by the method of Broekhuyse [30].

Fluorescence microscopy

Fluorescence microscopy was performed to localize the C-terminally GFP-tagged proteins Gph1p, Rsb1p and Gpm2p within the cell. The yeast strains carrying fusion proteins were grown at 30°C in uracil- and methionine-free minimal medium. At different time points samples were taken. Fluorescence microscopic analysis was performed using a Zeiss Axiovert 35 microscope with a 100-fold oil immersion objective, a UV lamp and a detection range between 450 and 490 nm. Images were taken with a CCD camera.

Results

Influence of PSD1 deletion on gene expression in Saccharomyces cerevisiae

The present study was aimed at identifying genes of the yeast *Saccharomyces cerevisiae* whose expression levels were changed in response to PE depletion and deletion of the major producer of PE in the cell, Psd1p. The genome-wide expression pattern was analyzed using cDNA microarrays (Affymetrix gene chip system). Among 5,841 open reading frames (transcripts) examined, 54 genes were significantly up-regulated in a Δ*psd1* deletion strain. Significant down-regulation of genes was not observed under these conditions. The list of genes and their expression levels (x-fold change values) are shown in the Supplementary Table S1. The logFC (log *fold change*) values were in the range from -6.09 (*PSD1*) to +2.72 (*DAN1*). All 54 up-regulated ORFs encode for non-essential proteins.

Analysis of ORFs affected by PSD1 deletion

ORFs detected by the above mentioned assay were bioinformatically analyzed using SGD GO slim mapping [28]. This bioinformatic tool sorted the 54 ORFs according to their contribution to biological processes, molecular function and subcellular localization (Tables 3-5). Among the up-regulated genes a large fraction was related to transport, carbohydrate metabolism, generation of precursor metabolites and energy, and response to stress (Table 3). 23 ORFs with unknown biological process were also up-regulated under PE limitation. As molecular function, 18 ORFs were related to catalytic enzyme activities, namely hydrolase, transferase, oxidoreductase, phosphatase and isomerase activity (Table 4). Five ORFs were assigned to transporter activity. Other molecular functions of genes identified were lipid binding, RNA binding, protein binding, transcription regulator activity, signal transducer activity, DNA binding and enzyme regulator activity. The molecular function of 28 ORFs is

unknown. The largest group of gene products was localized to the cytoplasm, followed by the nucleus, plasma membrane, cell wall and mitochondrion (Table 5). In addition, some ORFs are assigned to the vacuole, endoplasmic reticulum, cytoplasmic-membrane bound vesicles, ribosomes and lipid droplets. Subcellular localization of 14 gene products is unknown.

Taken together, the bioinformatic analysis described above showed that depletion of PE in yeast cells appears to affect a number of biological processes. The fact that all genes were up-regulated upon deletion of *PSD1* suggests compensatory gain-of-function. Such a response appears logical for organelle membrane associated gene products (see Table 5) whose activity may at least in part depend on the presence of PE in the membrane environment. Various enzymes and transport related proteins (see Table 4) might be affected in that way. How PE levels in the cell may affect the activity of cytosolic proteins is less obvious. In these cases, secondary effects may be regarded as the reason for this finding. Interestingly, a number of up-regulated genes belong to the so-called seripauperin multigene family (*PAU3, PAU15, PAU16, PAU19, PAU21* and *PAU22*). These gene products are mostly located in the subtelomeric regions of chromosomes [31]. Although the exact role of *PAU* genes and their gene products is still unclear, they are considered to be induced by stress and anaerobiosis [32]. *PAU* genes are extremely homologous to each other and also share homology with Tir and Dan proteins. *TIR4* and *DAN1* were also overexpressed in a Δ*psd1* deletion mutant. Tir and Dan proteins are also induced by anaerobiosis [33,34]. Due to the large number of protein family members most likely with overlapping functions single mutations of these genes do not lead to obvious phenotypes. The link of *PAU*, *TIR* and *DAN* genes to *PSD1* may reflect the importance of Psd1p for respiratory function. Recent work by Böttinger et al. [35] showed that respiration is compromised in a Δ*psd1* mutant. Overexpression of Pau, Tir and Dan proteins might be a compensatory reaction on this defect.

Table 3. GO slim terms Biological Process of the set of up-regulated genes in the absence of *PSD1*

Biological Process	**Genes**
Biological process unknown	*BDH2, FMP23, PAU3, GPM2, UGX2, PAU5, YGR066C, SET4, YJR005C-A, YKL071W, PAU16, YLR149C, TMA10, YMR317W, PAU19, YTP1, TIR4, YOR289W, FRE5, PAU21, PAU22, PAU15, YLR346C*
Transport	*PDR15, FIT1, HXK1, ARN1, ARN2, HXT5, DAN1, RSB1, HES1, FIT2, FIT3, RTC2*
Carbohydrate metabolic process	*GLC3, HXK1, AMS1, SOL4, TDH1, TSL1, PGM2, GPH1*
Cellular homeostasis	*ARN1, ARN2, TIS11 ,PGM2, FIT1, FIT2, FIT3*
Generation of precursor metabolites and energy	*GLC3, HXK1, TDH1, PGM2, GPH1*
Response to stress	*HSP26, HSP12, XBP1, TSL1, DDR2*
Cofactor metabolic process	*SOL4, ARN2, GTO3*
Membrane organization and biogenesis	*RSB1, HES1, HSP12*
RNA metabolic process	*RTC3, TIS11*
Response to chemical stimulus	*PDR15, HSP12*
Protein folding	*HSP26*
Cell wall organization and biogenesis	*ECM4*
Vitamin metabolic process	*SOL4*
Signal transduction	*GPG1*
Sporulation	*YNL194C*
Vesicle-mediated transport	*HES1*
Anatomical structure morphogenesis	*HES1*
Nuclear organization and biogenesis	*GSP2*
Response to starvation	*PHM8*

Table 4. GO slim terms Molecular Function of the set of up-regulated genes in the absence of *PSD1*

Molecular Function	**Genes**
Molecular function unknown	*FMP23, PAU3, GPM2, UGX2, FIT1, PAU5, YGR066C, RTC3, PAU15, SET4, YJR005C-A, DAN1, YKL071W, PAU16, YLR149C, TMA10, YLR346C, YMR317W, PAU19, YNL194C, YTP1, DDR2, TIR4, YOR289W, FIT2, FIT3, PAU21, PAU22*
Hydrolase activity	*PDR15, AMS1, SOL4, TSL1, RSB1, GSP2*
Transferase activity	*GLC3, HXK1, ECM4, TSL1, GTO3, GPH1*
Transporter activity	*PDR15, ARN1, ARN2, HXT5, RTC2*
Oxidoreductase activity	*BDH2, TDH1, FRE5*
Lipid binding	*HES1, HSP12*
RNA binding	*TIS11, HSP26*
Phosphatase activity	*PHM8, TSL1*
Isomerase activity	*PGM2*
Protein binding	*HSP26*
Transcription regulator activity	*XBP1*
Signal transducer activity	*GPG1*
DNA binding	*XBP1*
Enzyme regulator activity	*TSL1*

Table 5. GO slim terms Cellular Component of the set of up-regulated genes in the absence of *PSD1*

Cellular Component	**Genes**
Cytoplasm	*BDH2, FMP23, HSP26, RTC2, GPM2, GLC3, PHM8, HSP12, HXK1, AMS1, SOL4, ARN1, ARN2, RTC3, TDH1, YKL071W, ECM4, TIS11, TMA10, YLR346C, TSL1, PGM2, GTO3, YNL194C ,DDR2, RSB1, YOR289W, FRE5, GPH1, PAU5*
Cellular component unknown	*PAU3, UGX2, GPG1, YGR066C, PAU15, SET4, YJR005C-A, PAU16, YLR149C, YMR317W, PAU19, HES1, PAU21, PAU22*
Nucleus	*BDH2, HSP26, PHM8, HSP12, SOL4, RTC3, XBP1, TIS11, TMA10, GSP2, YOR289W*
Plasma membrane	*HSP12, ARN2, HXT5, RSB1, ARN1, TDH1, YNL194C*
Cell wall	*FIT1, TDH1, DAN1, TIR4, FIT2, FIT3*
Mitochondrion	*FMP23, RTC2, TDH1, YLR346C, FRE5*
Vacuole	*AMS1 ,DDR2, RTC2*
Endoplasmic reticulum	*YNL194C, RSB1*
Cytoplasmic membrane-bounded vesicle	*ARN1, ARN2*
Ribosome	*TMA10*
Lipid droplet	*TDH1*

Phenotype analysis of deletion mutants compromised in genes up regulated in Δpsd1

Mutants deleted of genes which were found to be up-regulated in Δ*psd1* were tested for their growth phenotype on different media. Fermentable and non-fermentable carbon sources were used for these tests. It has to be noted that on non-fermentable carbon sources like lactate and glycerol the requirement for PE increases due to intense proliferation of mitochondria and the importance of PE for cell respiration [1]. Under these conditions even a single deletion of *PSD1* leads to a growth defect (Figure 1). Additionally, osmotic stability of strains on sorbitol and SDS sensitivity was analyzed. Surprisingly, most of the 54 mutants tested did not show changes in their growth behavior compared to wild type (data not shown). Only Δ*gpm2* and Δ*gph1* deletion mutants exhibited growth defects on certain media. Whereas Δ*gpm2* grew on YPD with glucose as carbon source as wild type, a slight growth defect was observed on non-fermentable, suggesting a respiratory defect in these cells. A strong growth defect of Δ*gpm2* was observed in the presence of SDS indicating decreased stability of the plasma membrane and/or cell wall. Gph1p seems to play also a role in respiration and cell wall/plasma membrane organization because of the strongly reduced growth of the deletion mutant on lactate and SDS containing media. The Δ*gph1* deletion mutant also exhibits osmotic instability on sorbitol.

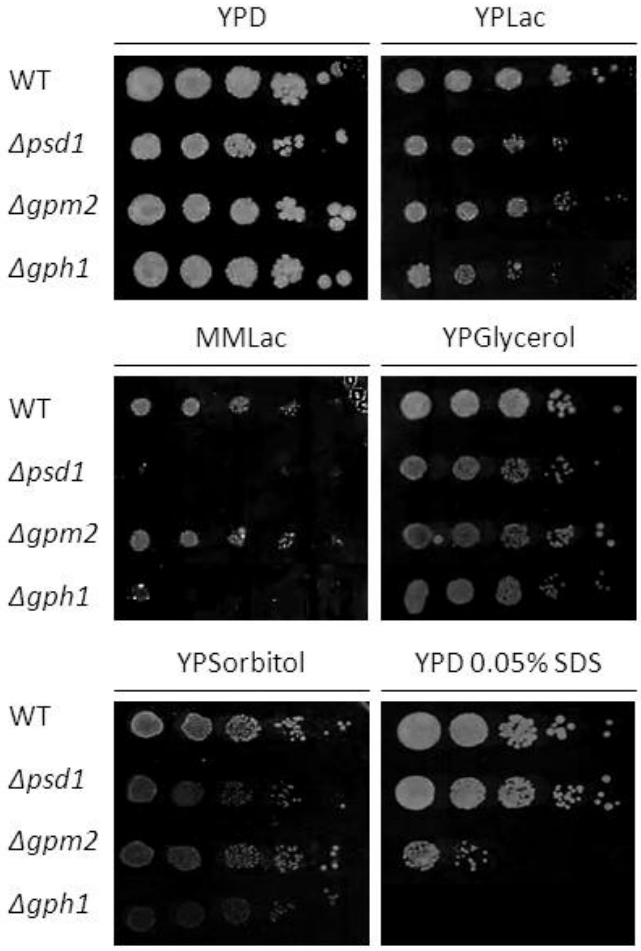

Figure 1. Growth analysis of yeast strains deleted of genes up-regulated in the absence of *PSD1*.
Strains as indicated were grown on YPD, YPLac, MMLac, YPGlycerol, YPSorbitol, and YPD with 0.05% SDS. Cell suspensions of strains listed were spotted at dilutions 1, 1/10, 1/100, 1/1000, and 1/10000. Incubation was carried out at 30°C.

Phospholipid analysis of deletion mutants compromised in genes up-regulated in Δpsd1

To estimate the possible involvement of the up-regulated genes in phospholipid metabolism we established phospholipid profiles of all deletion mutants. In wild type homogenate, the major phospholipids were phosphatidylcholine (PC), PE and phosphatidylinositol (PI) (Table 6). Lysophospholipids (LPL), phosphatidic acid (PA), cardiolipin (CL) and dimethylphosphatidylethanolamine (DMPE) were present only at minor amounts. As shown before [2,3,15,36,37] deletion of *PSD1* causes a depletion of PE in the cell homogenate and mitochondria compared to wild type. Interestingly, no major changes in the PE level were observed in total cell extracts of mutants detected of genes which were up-regulated in the absence of *PDS1*. Only in a strain deleted of *RSB1* a slight decrease of PE

Table 6. Phospholipid composition of cell-free homogenate, mitochondria and plasma membrane from cells grown on YPD. CF, cellular fraction; LPL, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid. Mean values of at least three measurements and standard deviations are shown.

		Phospholipids in cell-free homogenate, mitochondria and plasma membrane (mol %)							
CF	Strain	LPL	PI	PS	PC	PE	CL	DMPE	PA
Homogenate	WT	1.52±0.21	9.93±3.53	8.75±0.51	45.08±1.82	26.61±2.53	3.35±0.29	4.43±0.70	0.71±0.38
	Δpsd1	2.15±0.76	16.00±1.17	11.28±2.38	46.68±2.34	18.23±1.23	1.50±0.64	2.75±0.44	0.95±0.23
	Δgpm2	1.03±0.64	13.07±5.34	7.73±1.83	46.63±3.92	23.40±2.46	3.94±0.84	2.75±1.19	0.90±0.50
	Δgph1	0.62±0.33	14.04±0.74	9.18±0.85	39.30±1.01	25.66±0.47	2.13±0.37	6.85±0.52	1.73±1.30
	Δrsb1	1.04±0.13	17.16±1.14	10.50±1.03	40.36±0.08	22.64±0.91	2.84±0.50	3.51±0.62	1.76±0.62
Mitochondria	WT	1.92±1.11	8.06±1.72	4.07±0.60	40.65±2.38	30.35±1.36	4.97±3.64	6.58±2.54	2.38±0.60
	Δpsd1	2.12±0.46	8.80±3.67	7.31±1.54	46.98±5.42	23.86±3.96	2.87±1.77	3.23±1.56	4.78±2.21
	Δgpm2	1.53±0.44	10.79±2.45	3.68±1.02	45.14±2.27	26.98±1.13	7.34±1.79	1.56±0.35	2.73±0.62
	Δgph1	1.58±0.68	9.49±2.30	6.94±1.00	35.90±2.32	33.94±0.38	6.17±1.51	3.66±0.26	2.10±0.58
	Δrsb1	1.12±0.11	13.33±0.52	5.29±0.44	34.86±1.24	29.15±1.13	8.18±0.26	5.70±0.25	2.05±0.20
Plasma membrane	WT	2.26±0.96	12.38±2.12	26.18±2.79	18.18±1.38	32.06±4.14	0.69±0.10	2.23±0.43	5.76±0.61
	Δpsd1	2.80±0.53	17.11±4.11	27.27±3.20	21.27±3.32	23.47±3.61	0.64±0.32	1.90±0.30	5.30±2.18
	Δgpm2	2.19±0.70	12.90±1.71	24.31±3.02	21.36±1.93	30.94±3.25	0.73±0.34	1.91±0.64	5.42±1.26
	Δgph1	1.88±0.96	14.82±2.93	24.90±3.96	11.04±2.25	36.27±2.77	0.58±0.27	2.44±0.55	7.84±3.13
	Δrsb1	1.49±0.40	13.63±0.44	24.74±2.98	20.17±1.44	31.47±2.71	0.12±0.17	2.21±0.62	5.72±0.60

was measured which was accompanied by an increase in PI, PS and PA (see Table 6). However, changes in the phospholipid pattern were also detected in Δ*gpm2* and Δ*gph1*. In Δ*gpm2* these changes were moderate, but in Δ*gph1* markedly increased levels of PI, DMPE and PA mainly at the expense of PC were detected.

To address possible effects of the lipid profile on respiration and osmotic stability as suggested by the growth phenotype analysis (see Figure 1) we analyzed isolated mitochondrial and plasma membrane fractions of the respective strains. In the Δ*gpm2* mutant, a 75% reduction of the DMPE level in mitochondria was observed resembling the effect in Δ*psd1*. DMPE is an intermediate product in the methylation pathway from PE to PC catalyzed by Cho2p and Opi3p. Surprisingly, PC levels in the homogenate and especially in mitochondria of Δ*gpm2* were increased over the wild type suggesting that production of PC through the alternative biosynthetic route, the so-called Kennedy pathway, was enhanced. As another marked feature of the Δ*gpm2* strain the CL level in mitochondria was increased. No significant changes were observed in the phospholipid pattern of the plasma membrane.

Major changes in mitochondria of the Δ*gph1* mutant were reduction of the PC and DMPE levels and an increase of PI and CL. The reduction of PC was even more pronounced in the plasma membrane, where it was reduced to 60% of wild type. The decrease in the PC level was compensated by increased amounts of PE, PI and PA. Deletion of the *RSB1* gene caused a dramatic increase in PI in the homogenate over the wild type at the expense of PC and PE. Changes in PI and PC levels were also detected in mitochondria, whereas the plasma membrane was not affected.

Subcellular localization of Gph1p, Gpm2p and Rsb1p analyzed by fluorescence microscopy

According to the SGD GO Slim Mapper (see Methods section), Gph1p, Gpm2p and Rsb1p are localized to the cytosol of yeast cells. This view was only partially confirmed in

our fluorescence microscopy analysis of yeast strains bearing the respective GFP-tagged hybrids. Figure 2 demonstrates localization of GFP-Gpm2p throughout the cytoplasm. In contrast, GFP-Rsb1p was localized to the cell periphery in the form of distinct spots in or close to the plasma membrane. These spots may be specific domains of the plasma membrane or parts of the ER which are closely associated with the plasma membrane (plasma membrane associated membranes; PAM) [19].

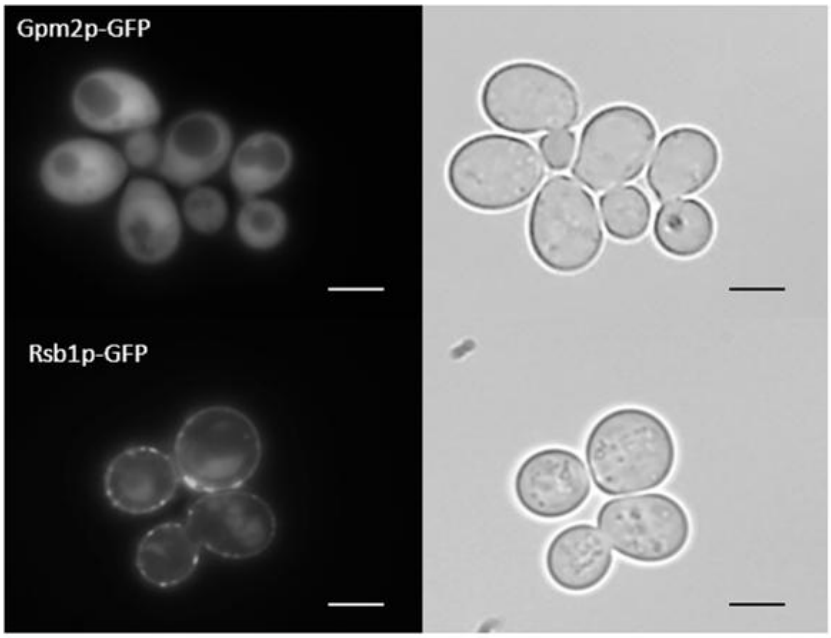

Figure 2. Subcellular localization of Gpm2p-GFP and Rsb1p-GFP by fluorescence microscopy.
Fluorescence microscopy was carried out as described in the Methods section. Cells were grown in minimal medium –ura –met at 30°C to the late exponential phase and GFP fluorescence (left panel) was detected as described. Corresponding transmission microscopy of cells is shown in the right panel Subcellular distribution of Gpm2p-GFP (upper lane) and Rsb1p-GFP (lower lane) is shown. The size of the scale bar is 1 µm.

Fluorescence microscopy revealed that GFP-Gph1p is localized to distinct spots in the cytoplasm (Figure 3). Gph1p is a glycogen phosphorylase, which degrades glycogen as carbohydrate source under conditions of nutrient limitation in the stationary phase and was reported to be associated with glycogen particles [38,39]. The present study confirms this

localization of GFP-Gph1p. We also studied the subcellular distribution of GFP-Gph1p during various growth phases of the cell, namely in the lag phase (5h), middle (15h) and late (22h) exponentially phase and late stationary phase (55 h). Whereas only single dots of the fluorescent signal were detected in cells during the early growth phases, older cells (55h cultures) contained more of these particles. Additionally, diffuse fluorescence was observed throughout the cytoplasm, but not in the vacuole and in the nucleus. We excluded GFP-Gph1p accumulation in lipid droplets because staining of cells with Nile Red did not overlap with the signals of GFP-Gph1p (data not shown).

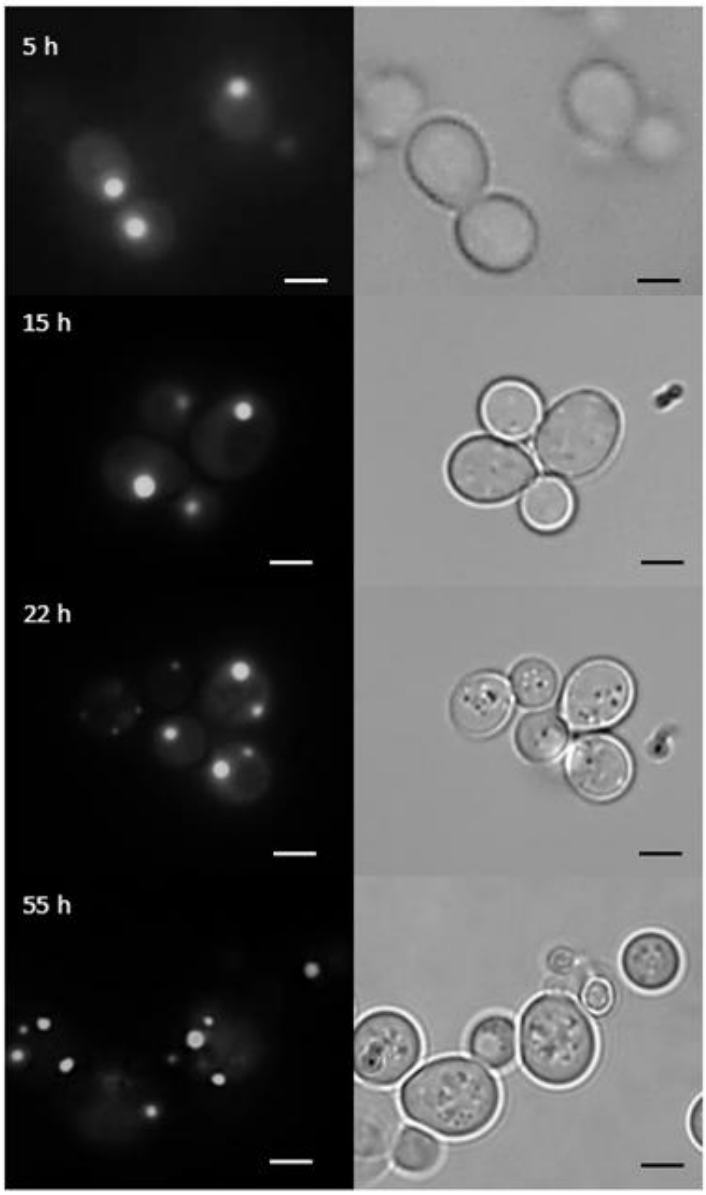

Figure 3. Subcellular localization of Gph1p-GFP by fluorescence microscopy.
Fluorescence microscopy was performed as described in the legend to Figure 2. GFP fluorescence (left panel) and transmission microscopy (right panel) of cells bearing a Gph1p-GFP hybrid are shown. Cells were grown in minimal medium –ura –met at 30°C and pictures were taken from cells at early (5h), middle (15h) and late (22h) exponentially phase and at the late stationary phase (55h). The size of the scale bar is 1 µm.

Discussion

In the yeast *Saccharomyces cerevisiae* as in most other eukaryotic cells, the major membrane phospholipids are PC, PE, PI and PS. Phospholipid metabolism is governed by a complex network of reactions which are subject to strict genetic and biochemical regulation (for recent review see [40]). ER and mitochondria are major sites of phospholipid synthesis [41] whereas other compartments such as the plasma membrane are devoid of phospholipid synthesizing enzymes [20]. Such membranes rely completely on the supply of lipids from other organelles.

The present study was focused on the global role of PE in the yeast and designed to study the genome wide response of *Saccharomyces cerevisiae* to PE depletion caused by deletion of *PSD1*. Psd1p is the major producer of PE in the yeast and localized to the inner mitochondrial membrane where it catalyzes decarboxylation of PS to PE [5,6,7]. For the present study a DNA-microarray analysis with a Δ*psd1* deletion mutant was performed. As described in the Results section, 54 genes were identified which were up-regulated in a Δ*psd1* deletion strain compared to wild type. The respective gene products serve several functions in diverse biological processes (see Tables 3-4). This large variety reflects the possible direct or indirect involvement of PE in many different cellular processes. As a matter of fact, DNA-microarray analysis provides information about genetic interactions, whereas functional links between reactions catalyzed by potentially interacting partner gene products are often hard to pinpoint. To narrow down the list of candidate genes which were detected in the DNA-microarray analysis we focused on growth phenotype and lipid analysis of mutants deleted of the identified genes. Surprisingly, only three mutants out of the collection of 54 candidates showed significant changes in growth phenotype and/or lipid profile, namely those affected in *GPM2*, *GPH1* and *RSB1*.

Gpm2p (glycerate phosphomutase 2) has been predicted as a non-functional homologue of Gpm1p which catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate in the early glycolytic pathway of the yeast [42]. However, the fact that *GPM2* was subject to transcriptional regulation and the Δ*gpm2* deletion mutant exhibited growth defects on fermentable and non-fermentable carbon sources rather qualified *GPM2* as a functional gene. Despite these findings, a straight forward role of Gpm2p in phospholipids synthesis or regulation was not detected by our analyses. A slight growth defect of the Δ*gpm2* deletion mutant on lactate may explain a possible involvement of Gpm2p in respiration or energy metabolism, supported by some changes in the mitochondrial phospholipid pattern. The strong sensitivity to SDS (see Figure 1) of Δ*gpm2* and the altered levels of DMPE and PC in bulk membranes may be an indication for a link to membrane lipid metabolism and function.

Gph1p (glycogen phosphorylase 1) is localized to particles present in the cytoplasm (see Figure 3). This finding is in line with the physiological role of Gph1p which catalyzes the release of glucose 1-phosphate from glycogen and associates with glycogen particles [38,39]. In the stationary phase the distribution of GFP-tagged Gph1p was changing and the number of spots increased. This observation is most likely due to glycogen particle degradation under condition of starvation. Gph1p is known to be activated by a phosphorylase kinase and a cAMP dependent protein kinase, whereas glucose 6-phosphate, a product of glycolysis, inhibits Gph1p [38]. Therefore, Gph1p becomes only active when glucose 6-phosphate levels are decreasing. Whether or not these known functions of Gph1p are correlated with the novel observation that this protein is obviously also involved in the regulation of lipid metabolism (see Table 6) remains to be demonstrated. The growth defect of Δ*gph1* on non-fermentable carbon sources and in the presence of SDS (see Figure 1) combined with the changes of the lipid profile both in bulk membranes and especially in the plasma membrane (see Table 6)

strongly supports this view. More detailed studies are currently in progress (Gsell et al., manuscript in preparation).

Among the short-listed candidate genes affected by *PSD1* deletion, *RSB1* is the only one with a direct link to lipid metabolism. It was suggested that Rsb1p (resistance to sphingoid long-chain base) is involved in lipid translocation across the plasma membrane [43,44,45]. It was suggested that Rsb1p may function as a transporter or flippase translocating LCBs (sphingoid long-chain bases) from the cytoplasmic side to the extracytoplasmic side of the plasma membrane [43]. Furthermore, it was found that the expression of Rsb1p was increased in cells with altered phospholipid asymmetry of the plasma membrane to compensate for compromised membrane functions by inappropriate distribution of LCBs between the inner and outer leaflet [46]. This finding may be linked to the observed up-regulation of *RSB1* under depletion of PE caused by *PSD1* deletion. Depletion of PE in Δ*psd1* causes changes in the phospholipid pattern of the plasma membrane. To compensate for possible defects due to these changes, expression of *RSB1* may be enhanced to maintain membrane stability. In a Δ*rsb1* mutant the phospholipid pattern of the plasma membrane was not changed (see Table 6). However, moderate changes in the total cell extract and also in mitochondria such as decrease of PE and PC accompanied by an increase in PI might indicate an influence of Rsb1p on cellular lipid homeostasis.

In summary, our study identified a network of genes linked to function of *PSD1* in the yeast. Processes affected by depletion of PE appear to wide spread (see Table 3-5) although defects may be secondary effects of changes in membrane behavior caused by PE depletion. At least in the cases of *GPM2*, *GPH1* and *RSB1*, a direct link to lipid metabolism and growth phenotype was established.

Acknowledgement

This work was supported by the Austrian Science Fund FWF (project 21429 and DK Molecular Enzymology W901-B05 to GD).

References

1. Birner R, Bürgermeister M, Schneiter R, Daum G (2001) Roles of phosphatidyl-ethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*. Mol Biol Cell 12: 997-1007.

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

3. Storey MK, Clay KL, Kutateladze T, Murphy RC, Overduin M, et al. (2001) Phosphatidylethanolamine has an essential role in *Saccharomyces cerevisiae* that is independent of its ability to form hexagonal phase structures. J Biol Chem 276: 48539-48548.

4. Hui SW, Stewart TP, Yeagle PL, Albert AD (1981) Bilayer to non-bilayer transition in mixtures of phosphatidylethanolamine and phosphatidylcholine: implications for membrane properties. Arch Biochem Biophys 207: 227-240.

5. Carson MA, Emala M, Hogsten P, Waechter CJ (1984) Coordinate regulation of phosphatidylserine decarboxylase activity and phospholipid N-methylation in yeast. J Biol Chem 259: 6267-6273.

6. Trotter PJ, Pedretti J, Voelker DR (1993) Phosphatidylserine decarboxylase from Saccharomyces cerevisiae. Isolation of mutants, cloning of the gene, and creation of a null allele. J Biol Chem 268: 21416-21424.

7. Kuchler K, Daum G, Paltauf F (1986) Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in *Saccharomyces cerevisiae*. J Bacteriol 165: 901-910.

8. Trotter PJ, Voelker DR (1995) Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (*PSD2*) in the yeast *Saccharomyces cerevisiae*. J Biol Chem 270: 6062-6070.

9. Kennedy EP, Weiss SB (1956) The function of cytidine coenzymes in the biosynthesis of phospholipids. J Biol Chem 222: 193-214.

10. Birner R, Daum G (2003) Biogenesis and cellular dynamics of aminoglycerophospholipids. Int Rev Cytol 225: 273-323.

11. Daum G, Lees ND, Bard M, Dickson R (1998) Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast 14: 1471-1510.

12. Riekhof WR, Wu J, Jones JL, Voelker DR (2007) Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in *Saccharomyces cerevisiae*. J Biol Chem 282: 28344-28352.

13. Riekhof WR, Voelker DR (2006) Uptake and utilization of lyso-phosphatidylethanolamine by *Saccharomyces cerevisiae*. J Biol Chem 281: 36588-36596.

14. Schuiki I, Schnabl M, Czabany T, Hrastnik C, Daum G (2010) Phosphatidylethanolamine synthesized by four different pathways is supplied to the plasma membrane of the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1801: 480-486.

15. Nebauer R, Schuiki I, Kulterer B, Trajanoski Z, Daum G (2007) The phosphatidylethanolamine level of yeast mitochondria is affected by the mitochondrial components Oxa1p and Yme1p. FEBS J 274: 6180-6190.

16. Güldener U, Heck S, Fiedler T, Beinhauer J, Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24: 2519-2524.

17. Serrano R (1988) H^+-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. Methods Enzymol 157: 523-544.

18. van den Hazel HB, Pichler H, do Valle Matta MA, Leitner E, Goffeau A, et al. (1999) *PDR16* and *PDR17*, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs. J Biol Chem 274: 1934-1941.

19. Pichler H, Gaigg B, Hrastnik C, Achleitner G, Kohlwein SD, et al. (2001) A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids. Eur J Biochem 268: 2351-2361.

20. Zinser E, Sperka-Gottlieb CDM, Fasch EV, Kohlwein SD, Paltauf F, et al. (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. J Bacteriol 173: 2026-2034.

21. Daum G, Böhni PC, Schatz G (1982) Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. J Biol Chem 257: 13028-13033.

22. Zinser E, Daum G (1995) Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*. Yeast 11: 493-536.

23. Lowry CV, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

24. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

25. Haid A, Suissa M (1983) Immunochemical identification of membrane proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Methods Enzymol 96: 192-205.

26. Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy - analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20: 307-315.

27. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article 3.

28. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. (2000) Gene Ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25: 25-29.

29. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226: 497-509.

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

31. Luo Z, van Vuuren HJ (2009) Functional analyses of *PAU* genes in *Saccharomyces cerevisiae*. Microbiology 155: 4036-4049.

32. Rachidi N, Martinez MJ, Barre P, Blondin B (2000) *Saccharomyces cerevisiae PAU* genes are induced by anaerobiosis. Mol Microbiol 35: 1421-1430.

33. Sertil O, Cohen BD, Davies KJ, Lowry CV (1997) The *DAN1* gene of *S. cerevisiae* is regulated in parallel with the hypoxic genes, but by a different mechanism. Gene 192: 199-205.

34. Donzeau M, Bourdineaud JP, Lauquin GJ (1996) Regulation by low temperatures and anaerobiosis of a yeast gene specifying a putative GPI-anchored plasma membrane. Mol Microbiol 20: 449-459.

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

36. Bürgermeister M, Birner-Grünberger R, Nebauer R, Daum G (2004) Contribution of different pathways to the supply of phosphatidylethanolamine and

phosphatidylcholine to mitochondrial membranes of the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1686: 161-168.

37. Gohil VM, Thompson MN, Greenberg ML (2005) Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine and cardiolipin biosynthetic pathways in *Saccharomyces cerevisiae*. J Biol Chem 280: 35410-35416.

38. Hwang PK, Tugendreich S, Fletterick RJ (1989) Molecular analysis of *GPH1*, the gene encoding glycogen phosphorylase in *Saccharomyces cerevisiae*. Mol Cell Biol 9: 1659-1666.

39. Wilson WA, Boyer MP, Davis KD, Burke M, Roach PJ (2010) The subcellular localization of yeast glycogen synthase is dependent upon glycogen content. Can J Microbiol 56: 408-420.

40. Henry SA, Kohlwein SD, Carman GM (2012) Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. Genetics 190: 317-349.

41. Daum G, Vance JE (1997) Import of lipids into mitochondria. Prog Lipid Res 36: 103-130.

42. Heinisch J, Müller S, Schlüter E, Jacoby J, Rodicio R (1998) Investigation of two yeast genes encoding putative isoenzymes of phosphoglycerate mutase. Yeast 14: 203-213.

43. Kihara A, Igarashi Y (2002) Identification and characterization of a *Saccharomyces cerevisiae* gene, *RSB1*, involved in sphingoid long-chain base release. J Biol Chem 277: 30048-30054.

44. Johnson SS, Hanson PK, Manoharlal R, Brice SE, Cowart LA, et al. (2010) Regulation of yeast nutrient permease endocytosis by ATP-binding cassette transporters and a seven-transmembrane protein, RSB1. J Biol Chem 285: 35792-35802.

45. Manente M, Ghislain M (2009) The lipid-translocating exporter family and membrane phospholipid homeostasis in yeast. FEMS Yeast Res 9: 673-687.

46. Kihara A, Igarashi Y (2004) Cross talk between sphingolipids and glycerophospholipids in the establishment of plasma membrane asymmetry. Mol Biol Cell 15: 4949-4959.

Supplement Table S1. List of differentially expressed genes in *Δpsd1* versus wild type

Gene ID	Gene Symbols	Gene Title	logFC	*p* value
YNL169C	*PSD1*	Phosphatidylserine decarboxylase of the mitochondrial inner membrane, converts phosphatidylserine to phosphatidylethanolamine	-6,91	2,51E-11
YLR327C	*TMA10*	Protein of unknown function that associates with ribosomes; putative homolog of the F1F0-ATPase synthase regulator Stf2p	1,77	3,21E-05
YEL011W	*GLC3*	Glycogen branching enzyme, involved in glycogen accumulation; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern	1,07	0,00053906
YBR047W	*FMP23*	Putative protein of unknown function; proposed to be involved in iron or copper homeostasis; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies	1,12	0,00071439
YBR147W	*RTC2*	Putative vacuolar membrane transporter for cationic amino acids; likely contributes to amino acid homeostasis by exporting cationic amino acids from the vacuole; positive regulation by Lys14p suggests that lysine may be the primary substrate; member of the PQ-loop family, with seven transmembrane domains; similar to mammalian PQLC2 vacuolar transporter	1,14	0,00072053
YIL101C	*XBP1*	Transcriptional repressor that binds to promoter sequences of the cyclin genes, CYS3, and SMF2; expression is induced by stress or starvation during mitosis, and late in meiosis; member of the Swi4p/Mbp1p family; potential Cdc28p substrate	2,09	0,0011918
YDL169C	*UGX2*	Protein of unknown function, transcript accumulates in response to any combination of stress conditions	1,16	0,00154061
YMR251W	*GTO3*	Omega class glutathione transferase; putative cytosolic localization	1,58	0,00154115
YNL237W	*YTP1*	Probable type-III integral membrane protein of unknown function, has regions of similarity to mitochondrial electron transport proteins	1,2	0,00156358
YDL021W	*GPM2*	Homolog of Gpm1p phosphoglycerate mutase which converts 3-phosphoglycerate to 2-phosphoglycerate in glycolysis; may be non-functional derivative of a gene duplication event	1,09	0,00168495
YML100W	*TSL1*	Large subunit of trehalose 6-phosphate synthase (Tps1p)/phosphatase (Tps2p) complex, which converts uridine-5'-diphosphoglucose and glucose 6-phosphate to trehalose, similar to Tps3p and may share function; mutant has aneuploidy tolerance	1,41	0,00179942
YKR076W	*ECM4*	Omega class glutathione transferase; not essential; similar to Ygr154cp; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm	1,01	0,00198524

YOR185C	*GSP2*	GTP binding protein (mammalian Ranp homolog) involved in the maintenance of nuclear organization, RNA processing and transport; interacts with Kap121p, Kap123p and Pdr6p (karyophilin betas); Gsp1p homolog that is not required for viability	1,01	0,00199361
YAL061W	*BDH2*	Putative medium-chain alcohol dehydrogenase with similarity to BDH1; transcription induced by constitutively active *PDR1* and *PDR3*	1,81	0,00210168
YLR149C	-	Putative protein of unknown function; overexpression causes a cell cycle delay or arrest; null mutation results in a decrease in plasma membrane electron transport	1,3	0,00224792
YOR384W	*FRE5*	Putative ferric reductase with similarity to Fre2p; expression induced by low iron levels; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies	2,09	0,00251527
YLR346C	-	Putative protein of unknown function found in mitochondria; expression is regulated by transcription factors involved in pleiotropic drug resistance, Pdr1p and Yrr1p; YLR346C is not an essential gene	1,99	0,00255215
YDR534C	*FIT1*	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall	1,72	0,00360081
YHR087W	*RTC3*	Protein of unknown function involved in RNA metabolism; has structural similarity to SBDS, the human protein mutated in Shwachman-Diamond Syndrome (the yeast SBDS ortholog = *SDO1*); null mutation suppresses cdc13-1 temperature sensitivity	1,58	0,00408903
YGL121C	*GPG1*	Proposed gamma subunit of the heterotrimeric G protein that interacts with the receptor Gpr1p; involved in regulation of pseudohyphal growth; requires Gpb1p or Gpb2p to interact with Gpa2p; overproduction causes prion curing	1,5	0,00470336
YPR160W	*GPH1*	Non-essential glycogen phosphorylase required for the mobilization of glycogen, activity is regulated by cyclic AMP-mediated phosphorylation, expression is regulated by stress-response elements and by the HOG MAP kinase pathway	1,28	0,00480749
YNL194C	-	Integral membrane protein required for sporulation and plasma membrane sphingolipids content; has sequence similarity to *SUR7* and *FMP45* ; GFP-fusion protein is induced in response to the DNA-damaging agent MMS	1,18	0,00498808
YJR005C-A	-	Putative protein of unknown function, originally identified as a syntenic homolog of an *Ashbya gossypii* gene	2,46	0,00510371
YOR289W	-	Putative protein of unknown function; transcription induced by the unfolded protein response; green fluorescent protein (GFP)-fusion protein localizes to both the cytoplasm and the nucleus	1,15	0,0052983

YKL071W	-	Putative protein of unknown function; expression induced in cells treated with the mycotoxin patulin, and also the quinone methide triterpene celastrol; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm	1	0,00538264
YHL047C	*ARN2*	Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; responsible for uptake of iron bound to the siderophore triacetylfusarinine C	1,51	0,00553745
YFR053C	*HXK1*	Hexokinase isoenzyme 1, a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-induced repression involves the hexokinase Hxk2p	1,22	0,00574348
YJL105W	*SET4*	Protein of unknown function, contains a SET domain	1,5	0,00588631
YOR383C	*FIT3*	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall	1,11	0,00627485
YOR049C	*RSB1*	Suppressor of sphingoid long chain base (LCB) sensitivity of an LCB-lyase mutation; putative integral membrane transporter or flippase that may transport LCBs from the cytoplasmic side toward the extracytoplasmic side of the membrane	1,58	0,00642199
YDR406W	*PDR15*	Plasma membrane ATP binding cassette (ABC) transporter, multidrug transporter and general stress response factor implicated in cellular detoxification; regulated by Pdr1p, Pdr3p and Pdr8p; promoter contains a PDR responsive element	1,22	0,00642509
YMR105C	*PGM2*	Phosphoglucomutase, catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate, which is a key step in hexose metabolism; functions as the acceptor for a Glc-phosphotransferase	1,18	0,00662036
YER037W	*PHM8*	Lysophosphatidic acid (LPA) phosphatase involved in LPA hydrolysis in response to phosphate starvation; phosphatase activity is soluble and Mg2+ dependent; expression is induced by low phosphate levels and by inactivation of Pho85p	1,03	0,00663793
YIR041W	*PAU15*	Part of 23-member seripauperin multigene family encoded mainly in subtelomeric regions, active during alcoholic fermentation, regulated by anaerobiosis, negatively regulated by oxygen, repressed by heme /// Hypothetical protein /// Putative protein of unknown function	1,59	0,00697615
YCR104W	*PAU3*			
YKL224C	*PAU16*			
YMR325W	*PAU19*			
YOR394W	*PAU21*			
YPL282C	*PAU22*			

YGL156W	*AMS1*	Vacuolar alpha mannosidase, involved in free oligosaccharide (fOS) degradation; delivered to the vacuole in a novel pathway separate from the secretory pathway	1,43	0,00717477
YBR072W	*HSP26*	Small heat shock protein (sHSP) with chaperone activity; forms hollow, sphere-shaped oligomers that suppress unfolded proteins aggregation; oligomer activation requires a heat-induced conformational change; also has mRNA binding activity	1,8	0,00788137
YFL020C	*PAU5*	Member of the seripauperin multigene family encoded mainly in subtelomeric regions; induced during alcoholic fermentation; induced by low temperature and also by anaerobic conditions; negatively regulated by oxygen repressed by heme	1,22	0,00831995
YJL052W	*TDH1*	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 1, involved in glycolysis and gluconeogenesis; tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; detected in the cytoplasm and cell-wall	1,06	0,00832004
YHL040C	*ARN1*	Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; responsible for uptake of iron bound to ferrirubin, ferrirhodin, and related siderophores	1,05	0,00885204
YHR096C	*HXT5*	Hexose transporter with moderate affinity for glucose, induced in the presence of non-fermentable carbon sources, induced by a decrease in growth rate, contains an extended N-terminal domain relative to other HXTs	1,74	0,00899573
YGR066C	-	Putative protein of unknown function	1,57	0,00974168
YOL052C-A	*DDR2*	Multistress response protein, expression is activated by a variety of xenobiotic agents and environmental or physiological stresses; also known as: DDRA2	1,56	0,01090044
YGR248W	*SOL4*	6-phosphogluconolactonase with similarity to Sol3p	1,14	0,01197417
YJR150C	*DAN1*	Cell wall mannoprotein with similarity to Tir1p, Tir2p, Tir3p, and Tir4p; expressed under anaerobic conditions, completely repressed during aerobic growth	2,72	0,01355517
YOR382W	*FIT2*	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall	1,34	0,01460368
YMR317W	-	Putative protein of unknown function with some similarity to sialidase from Trypanosoma	1,33	0,01474489
YFL014W	*HSP12*	Plasma membrane protein involved in maintaining membrane organization in stress conditions; induced by heat shock, oxidative stress, osmostress, stationary phase, glucose depletion, oleate and alcohol; regulated by HOG and Ras-Pka pathways	2,05	0,01889002

YOR009W	*TIR4*	Cell wall mannoprotein of the Srp1p/Tip1p family of serine-alanine-rich proteins; expressed under anaerobic conditions and required for anaerobic growth; transcription is also induced by cold shock	1,18	0,03393083
YOR237W	*HES1*	Protein implicated in the regulation of ergosterol biosynthesis; one of a seven member gene family with a common essential function and non-essential unique functions; similar to human oxysterol binding protein (OSBP)	1,07	0,03526453
YLR136C	*TIS11*	mRNA-binding protein expressed during iron starvation; binds to a sequence element in the 3'-untranslated regions of specific mRNAs to mediate their degradation; involved in iron homeostasis	1,27	0,04999745

Chapter 3

A yeast mutant deleted of *GPH1* bears defects in lipid metabolism

Martina Gsell[a], Gerald Mascher[a], Günther Zellnig[b] and Günther Daum[a,*]

[a]Institute of Biochemistry, Graz University of Technology,

Petersgasse 12/2, 8010 Graz, Austria

[b]Institute of Plant Sciences, Karl Franzens University Graz, Austria

*Corresponding author:
Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria.
e-mail: guenther.daum@tugraz.at; Tel.: +43 316 873 6462; Fax.: +43 316 873 6952

Keywords: phosphatidylethanolamine, phosphatidylserine decarboxylase, glycogene phosphorylase, triacylglycerol, lipid droplet, yeast

Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPE, dimethylethanolamine; TG, triacylglycerol; DG, diacylglycerol; SE, steryl ester

March 2013, Manuscript in preparation

Abstract

In a previous study we identified up-regulation of the yeast *GPH1* gene under conditions of phosphatidylethanolamine depletion caused by deletion of the mitochondrial phosphatidylserine decarboxylase 1 (Gsell et al., manuscript submitted). Gph1p has originally been identified as a glycogen phosphorylase catalyzing degradation of glycogen to glucose in the stationary growth phase of the yeast. Here we show that deletion of this gene causes decreased levels of phosphatidylcholine, triacylglycerols and steryl esters. Depletion of the two non-polar lipids in a Δ*gph1* strain leads to a lack of lipid droplets. Moreover, in such mutants the stability of the plasma membrane appears to be compromised. *In vivo* labeling experiments revealed that both pathways of phosphatidylcholine biosynthesis, the CDP-choline and the methylation route, are negatively affected by a Δ*gph1* mutation. These findings suggest that Gph1p plays a regulatory role in yeast lipid metabolism, although its specific molecular function remained unclear.

Introduction

Cellular lipids fulfill three major functions. First, they provide a depot and source of energy especially in the form of triacylglycerols (TG) and steryl esters (SE) which are stored in lipid droplets [1]. Secondly, certain classes of lipids such as glycerophospholipids, sterols, sphingolipids and glycolipids are important components of biological membrane. Finally, lipids can act as cellular messengers [2]. Depending on the environmental and nutritional conditions one or the other function of lipids becomes paramount. Importantly, pathways of lipid storage/mobilization and membrane lipid biosynthesis are interlinked thus providing a means to switch within lipid metabolism to the required branches. As examples, fatty acids from TG and/or SE can either be used for energy production through β-oxidation or as building blocks for the synthesis of membrane lipids. Diacylglycerol (DG), the other degradation product of TG hydrolysis, can be utilized as a substrate for phospholipid synthesis and consequently a key intermediate in membrane lipid formation [2,3,4,5,6] but also as a second messenger.

As a consequence of the above mentioned scenario, lipid metabolism is a highly complex network of reactions which are subject to sophisticated regulation. During the last few years our laboratory focused on the central role of the phospholipid phosphatidylethanolamine (PE) in lipid metabolism. PE is essential in the yeast *Saccharomyces cerevisiae* and one of the major phospholipids besides phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS). The mitochondrial phosphatidylserine decarboxylase 1 (Psd1p) is the major producer of PE catalyzing the decarboxylation of PS to form PE [7,8]. The other three pathways of PE synthesis, namely decarboxylation of PS by Psd2p [9], the CDP-ethanolamine branch of the so-called Kennedy pathway [10], and synthesis of PE through acylation of lyso-PE catalyzed by the acyl-CoA-

dependent acyltransferase Ale1p [11] are less efficient under standard growth conditions. Inactivation or deletion of the *PSD1* gene leads to a considerable decrease of PE in total cellular and mitochondrial membranes and to a number of cellular defects [12,13].

To investigate effects of PE depletion caused by Δ*psd1* deletion on a genome wide basis we performed DNA microarray analysis of a Δ*psd1* mutant and compared its gene expression pattern with wild type (Gsell et al. manuscript submitted). This analysis revealed up-regulation of 54 genes in the Δ*psd1* mutant. One of the genes highlighted in this analysis was *GPH1*. Further analysis of the growth phenotype and phospholipid composition suggested a possible involvement of *GPH1* in lipid metabolism. Gph1p was originally identified as glycogen phosphorylase 1 catalyzing the release of glucose 1-phosphate from glycogen in the late stationary growth phase of the yeast to maintain the required energy for cell activity and growth during periods of nutrient starvation. The activity of glycogen phosphorylase is regulated by cyclic AMP-mediated phosphorylation of the enzyme. *GPH1* is not essential in yeast, but Δ*gph1* mutants lacking the phosphorylase activity exhibit increased levels of intracellular glycogen [14].

In previous studies it has been shown that Gph1p is localized on so-called glycogen particles [14,15] whose size and amount are changing during the growth phases of the yeast cell (Gsell et al., manuscript submitted). Glycogen, which is a storage form of carbon and energy, consists of branched glucose polymers and is synthesized by many different organisms [15]. Degradation of glycogen occurs when energy is required for cell activity and growth during stationary phase. In the yeast, expression of *GPH1* is induced at the end of the logarithmic growth phase. Almost simultaneously intracellular glycogen starts to accumulate as long as carbon sources are present. This finding suggested an important role of Gph1p in glycogen utilization as a reserve energy source during periods of nutrient starvation. Gph1p is inhibited by glucose 6-phosphate, and its activity is regulated by reversible phosphorylation

[14]. The Δ*gph1* deletion mutant accumulates large amounts of glycogen during the stationary phase and shows rapid chronological aging and low stress tolerance [16].

A more detailed analysis of *GPH1* and its gene product presented here shows that the Δ*gph1* deletion mutant exhibits decreased PC levels in total cell homogenates and especially in the plasma membrane. Moreover, deletion of *GPH1* caused changes in the metabolism of non-polar lipids. It appears that Gph1p is involved in specific branches of lipid metabolism but at the same time has more global effects on cell metabolism. The multiple function of Gph1p with emphasis on regulating aspects in lipid metabolism is discussed.

Materials and Methods

Strains and media

The yeast strains used in this study are listed in Table 1. Cells were grown in liquid YPD media (1% yeast extract, 2% peptone and 2% glucose) under aerobic conditions with shaking at 30°C. Growth tests on solid media were performed on 1% yeast extract, 2% peptone, and 2% agar supplemented with 2% glucose, 2.66% lactate (adjusted to pH 5.5 with KOH), 2% glycerol or 8 mM sorbitol, respectively. For SDS resistance assays on solid media, 0.05% SDS was added to the media immediately prior to pouring plates. MMLac (minimal medium with lactate) culture plates contained 0.67% yeast nitrogen base without amino acids, 0.073% amino acid mix, 2.66% lactate, adjusted to pH 5.5 with KOH and 2% agar.

For growth phenotype analysis cell suspensions of overnight cultures grown on YPD were spotted at dilutions 1, 1/10, 1/100, 1/1000 and 1/10000 on YPD, YPLac, MMLac, YPGlycerol, YPSorbitol and YPD with 0.05% SDS. Incubations were carried out at 30°C.

Table 1
Yeast strains used in this study

Strain	Genotype	Source/reference
BY4741	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	Euroscarf
Δ*gph1*	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *gph*1Δ::KanMX4	Euroscarf
Δ*psd1*	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *psd1*Δ::KanMX4	Euroscarf
Δ*gph1*Δ*psd1*	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *gph1*Δ::KanMX4 *psd1*Δ::His3MX6	This study

Strain constructions

Inactivation of the *PSD1* gene in a Δ*gph1* deletion mutant was performed by transformation of the Δ*psd1*::His3MX6 disruption cassette as described by Longtine et al. [17]. The His3MX6 disruption cassette was amplified from the pFA6a-His3MX6 plasmid using the primers PSD1-koF 5'-gccagttaagaacgccttggcgcaagggaggacgctcctccggatccccgggttaattaa-3' and PSD1-koR 5'-caggtatgtggttccaagtgtttgtcgc -3'.

The disruption cassette was amplified by PCR under standard conditions using the proof reading *Ex Taq*^{TM}-DNA polymerase (Takara). The cassette was introduced into the respective strain by lithium acetate transformation [18]. Correct insertion of the cassette was tested by growing strains on selective media. Identity of strains was confirmed by marker-dependent growth, colony PCR and sequencing.

Isolation of plasma membrane and mitochondria

Yeast cells were grown aerobically in YPD to the early stationary growth phase at 30°C. Then, cells were disrupted with glass beads using a Merckenschlager homogenizer under CO_2-cooling. Cell extracts were cleared of glass beads, unbroken cells and cell debris by centrifugation at 2,500 x g for 5 min. The supernatant fraction represented the homogenate.

Crude plasma membrane was isolated essentially as described by Serrano [19] and further purified as reported by van den Hazel et al. [20] and Pichler et al. [21].

To isolate mitochondria, spheroplasts were prepared and homogenized in breaking buffer consisting of 0.6 M mannitol, 10 mM Tris, pH 7.4 and 1 mM PMSF by using a Dounce homogenizer as described previously [22]. Unbroken cells and debris were removed by centrifugation at 3,000 x g for 5 min. The resulting supernatant was used to isolate mitochondria by published procedures [23].

Relative enrichment of markers and cross-contamination of subcellular fractions were assessed as described by Zinser and Daum [24]. Protein was quantified by the method of Lowry et al. [25] using bovine serum albumin (BSA) as standard. SDS-PAGE was carried out as published by Laemmli [26]. Western blot analysis of proteins from subcellular fractions was performed as described by Haid and Suissa [27]. Immunoreactive bands were visualized by enzyme-linked immunosorbent assay using a peroxide-linked secondary antibody (Sigma-Aldrich, St. Louis, MO) following the manufacturer´s instructions.

Lipid analysis

Lipids from yeast cells were extracted as described by Folch et al. [28]. For phospholipid analysis 3 mg protein from total cell homogenate or 2 mg protein from mitochondria and plasma membrane fractions, respectively, were extracted using 3 ml chloroform/methanol (2:1; per vol.). 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 (50:25:6; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second developing solvents. Phospholipids were stained with iodine vapor, scraped off the plate, and quantified by the method of Broekhuyse [29].

For quantification of neutral lipids, lipid extracts were applied to Silica Gel 60 plates, and chromatograms were developed in an ascending manner by a two-step developing system [30]. First, chromatograms were developed using light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) and then light petroleum:diethyl ether (49:1; per vol.) as solvents. To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g $MnCl_2$ x $4H_2O$, 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 triolein, cholesteryl ester and ergosterol as standards, using a Shimadzu dual-wave length chromatoscanner CS-930. For DG analysis chromatograms were developed in chloroform/acetone/acetic acid (45:4:0.5; per vol.) with diolein as standard.

Metabolic labeling of phospholipids and neutral lipids

Labeling of aminoglycerophospholipids *in vivo* was determined by following the incorporation of L-[^{3}H]serine or [methyl-^{14}C]choline chloride, respectively, into PS, PE and PC as described [13,31]. An equivalent of 10 OD_{600} from an overnight culture (~1 ml, corresponding to 1.45 x 10^8 cells) was harvested in a Pyrex tube, washed once, suspended in 500 µl YPD and incubated for 30 min at 30°C. Cells were labeled with 10 µCi [^{3}H]serine (21.99 Ci·mmol^{-1}, Perkin-Elmer, Boston, MA) or 10 µCi [methyl-^{14}C]choline chloride (54 mCi·mmol^{-1}, Perkin Elmer Boston, MA), respectively, for 1 hour at 30°C. Samples were put on ice, harvested by centrifugation and shock frozen with liquid nitrogen. Chloroform/methanol (2:1; per vol.) and glass beads were added to the cell pellets and samples were vigorously shaken on an IKA® Vibrax VXR for 1 hour. Then, lipids were extracted by the method of Folch et al. [28]. Individual phospholipids were separated by TLC on Silica gel 60 plates (Merck, Darmstadt, Germany) with chloroform/methanol/25% NH_3 (50:25:6, per vol.) as developing solvent. Spots on TLC plates were stained with iodine vapor,

scraped off and suspended in 8 ml scintillation cocktail (Packard Bio-Science, Groningen, the Netherlands) containing 5% water. Radioactivity was determined by liquid scintillation counting using a Packard TriCarb® Liquid Scintillation Analyzer.

To estimate the incorporation of [1-^{14}C]acetic acid *in vivo* into total phospholipids and non-polar lipids an equivalent of 10 OD_{600} from an overnight culture was harvested, washed and suspended in 500 µl YPD. After an incubation of 30 min at 30°C, cells were labeled with 0.5 µCi [1-^{14}C]acetic acid (55.3 mCi mmol^{-1}, Perkin Elmer, Boston, MA) for 0, 10, 20, 30, 60 and 120 min, respectively. Lipids were extracted as described above and individual lipids were separated by TLC with light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) and then light petroleum/diethyl ether (49:1; per vol.) as solvents. Radioactivity was determined as described above.

Electron microscopy of yeast cells

For ultrastructural inspection, cells were harvested in the early stationary growth phase by centrifugation and washed three times with double-distilled water. Subsequently, cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with distilled water, and fixed in a 1% aqueous solution of $KMnO_4$ for 20 min again. Fixed cells were washed three times in distilled water and incubated in 0.5% aqueous uranylacetate overnight at 4°C. Samples were dehydrated in a graded series of acetone (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.

Fluorescence microscopy of lipid particles

Nile Red staining was performed as described by Greenspan et al. [32]. Yeast strains were grown to the early stationary growth phase and stained with Nile Red dissolved in ethanol. Microscopic pictures visualized using a fluorescence microscope (Axiovert 35, Carl Zeiss, Jena, Germany) with the filter set 14 (Zeiss). Nile Red fluorescence of lipid droplets was detected at an emission wavelength of 590 nm with a 100-fold oil immersion objective.

In vivo mobilization of neutral lipids

To measure the mobilization of neutral lipids, cells were pre-grown for 24 h in minimal medium containing 0.67% yeast nitrogen base without amino acids, 0.073% amino acid mix and 2% glucose as the carbon source. Then, fresh minimal medium was inoculated with the pre-grown culture to an OD_{600} of 3, and cerulenin (final concentration 10 µg/ml) was added from an ethanolic stock solution. Control incubations contained the equivalent volume of ethanol only. At time points indicated, aliquots of the culture were withdrawn, and an equivalent of 10 OD_{600} were harvested by centrifugation on a table-top centrifuge. The pellet was washed and shock frozen with liquid nitrogen. Lipids were extracted and analyzed as described above.

Results

Growth phenotype analysis

To obtain more insight into the possible involvement of the *GPH1* gene product in lipid metabolism and especially in PE synthesis we performed a number of tests addressing growth behavior and lipid profiling of a Δ*gph1* mutants strain. First, Δ*gph1* and Δ*psd1* single deletion mutants as well as a Δ*gph1*Δ*psd1* double mutant were tested for their growth phenotype on different media. Fermentable and non-fermentable carbon sources were used for these tests. Additionally, osmotic stability of strains on sorbitol and SDS sensitivity was analyzed.

As shown in Figure 1, Δ*gph1* and Δ*psd1* mutants grow like wild type on glucose-containing media. Growth of the Δ*gph1*Δ*psd1* double mutant on this medium is slightly decreased. Previous work from our laboratory has shown that the requirement for PE in *Saccharomyces cerevisiae* is more stringent on non-fermentable carbon sources (lactate, glycerol) than on glucose due to intense proliferation of mitochondria and the importance of PE for cell respiration [12,31]. The expected effect with a single deletion of *PSD1* was confirmed (see Figure 1). In contrast, the Δ*gph1* mutant grew like wild type on YPLac. Growth inhibition of the Δ*gph1*Δ*psd1* double mutant on lactate full medium appears to reflect the effect of the Δ*psd1* deletion. Growth of the three strains on glycerol full medium was roughly the same as on YPLac. On lactate minimal medium with (MMLac) neither the two single mutants nor the double mutant were viable indicating that under these stringent conditions defects became evident. Growth on sorbitol containing plates revealed that Δ*gph1* as well as Δ*psd1* mutants became slightly instable. Most interestingly, the Δ*gph1* deletion mutant was highly sensitive to SDS. Such an effect was not observed with the Δ*psd1* strain. This result suggested that in Δ*gph1* the plasma membrane and/or the cell wall were compromised.

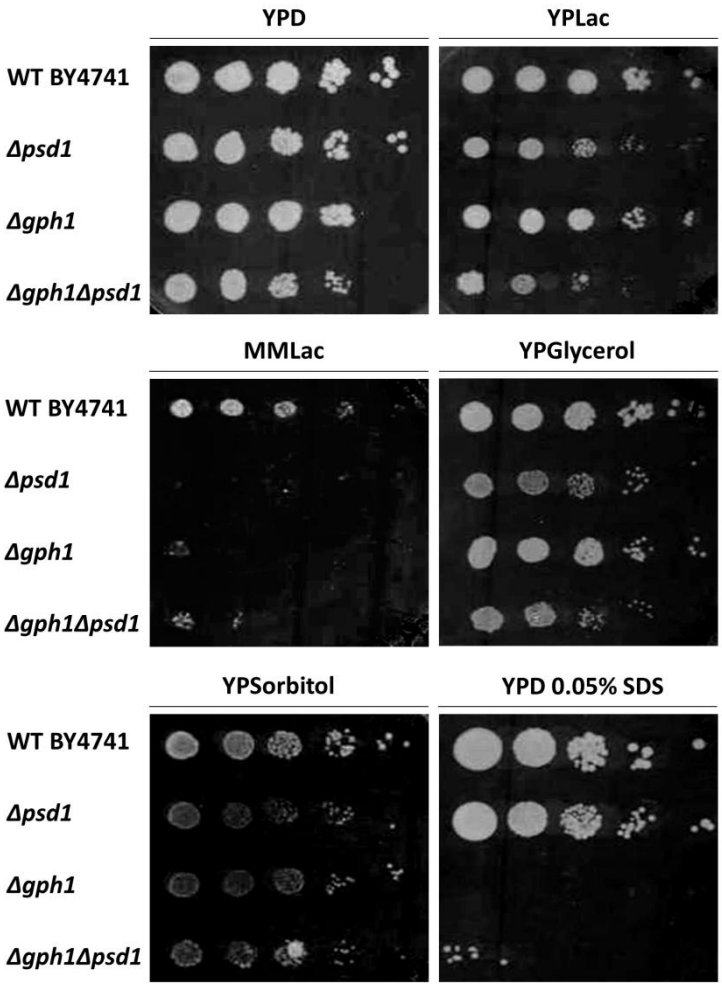

Figure 1. Growth analysis of Δ*psd1*, Δ*gph1* and Δ*gph1*Δ*psd1* deletion mutants.
Strains as indicated were grown on YPD, YPLac, MMLac, YPGlycerol, YPSorbitol, and YPD with 0.05% SDS. Cell suspensions of strains listed were spotted at dilutions 1, 1/10, 1/100, 1/1000, and 1/10000. Incubation was carried out at 30°C.

Phospholipid analysis of total cell homogenate and subcellular fractions

To estimate the possible involvement of Gph1p in phospholipid metabolism we measured phospholipid profiles of Δ*gph1*, Δ*psd1* and Δ*gph1*Δ*psd1* and compared to wild type. Since phenotype analysis suggested possible effects of the *GPH1* deletion on respiration and osmotic stability (see Figure 1) we analyzed lipids from mitochondrial and plasma membrane fractions of those strains.

In wild type homogenate, the major phospholipids were PC, PE and PI (Table 2). Lysophospholipids (LPL), phosphatidic acid (PA), cardiolipin (CL) and dimethylphosphatidylethanolamine (DMPE) were present only at minor amounts. In the homogenate from the Δ*gph1* mutant the amount of PC was reduced by ~15% compared to wild type. This decrease was compensated by an increase in PI and DMPE. As shown previously [13,33,34,35,36], deletion of *PSD1* caused a depletion of PE in the cell homogenate. Also in this case, the level of PI was increased. The Δ*gph1*Δ*psd1* double mutant exhibited a similar phospholipid pattern as the Δ*psd1* single mutant, although the PC level was also slightly reduced.

A decrease of PC was also observed in mitochondria and in the plasma membrane from Δ*gph1* deletion mutant which was accompanied by a slight increase of PE and PI. The marked effect observed in these two subcellular fractions of Δ*psd1* was the decrease of PE. The phospholipid pattern of the Δ*gph1*Δ*psd1* double mutant was similar to the Δ*psd1* single mutant. Since the plasma membrane does not contain phospholipid synthesizing enzymes [22] all phospholipids need to be imported from other cellular compartments. The fact that the ratio of PC to PE in the plasma membrane of the Δ*gph1* mutant (0.30) is markedly lower than in wild type plasma membrane (0.56), and that there are comparable values in the homogenate (1.53 in Δ*gph1* and 1.69 in wild type), indicate that transport of both phospholipids to the plasma membrane does not occur randomly. It may be hypothesized that the more pronounced depletion of PC in the plasma membrane is due to disturbed transport processes. Interestingly, however, the total amount of phospholipids was significantly increased in the Δ*gph1* mutant over the wild type (Figure 2). Specific enhancement of membrane proliferation was not found in Δ*gph1* as will be discussed below.

Table 2: Phospholipid composition of cell-free homogenate, plasma membrane and mitochondria from cells grown on YPD. CF, cellular fraction; LPL, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; PA, phosphatic acid. Mean values of at least three measurements and standard deviations are shown.

		Phospholipids in cell-free homogenate, plasma membrane, mitochondria and ER (mol%)							
CF	Strain	LPL	PI	PS	PC	PE	CL	DMPE	PA
Homogenate	WT	1.52±0.21	9.93±3.53	8.75±0.51	45.08±1.82	26.61±2.53	3.35±0.29	4.43±0.70	0.71±0.38
	Δ*psd1*	2.15±0.76	16.00±1.17	11.28±2.38	46.68±2.34	18.23±1.23	1.50±0.64	2.75±0.44	0.95±0.23
	Δ*gph1*	0.62±0.33	14.04±0.74	9.18±0.85	39.30±1.01	25.66±0.47	2.13±0.37	6.85±0.52	1.73±1.30
	Δ*gph1* Δ*psd1*	1.13±0.31	17.22±2.82	12.66±1.91	43.04±2.79	18.64±1.85	2.00±0.57	4.33±0.48	1.14±0.48
Mitochondria	WT	1.92±1.11	8.06±1.72	4.07±0.60	40.65±2.38	30.35±1.36	4.97±3.64	6.58±2.54	2.38±0.60
	Δ*psd1*	2.12±0.46	8.80±3.67	7.31±1.54	46.98±5.42	23.86±3.96	2.87±1.77	3.23±1.56	4.78±2.21
	Δ*gph1*	1.58±0.68	9.49±2.30	6.94±1.00	35.90±2.32	33.94±0.38	6.17±1.51	3.66±0.26	2.10±0.58
	Δ*gph1* Δ*psd1*	2.34±0.09	10.59±0.84	8.82±0.76	48.46±1.53	21.64±1.64	2.83±0.53	3.45±0.42	1.79±0.59
Plasma membrane	WT	2.26±0.96	12.38±2.12	26.18±2.79	18.18±1.38	32.06±4.14	0.69±0.10	2.23±0.43	5.76±0.61
	Δ*psd1*	2.80±0.53	17.11±4.11	27.27±3.20	21.27±3.32	23.47±3.61	0.64±0.32	1.90±0.30	5.30±2.18
	Δ*gph1*	1.88±0.96	14.82±2.93	24.90±3.96	11.04±2.25	36.27±2.77	0.58±0.27	2.44±0.55	7.84±3.13
	Δ*gph1* Δ*psd1*	2.31±0.37	14.39±1.64	35.46±6.64	15.71±5.82	22.79±2.13	1.15±1.05	2.17±0.70	5.85±1.73

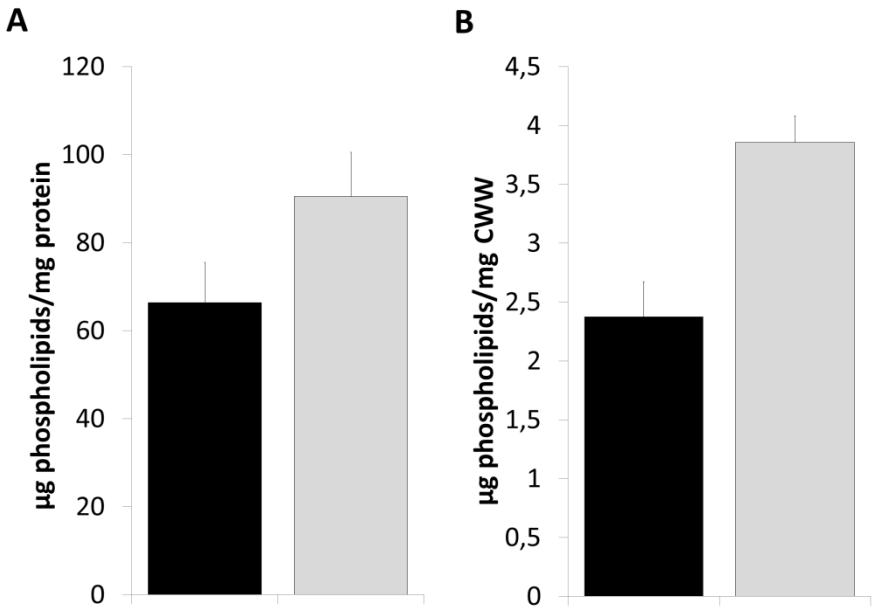

Figure 2. Total amount of phospholipids in cell-free homogenate in BY4741 and Δ*gph1* deletion mutant.
Cells were grown on YPD until early stationary growth phase at 30°C and disrupted with glass beads. Lipids were extracted with chloroform/methanol (2:1; per vol.). Black bar, BY4741; grey bar, Δ*gph1* deletion mutant. A) total amount of phospholipids were related to the amount of proteins. B) total amount of phospholipids were related to cell wet weight. Inorganic phosphate was used as standard.

Synthesis of PC is down-regulated in the Δgph1 deletion mutant in vivo

As described above, the PC level in the Δ*gph1* deletion mutant was markedly decreased over wild type. Since there are two pathways of PC synthesis in *Saccharomyces cerevisiae* we wished to estimate which one was affected by the Δ*gph1* deletion. PC can be synthesized i) via the CDP-choline branch of the Kennedy pathway which utilizes choline as a substrate [10]; and ii) through a three step methylation of PE catalyzed by Cho2p and Opi3p [37,38,39]. To analyze the CDP-choline pathway we labeled cells with [methyl-^{14}C]choline chloride and measured incorporation of the label into PC. As shown in Figure 3A, the synthesis of PC via this pathway was reduced to 60% of wild type. Interestingly, however, also the methylation

pathway was affected by the Δ*gph1* deletion (Figure 3B). In this assay, cells were labeled with L-[^{3}H]serine, and sequential incorporation of the label into PS, PE (catalyzed by Psd1p or Psd2p), and PC (catalyzed by Cho2p and Opi3p) was measured. Whereas the first two steps in the biosynthetic route of aminoglycerophospholipids were reduced by 20%, the methylation of PE to PC in Δ*gph1* was approximately only 50% of wild type. In summary, both pathways of PC synthesis are strongly decreased in the mutant.

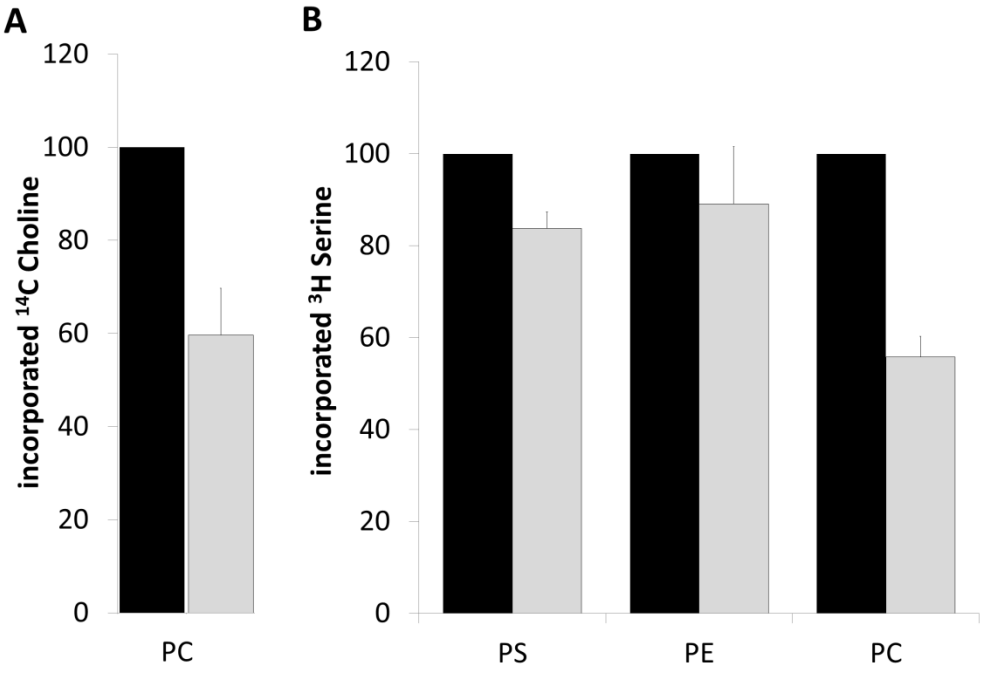

Figure 3. Synthesis of PC is down-regulated in the Δ*gph1* deletion mutant *in vivo*.
Black bar, BY4741; grey bar, Δ*gph1* deletion mutant; A) The so-called Kennedy pathway was analyzed by counting the incorporation of [methyl-^{14}C]choline chloride into PC. B) To analyze the methylation pathway the incorporation of L-[^{3}H]serine from PS to PE, catalyzed by Psd1p or Psd2p, to PC catalyzed by Cho2p and Opi3p was counted. BY4741 was set to 100%. Synthesis of PC via both pathways is reduced to ~60% of wild type in the Δ*gph1* mutant.

The amount of triacylglycerols and steryl esters are reduced in the Δgph1 deletion mutant

Changes in the phospholipid profile of Δ*gph1* tempted us to speculate that also non-polar lipid synthesis was affected in this deletion strain. As can be seen from Figure 4 the amounts of TG and SE were highly reduced in the Δ*gph1* mutant. At the same time, the level of DG in the Δ*gph1* mutant was slightly increased over wild type suggesting a possible involvement of Gph1p in synthesis of TG. The amount of ergosterol was more or less the same in the mutant and in wild type.

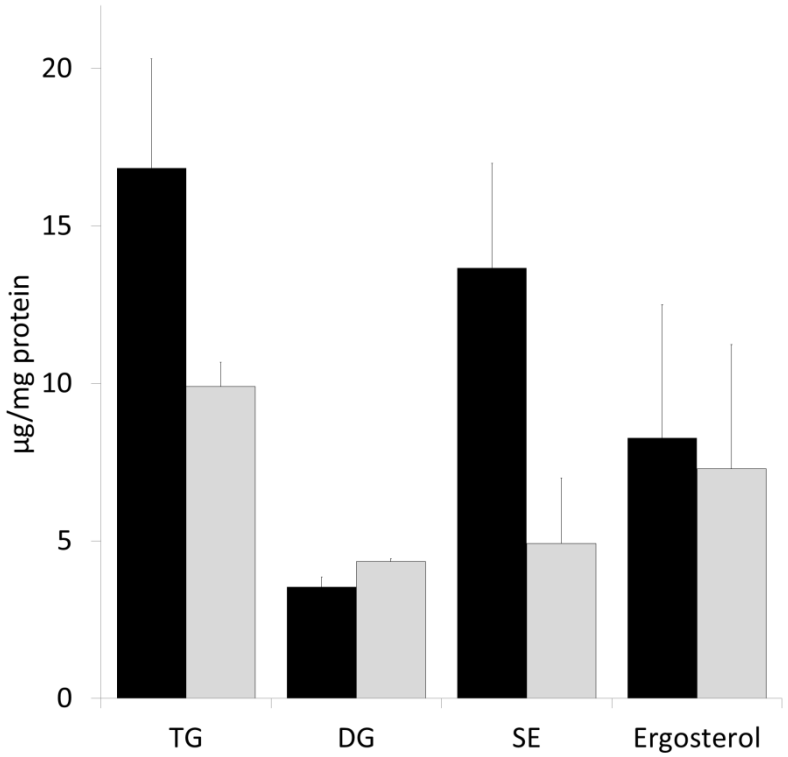

Figure 4. The amount of triacylglycerols and steryl esters are reduced in the Δ*gph1* deletion mutant.
The wild type and Δ*gph1* deletion mutant were grown aerobically until early stationary growth phase and triacylglycerols, diacylglycerols, steryl esters and ergosterol were analyzed. Black bar, BY4741; grey bar, Δ*gph1* deletion mutant.

Since TG and SE are the main components of lipid droplets we wished to investigate the effect of Δ*gph1* deletion on the formation of this compartment. Electron microscopic analysis

of the Δ*gph1* mutant showed to our surprise, that lipid droplets were more or less missing in the mutant strain (Figure 5A). In the wild type strain one to four distinct lipid droplets appeared in each cell, whereas the Δ*gph1* deletion mutant lacked lipid droplets or contained rare droplets at a very small size. This observation was confirmed by Nile Red staining and fluorescence microscopy (Figure 5B). The typical fluorescence signal of lipid droplets stained with Nile Red, which appears as distinct spots was only observed in wild type, whereas in Δ*gph1* only diffuse fluorescence signals in the cellular background were observed.

Electron microscopic inspection of the Δ*gph1* mutant (see Figure 5A) also revealed that the total cell structure including the plasma membrane was not changed. High sensitivity of the deletion mutant to SDS had been a hint for possible defects of the plasma membrane. However, such changes were not observed.

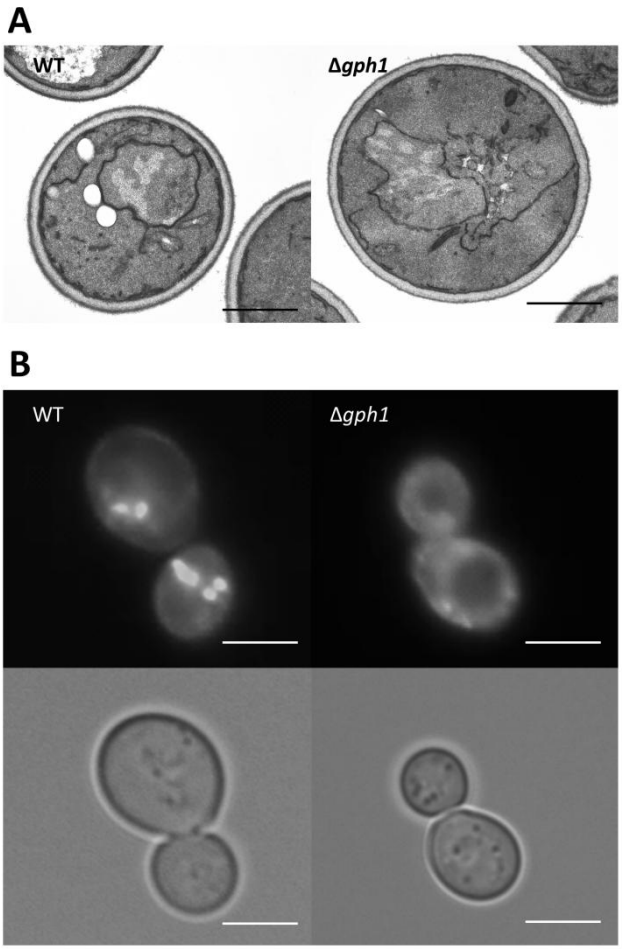

Figure 5
The amount and size of lipid particles are highly decreased in the Δ*gph1* deletion mutant
A) Transmission electron analysis of wild type BY4741 and Δ*gph1*. The wild type cell contains nicely developed lipid particles, whereas in the Δ*gph1* deletion mutant no lipid particles are visible. Scale bar 1µm. B) Nile Red staining and fluorescence microscopy of wild type BY4741 and Δ*gph1*. There are distinct fluorescent lipid particles in each wild type cell, whereas the Δ*gph1* deletion mutant cells appear in a diffuse fluorescent signal. Scale bar 2µm.

Metabolism of non-polar lipids in vivo

The imbalanced amounts of phospholipids and non-polar lipids in the Δ*gph1* mutant led to the assumption that Gph1p may be involved in the regulation of fatty acid distribution between the different lipid classes. To address this aspect performed *in vivo* assays addressing lipid synthesis and turnover in the Δ*gph1* mutant. First, we labeled cells with [1-^{14}C]acetic acid as lipid precursor and measured the incorporation of acetate into non-polar lipids and phospholipids (Figure 6). Over the time period tested there were no dramatic differences in the incorporation of [1-^{14}C]acetic acid into the different lipid classes of wild type and Δ*gph1* mutant. Even the incorporation of the label into TG from Δ*gph1* was not decreased although the amount of TG in the mutant is markedly lower than in wild type.

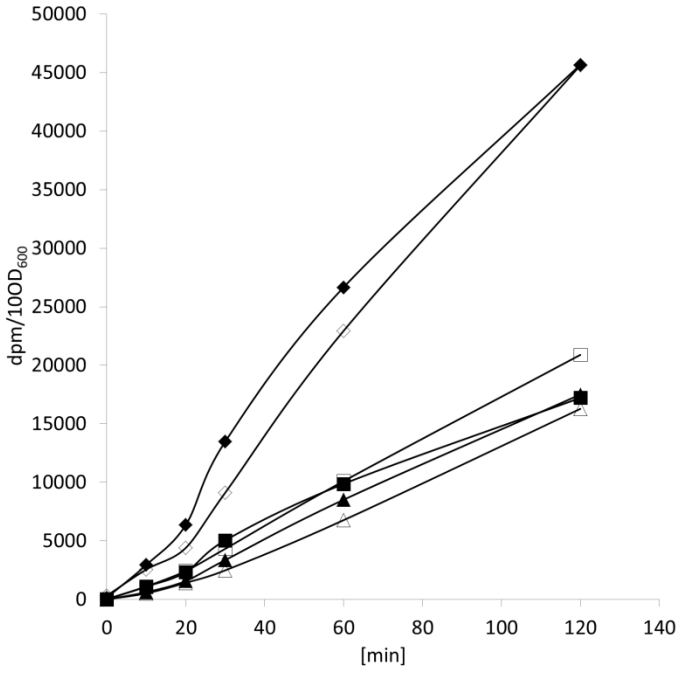

Figure 6. *In vivo* synthesis and turnover of lipids
[1-^{14}C]acetic acid incorporation into phospholipids (♦), triacylglycerols (■) and steryl esters (▲) are shown in BY4741 (filled symbols) and Δ*gph1* (open symbols). Data are shown of one performed experiment and are representative.

The question remained whether an increased turnover of TG in the Δ*gph1* mutant might be the reason for the decreased amount of this lipid in the mutant. To address this question, we followed mobilization of cellular TG *in vivo* in the presence of cerulenin, an inhibitor of fatty acid synthesis in yeast. Under these conditions, TG get mobilized, mainly for the synthesis of membrane phospholipids [40]. In both Δ*gph1* and wild type TG was properly mobilized, but the initial degradation rate in the mutant was slightly higher. Whereas in wild type 50% TG was degraded under these conditions within approximately 110 min, the half-live of TG in Δ*gph1* was only 95 min. Thus, the more efficient TG hydrolysis in Δ*gph1* may contribute to the lower level of this lipid in the mutant.

Discussion

Lipid metabolism of the yeast is a complex network of reactions with an even more complicated regulatory background. Besides genes encoding lipid metabolic enzymes a number of regulatory genes whose products are involved in synthesis and metabolic conversion of lipids have been identified (reviewed in [4]). Consequently, synthesis and metabolism of the major yeast lipid classes, e.g. phospholipids, fatty acids, triacylglycerols, sterols and sphingolipids are linked to each other [2]. Additionally, lipid synthesis in yeast is affected by growth conditions which influence the expression of enzymes and/or modulate their catalytic activities. As examples, expression of phospholipid biosynthetic genes in yeast is affected by carbon sources, availability of nutrient, growth phase, pH and temperature. Finally, posttranslational modifications of gene products, especially phosphorylation of key proteins involved in phospholipid synthesis, affect metabolism of phospholipids and the

balance between certain lipid precursors and final products of lipid biosynthetic pathways [5,41,42,43,44,45,46].

In previous studies from our laboratory (Gsell et al., manuscript submitted) *GPH1* was identified as possible regulator gene of yeast lipid metabolism. Gph1p has originally been identified as a glycogen phosphorylase which catabolizes the branched polysaccharide glycogen used as storage carbohydrate. In the present study we found more evidence that Gph1p serves as a regulator of lipid metabolism in yeast. As most remarkable lipid phenotypic features of a mutant deleted of *GPH1* we found decreased formation of TG and SE, increased synthesis of total phospholipids but a specific decrease of PC biosynthesis (see Figures 2, 3 and 4 and Table 2). The depletion of TG and SE in Δ*gph1* mutant cells leads to lack of lipid droplets, and the changes in the phospholipid composition, especially in the plasma membrane, to an increased sensitivity against SDS. At present it is not clear whether or not carbohydrate metabolism of the yeast and lipid metabolism are linked through the action of Gph1p. Alternatively, Gph1p may fulfill multiple independent functions which affect carbohydrate metabolism on one and lipid metabolism on the other hand.

Our results suggest that Gph1p may act as a moderate negative regulator of yeast TG lipases. The decreased TG level and the increased DG level in the deletion mutant as well as slightly more efficient TG mobilization in the Δ*gph1* deletion mutant may support this view. It has to be noted, however, that the expression levels of the three yeast genes encoding TG lipases, *TGL3*, *TGL4* and *TGL5*, are not up-regulated in the Δ*gph1* deletion mutant as estimated by qRT PCR (data not shown). Depletion of TG and SE are obviously the reason for the surprising observation that the Δ*gph1* mutant lacks lipid droplets. It is even more surprising that lipid droplets are not formed although some TG and SE are still present in the mutant cells (see Figures 4 and 5). It can only be speculated that the remaining TG and SE are spread over internal membranes without leading to initiation of lipid droplet formation.

The molecular role that is played by Gph1p in lipid metabolism is still obscure. Gph1p is a phosphatase and degrades glycogen leading to the formation of glucose-1-phosphate. The question remains whether or not Gph1p may also act as a protein phosphatase possibly regulating the activity of an enzyme involved in lipid metabolism. There is, however, some evidence that Gph1p has more than one function in cell metabolism. Examples for such enzymes in lipid metabolism are the yeast TG lipases Tgl3p, Tgl4p and Tgl5p which serve simultaneously as lipases and acyltransferases [47]. It has been published before that Gph1p is mostly active in the stationary phase of a yeast culture and not even expressed earlier [14]. In contrast, the study pinpointed a number of effects occurring already in earlier growth phases, e.g. sensitivity against SDS, changes in the lipid pattern and *in vivo* analysis of lipid formation and degradation. These results largely exclude that glycogen accumulation directly affects the lipid metabolism, because in early growth phases glycogen accumulation does not occur.

Finally, we may speculate that Gph1p is involved in the subcellular distribution of lipid components. The fact that TG, although formed at reduced quantities in Δ*gph1,* is not stored in lipid droplet may support this view. Additionally, deletion of *GPH1* affects the formation of PC but also its transport to the plasma membrane. It is presently not clear whether the reduced level of PC in Δ*gph1* is caused by the altered levels of TG or SE. Altogether, Gph1p appears to cause multiple effects in yeast lipid metabolism by molecular mechanisms which remain elusive.

Acknowledgement

This work was supported by the Austrian Science Fund FWF (project 21429 and DK Molecular Enzymology W901-B05 to GD)

References

[1] K. Athenstaedt, G. Daum, Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast *Saccharomyces cerevisiae* are localized to lipid particles J. Biol. Chem. 280 (2005) 37301–37309.

[2] S. Rajakumari, K. Grillitsch, G. Daum, Synthesis and turnover of non-polar lipids in yeast, Prog Lipid Res 47 (2008) 157-171.

[3] G. Daum, N.D. Lees, M. Bard, R. Dickson, Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*, Yeast 14 (1998) 1471-1510.

[4] S.A. Henry, S.D. Kohlwein, G.M. Carman, Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*, Genetics 190 (2012) 317-349.

[5] G.M. Carman, S.A. Henry, Phospholipid biosynthesis in the yeast *Saccharomyces cerevisiae* and interrelationship with other metabolic processes, Prog Lipid Res 38 (1999) 361-399.

[6] D.R. Voelker, Interorganelle transport of aminoglycerophospholipids, Biochim Biophys Acta 1486 (2000) 97-107.

[7] M.A. Carson, M. Emala, P. Hogsten, C.J. Waechter, Coordinate regulation of phosphatidylserine decarboxylase activity and phospholipid N-methylation in yeast, J Biol Chem 259 (1984) 6267-6273.

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

[9] P.J. Trotter, D.R. Voelker, Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (*PSD2*) in the yeast *Saccharomyces cerevisiae*, J. Biol. Chem. 270 (1995) 6062-6070.

[10] E.P. Kennedy, S.B. Weiss, The function of cytidine coenzymes in the biosynthesis of phospholipids, J. Biol. Chem. 222 (1956) 193-214.

[11] W.R. Riekhof, D.R. Voelker, Uptake and utilization of lyso-phosphatidylethanolamine by *Saccharomyces cerevisiae*, J. Biol. Chem. 281 (2006) 36588-36596.

[12] R. Birner, M. Bürgermeister, R. Schneiter, G. Daum, Roles of phosphatidylethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*, Mol. Biol. Cell 12 (2001) 997-1007.

[13] R. Nebauer, I. Schuiki, B. Kulterer, Z. Trajanoski, G. Daum, The phosphatidylethanolamine level of yeast mitochondria is affected by the mitochondrial components Oxa1p and Yme1p, FEBS J. 274 (2007) 6180-6190.

[14] P.K. Hwang, S. Tugendreich, R.J. Fletterick, Molecular analysis of *GPH1*, the gene encoding glycogen phosphorylase in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 9 (1989) 1659-1666.

[15] W.A. Wilson, M.P. Boyer, K.D. Davis, M. Burke, P.J. Roach, The subcellular localization of yeast glycogen synthase is dependent upon glycogen content, Can. J. Microbiol. 56 (2010) 408-420.

[16] C. Favre, P.S. Aguilar, M.C. Carrillo, Oxidative stress and chronological aging in glycogen-phosphorylase-deleted yeast, Free Radic. Biol. Med. 45 (2008) 1446-1456.

[17] M.S. Longtine, A. McKenzie, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, J.R. Pringle, Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*., Yeast 14 (1998) 953-961.

[18] D. Gietz, A. St Jean, R.A. Woods, R.H. Schiestl, Improved method for high efficiency transformation of intact yeast cells, Nucleic Acids Res 20 (1992) 1425.

[19] R. Serrano, H^+-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution, Methods Enzymol. 157 (1988) 523-544.

[20] H.B. van den Hazel, H. Pichler, M.A. do Valle Matta, E. Leitner, A. Goffeau, G. Daum, *PDR16* and *PDR17*, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs, J. Biol. Chem. 274 (1999) 1934-1941.

[21] H. Pichler, B. Gaigg, C. Hrastnik, G. Achleitner, S.D. Kohlwein, G. Zellnig, A. Perktold, G. Daum, A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids, Eur. J. Biochem. 268 (2001) 2351-2361.

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

[23] G. Daum, P.C. Böhni, G. Schatz, Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria, J. Biol. Chem. 257 (1982) 13028-13033.

[24] E. Zinser, G. Daum, Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*, Yeast 11 (1995) 493-536.

[25] C.V. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.

[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680-685.

[27] A. Haid, M. Suissa, Immunochemical identification of membrane proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Methods Enzymol. 96 (1983) 192-205.

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

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

[30] R. Schneiter, G. Daum, Analysis of yeast lipids, Methods Mol. Biol. 313 (2006) 75-84.

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

[32] P. Greenspan, E.P. Mayer, S.D. Fowler, Nile red: a selective fluorescent stain for intracellular lipid droplets, J. Cell. Biol. 100 (1985) 965-973.

[33] R. Birner, G. Daum, Biogenesis and cellular dynamics of aminoglycerophospholipids, Int. Rev. Cytol. 225 (2003) 273-323.

[34] M.K. Storey, K.L. Clay, T. Kutateladze, R.C. Murphy, M. Overduin, D.R. Voelker, Phosphatidylethanolamine has an essential role in *Saccharomyces cerevisiae* that is independent of its ability to form hexagonal phase structures, J. Biol. Chem. 276 (2001) 48539-48548.

[35] M. Bürgermeister, R. Birner-Grünberger, R. Nebauer, G. Daum, Contribution of different pathways to the supply of phosphatidylethanolamine and phosphatidylcholine to mitochondrial membranes of the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1686 (2004) 161-168.

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

[37] E.F. Summers, V.A. Letts, P. McGraw, S.A. Henry, *Saccharomyces cerevisiae cho2* mutants are deficient in phospholipid methylation and cross-pathway regulation of inositol synthesis, Genetics 120 (1988) 909-922.

[38] T. Kodaki, S. Yamashita, Yeast phosphatidylethanolamine methylation pathway. Cloning and characterization of two distinct methyltransferase genes, J Biol Chem 262 (1987) 15428-15435.

[39] P. McGraw, S.A. Henry, Mutations in the *Saccharomyces cerevisiae opi3* gene: effects on phospholipid methylation, growth and cross-pathway regulation of inositol synthesis, Genetics 122 (1989) 317-330.

[40] J. Inokoshi, H. Tomoda, H. Hashimoto, A. Watanabe, H. Takeshima, S. Omura, Cerulenin-resistant mutants of *Saccharomyces cerevisiae* with an altered fatty acid synthase gene, Mol Gen Genet 244 (1994) 90-96.

[41] M.L. Gaspar, M.A. Aregullin, S.A. Jesch, L.R. Nunez, M. Villa-García, S.A. Henry, The emergence of yeast lipidomics, Biochim Biophys Acta 1771 (2007) 241-254.

[42] M. Chen, L.C. Hancock, J.M. Lopes, Transcriptional regulation of yeast phospholipid biosynthetic genes, Biochim Biophys Acta 1771 (2007) 310-321.

[43] G.M. Carman, G.S. Han, Regulation of phospholipid synthesis in Saccharomyces cerevisiae by zinc depletion, Biochim Biophys Acta 1771 (2007) 322-330.

[44] J. Patton-Vogt, Transport and metabolism of glycerophosphodiesters produced through phospholipid deacylation, Biochim Biophys Acta 1771 (2007) 337-342.

[45] H. Santos-Rosa, J. Leung, N. Grimsey, S. Peak-Chew, S. Siniossoglou, The yeast lipin Smp2 couples phospholipid biosynthesis to nuclear membrane growth, EMBO J 24 (2005) 1931-1941.

[46] G. Li, S. Chen, M.N. Thompson, M.L. Greenberg, New insights into the regulation of cardiolipin biosynthesis in yeast: implications for Barth syndrome, Biochim Biophys Acta 1771 (2007) 432-441.

[47] S. Rajakumari, G. Daum, Multiple functions as lipase, steryl ester hydrolase, phospholipase, and acyltransferase of Tgl4p from the yeast *Saccharomyces cerevisiae*, J Biol Chem 285 (2010) 15769-15776.

Summary and General Discussion

Lipid metabolism of the yeast is a complex network of reactions with an even more complicated regulatory background. Besides genes encoding lipid metabolic enzymes a number of regulatory genes whose products are involved in synthesis and metabolic conversion of lipids have been identified. Consequently, synthesis and metabolism of the major yeast lipid classes, e.g., phospholipids, fatty acids, triacylglycerols, sterols and sphingolipids are linked to each other [1]. Additionally, lipid synthesis in yeast is affected by growth conditions which influence the expression of enzymes and/or modulate their catalytic activities. As examples, expression of phospholipid biosynthetic genes in yeast is affected by carbon sources, availability of nutrient, growth phase, pH and temperature. Finally, posttranslational modifications of gene products, especially phosphorylation of key proteins involved in phospholipid synthesis, affect metabolism of phospholipids and the balance between certain lipid precursors and final products of lipid biosynthetic pathways [2,3,4,5,6,7,8].

In the yeast *Saccharomyces cerevisiae* as in most other eukaryotic cells, the major membrane phospholipids are PC, PE, PI and PS. Phospholipid metabolism is governed by a network of reactions which are subject to strict genetic and biochemical regulation [9]. ER and mitochondria are major sites of phospholipid synthesis [10] whereas other compartments such as the plasma membrane are devoid of phospholipid synthesizing enzymes [11]. Such membranes rely completely on the supply of lipids from other organelles.

This Thesis was focused on the global role of PE in the yeast and designed to study the genome wide response of *Saccharomyces cerevisiae* to PE depletion caused by deletion of *PSD1*. Psd1p is the major producer of PE in the yeast and localized to the inner mitochondrial membrane where it catalyzes decarboxylation of PS to PE [12,13,14].

To obtain more insight into the genome wide effect of an unbalanced cellular and mitochondrial PE level and in particular the contribution of Psd1p to this depletion, DNA-microarray analysis with a Δ*psd1* deletion mutant was performed. This approach revealed that 54 genes were up-regulated in a Δ*psd1* deletion strain compared to wild type. The respective gene products serve several functions in diverse biological processes. This large variety reflects the possible direct or indirect involvement of PE in many different cellular processes and identified a network of genes linked to the function of *PSD1* in the yeast. Processes affected by depletion of PE are wide spread, although defects may be secondary effects of changes in membrane behavior caused by PE depletion. At least in the cases of *GPM2*, *GPH1* and *RSB1* a direct link to lipid metabolism and growth phenotype was established.

Further investigations of *GPH1* identified the respective gene product as a possible regulator of yeast lipid metabolism. Gph1p has originally been identified as a glycogen phosphorylase. A Δ*gph1* deletion mutant shows remarkable lipid phenotypic features. Depletions of neutral lipids (decreased TG and SE levels) as well as changes in the phospholipid profile (decreased PC level, increased total amount of phospholipids) were observed. These findings confirmed the close regulatory connection between neutral lipid and phospholipid synthesis. At present it is not clear whether or not carbohydrate metabolism of the yeast and lipid metabolism are directly linked through the action of Gph1p. Alternatively, Gph1 may fulfill multiple independent functions which affect carbohydrate metabolism on one and lipid metabolism on the other hand, although the molecular role of Gph1p in lipid metabolism remained unclear.

References

[1] S. Rajakumari, K. Grillitsch, G. Daum, Synthesis and turnover of non-polar lipids in yeast, Prog Lipid Res 47 (2008) 157-171.

[2] G.M. Carman, S.A. Henry, Phospholipid biosynthesis in the yeast *Saccharomyces cerevisiae* and interrelationship with other metabolic processes, Prog Lipid Res 38 (1999) 361-399.

[3] M.L. Gaspar, M.A. Aregullin, S.A. Jesch, L.R. Nunez, M. Villa-García, S.A. Henry, The emergence of yeast lipidomics, Biochim Biophys Acta 1771 (2007) 241-254.

[4] M. Chen, L.C. Hancock, J.M. Lopes, Transcriptional regulation of yeast phospholipid biosynthetic genes, Biochim Biophys Acta 1771 (2007) 310-321.

[5] G.M. Carman, G.S. Han, Regulation of phospholipid synthesis in Saccharomyces cerevisiae by zinc depletion, Biochim Biophys Acta 1771 (2007) 322-330.

[6] J. Patton-Vogt, Transport and metabolism of glycerophosphodiesters produced through phospholipid deacylation, Biochim Biophys Acta 1771 (2007) 337-342.

[7] H. Santos-Rosa, J. Leung, N. Grimsey, S. Peak-Chew, S. Siniossoglou, The yeast lipin Smp2 couples phospholipid biosynthesis to nuclear membrane growth, EMBO J 24 (2005) 1931-1941.

[8] G. Li, S. Chen, M.N. Thompson, M.L. Greenberg, New insights into the regulation of cardiolipin biosynthesis in yeast: implications for Barth syndrome, Biochim Biophys Acta 1771 (2007) 432-441.

[9] S.A. Henry, S.D. Kohlwein, G.M. Carman, Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*, Genetics 190 (2012) 317-349.

[10] G. Daum, J.E. Vance, Import of lipids into mitochondria, Prog. Lipid Res. 36 (1997) 103-130.

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

[12] M.A. Carson, M. Emala, P. Hogsten, C.J. Waechter, Coordinate regulation of phosphatidylserine decarboxylase activity and phospholipid N-methylation in yeast, J Biol Chem 259 (1984) 6267-6273.

[13] P.J. Trotter, J. Pedretti, D.R. Voelker, Phosphatidylserine decarboxylase from *Saccharomyces cerevisiae.* Isolation of mutants, cloning of the gene, and creation of a null allele, J Biol Chem 268 (1993) 21416-21424.

[14] K. Kuchler, G. Daum, F. Paltauf, Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in *Saccharomyces cerevisiae*, J. Bacteriol. 165 (1986) 901-910.

CURRICULUM VITAE

Personal Details

Name	Martina Gsell
Academic title	Mag. rer. nat
Date of Birth	11 August 1983
Nationality	Austrian
Telephone	+43 650 8308111
E-mail	martina.gsell@gmx.at

Education and Qualifications

05/2009 – 2013	Phd Program DK Molecular Enzymology Institute of Biochemistry, TU Graz, supervised by Prof. Günther Daum
03/2009	Master´s degree in Molecular Microbiology
07/2007 – 05/2008	Master thesis Lund University, Sweden Department of Cell- and Organism biology supervised by Prof. Marita Cohn
09/2006 – 03/2009	Master´s program Molecular Microbiology Karl Franzens University of Graz
09/2006	Bachelor´s degree in Molecular Biology
10/2002 – 09/2006	Bachelor´s program Molecular Biology, Karl Franzens University of Graz
1997 – 2002	HLW-Schrödinger, Graz High school for business and administration
1989 - 1997	Elementary School Wolfsberg i. Schw.

Professional experience

04/2012 – 08/2012	Amyris Inc., Emeryville, California, USA
06/2008 – 09/2008	Research assistant Lund University, Sweden Department of Cell- and Organism biology

Teaching activities

2010 and 2011	Teaching assignment for lab course "Methods in Immunology", TU Graz

Poster and oral presentations

09/2011	ICBL Expanding the Horizons of Lipidomics, Warsaw, Poland
03/2011	Graduate Seminar 2011 DK Molecular Enzymology, Graz, Austria
06/2010	FEBS Workshop, Eukaryotic Lipids, Treasure Regulatory information, Spetses, Greece
05/2010	FEBS Workshop Microbial Lipids, Vienna, Austria
04/2010	Graduate Seminar 2010 DK Molecular Enzymology, Loipersdorf, Austria
03/2010	Lipotox 3rd International Graz Symposium on Lipid and Membrane Biology, Graz, Austria