Melanie Connerth

Lipid Traffic in the Yeast

DISSERTATION

zur Erlangung des akademischen Grades einer

Doktorin der Naturwissenschaften

erreicht an der

Technischen Universität Graz

Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. Günther Daum

Institut für Biochemie

Technische Universität Graz

2010

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: 23.04.2010

(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: 23.04.2010

(signature)

DANKSAGUNG

An dieser Stelle möchte ich mich gerne bei den vielen lieben Menschen bedanken, die mich während meiner Dissertation begleitet und unterstützt haben.

Mein besonderer Dank gilt Prof. Günther Daum für die engagierte Betreuung meiner Dissertation und vor allem für die uneingeschränkte Unterstützung in fachlichen und persönlichen Dingen.

Ein herzlicher Dank gilt meinen jetzigen und natürlich ehemaligen ArbeitskollegInnen der „AG Daum“ für die freundliche und hilfsbereite Atmosphäre und die vielen lustigen Momente innerhalb und außerhalb des Labors.

Des weiteren möchte ich mich bei Prof. Anna Stina Sandelius und meiner „Gast-Arbeitsgruppe“ in Schweden für die herzliche Aufnahme und Unterstützung bedanken: tack så mycket för allt.

Ganz besonders möchte ich mich auch bei meiner Familie und Max dafür bedanken, dass sie mich während meines gesamten Studiums stets unterstützt haben.

Table of Contents

Zusammenfassung

Die Hefe *Saccharomyces cerevisiae* ist ein sehr gut geeigneter Modellorganismus, um zelluläre Lipid-Homöostase zu untersuchen. Prozesse, die an der Biosynthese von Fettsäuren, Neutrallipiden oder Phospholipiden beteiligt sind, befinden sich in verschiedenen zellulären Kompartimenten. Zwei Organellen, die am Fettsäure-Metabolismus beteiligt sind, sind Peroxisomen (Px), die Schritte der β-Oxidation beherbergen, sowie Lipidpartikel (LP), die überschüssige Fettsäuren speichern. Die Biosynthese beider Organellen ist nur unzureichend verstanden, und über Lipidtransport zu beiden Organellen ist wenig bekannt. Das Ziel dieser Dissertation war es daher, Lipidtransportwege zu und von Px und LP genauer zu ergründen. Zunächst wurde der Transport von Phospholipiden, insbesondere von Phosphatidylethanolamin (PE) zu Px eingehend untersucht. Analysen von verschiedenen Mutanten zeigten, dass die vier PE Biosynthesewege in unterschiedlichem Maß zu diesem Prozess beitragen. Zudem wurde ein Hybrid der PE-Methyltransferase Opi3p in Px einer *opi3*Δ Mutante eingebaut, welches uns erlaubte, Import von PE in Px durch das Erscheinen von Phosphatidylcholin (PC) nachzuweisen. Die wichtigste Erkenntnis dieser Studien war, dass wahrscheinlich direkter Membrankontakt den Lipidtransport zu Px ermöglicht. Des weiteren wurde während der Studien eine Verbindung von Px und der Neutrallipid-Speicherung in LP gefunden. Ölsäure aus dem Medium wurde bevorzugt in Form von Triacylglyceriden (TAG) gespeichert, welche dann durch bekannte und neue Hydrolasen mobilisiert und Px zugeführt wurden. Im Zuge einer Lipidom- und Proteomanalyse von LP wurde ein neuer Effekt von Ölsäure gefunden, welche spezifisch zur Hemmung der Enzymaktivität der Sterolsynthase Are2p führt und eine neue Facette der Lipotoxizität in Hefe darstellt. Zusammenfassend zeigte diese Dissertation, dass in der Zelle bestimmte Fettsäure-Depots vorliegen, die für unterschiedliche Prozesse herangezogen werden können, und dass LP eine entscheidende Rolle bei der Lipid-Homöostase spielen. Die Untersuchungen dieser Dissertation tragen daher grundlegend zu unserem Verständnis des intrazellulären Lipidtransports bei, die sich auch auf höhere Eukaryoten wie Pflanzen oder Säuger übertragen lassen sollten.

Summary

The yeast *Saccharomyces cerevisiae* is a suitable organism for studies on lipid homeostasis in the cell. Processes involved in the biosynthesis of lipid compounds such as fatty acids, neutral lipids and phospholipids are well understood and located to different subcellular compartments. Two major yeast organelles involved in fatty acid turnover are peroxisomes (Px) harboring steps of β-oxidation and lipid particles (LP) which are the storage organelle for excess fatty acid in the cell. Biogenesis of both compartments is not completely understood yet and little is known about mechanisms of lipid supply to either of these organelles. This thesis was aimed at elucidation of lipid traffic routes towards and from Px and LP and the interplay of fatty acid utilization processes in both compartments. Initially, phospholipid supply to peroxisomal membranes was investigated in some detail with focus on phosphatidylethanolamine (PE) transport to peroxisomes. These studies revealed that the four different PE biosynthetic pathways contributed with different efficiency to this process. Moreover, a hybrid of the PE methyltransferase Opi3p was introduced into peroxisomes of an *opi3*Δ mutant which allowed us to measure transport of PE to peroxisomes by appearance of phosphatidylcholine (PC) in peroxisomal membranes. The major take-home message from these experiments was that direct membrane contact most likely accounts for delivery of lipids from their sites of synthesis to peroxisomes. During these studies, a link of peroxisomes with LP, the neutral lipid storage compartment, was also discovered. It was shown that exogenous oleic acid was primarily stored in LP in the form of triacylglycerols (TAG) and subsequently mobilized by a subset of already known as well as novel lipases/hydrolases for supply to peroxisomes. In the course of complete lipidome and proteome analyses of LP a novel and specific inhibitory effect of oleate on the activity of the steryl ester synthase Are2p was discovered thus revealing a new aspect of yeast lipotoxicity. In summary, data from this thesis showed that distinct pools of fatty acids serving for different cellular processes exist in the cell, and LP play a major role in regulating lipid homeostasis. These data are a step forward in our understanding of cellular lipid traffic which may also be applicable to higher eukaryotic cells such as plants and mammals.

General Introduction

Lipid homeostasis resembles a complex, highly important and regulated network in the cell which has been of increasing interest in recent years. The significance of functional lipid homeostasis becomes obvious as some of the most common diseases in developed countries such as obesity, type 2 diabetes and atherosclerosis can be related to disturbed lipid processes. The yeast *Saccharomyces cerevisiae* has become a powerful model organism for studying the molecular biological as well as biochemical and cell biological mechanisms involved in lipid turnover. Many lipid synthesizing and degradation processes are conserved from yeast to mammals. Furthermore, the unicellular organism exhibits similar subcellular organization of cellular processes as in the mammalian system. Finally, knowledge of the complete genome as well as convenient molecular biological handling make the yeast a suitable model for studies of cell biological mechanisms.

The three major yeast lipid classes are fatty acids, phospholipids and neutral lipids which harbor distinct cellular functions. Although the underlying pathways of lipid synthesis and turnover have been investigated in some detail in recent years one of the most important aspects is not yet fully understood. As the metabolic processes are located to different organelles, substrates and product compounds have to be shuttled between these compartments. Several attempts have been made to elucidate these processes but many questions still remain open.

The aim of this Thesis was shedding more light on lipid homeostasis in the yeast and elucidation of the underlying lipid traffic routes. Emphasis was put on storage and degradation of fatty acids which involves the action of β-oxidation in peroxisomes and neutral lipid depot formation in lipid particles. The link between these two metabolic routes and the crosstalk of the two organelles were a central aspect of this study.

Fatty acids

Fatty acids (FA) are building blocks of complex lipids such as phospholipids and neutral lipids in the cell. Chain length and degree of saturation play an important role for membrane fluidity and functionality of membrane bound enzymes. In addition, fatty acids can serve as signaling molecules as e.g. myristic acid or palmitic acid are involved in Ras signaling. Also, fatty acids such as oleic acid and palmitic acid can act as transcription factor activators thus regulating the expression of several proteins. Finally, fatty acids can serve as energy source via β-oxidation. In the yeast, this process is restricted to peroxisomes whereas in mammals

only very long chain fatty acids (VLCFAs) are decomposed in this organelle and further oxidized in the mitochondria (Kunau *et al.*, 1995). These features make the yeast a suitable model organism for fatty acid oxidation and peroxisome biogenesis and function. In human cells, defects in peroxisomes can lead to severe diseases such as X-linked adrenoleukodystrophy (X-ALD), Zellweger Syndrome (ZS) or Infantile refsum disease (IRD) (Thoms *et al.*, 2009).

Fatty acid synthesis in the yeast takes place in the cytosol as well as in the mitochondria, facilitated by acetyl-CoA carboxylase, encoded by *ACC1* (Al-Feel *et al.*, 1992; Hasslacher *et al.*, 1993) and *HFA1*, respectively (for a recent review see: (Tehlivets *et al.*, 2007)). Fatty acid synthesis is then carried out by FAS complex consisting of *FAS1* (β-subunit) and *FAS2* (α-subunit) (Schweizer *et al.*, 1984; Chirala *et al.*, 1987; Mohamed *et al.*, 1988) which is responsible for the bulk fatty acid synthesis in the absence of exogenous fatty acids. Synthesis of VLCFAs (C20-C26) occurs via chain elongation of C16 or C18 compounds in the ER, facilitated by three proteins encoded by *ELO1, ELO2, ELO3* (Toke and Martin, 1996; Oh *et al.*, 1997; Dittrich *et al.*, 1998).

Fatty acid transport via diffusion has been discussed to proceed in three essential steps: (i) adsorption, (ii) transmembrane movement and (iii) desorption (Hamilton, 1998). This process is influenced by a couple of factors such as pH gradient (Maloy *et al.*, 1981), relative distribution of FA binding sites in both membrane sites (Van Nieuwenhoven *et al.*, 1996), modification of FA to acyl-CoA esters (Klein *et al.*, 1971; Schaffer and Lodish, 1994) and utilization of FA for metabolic processes thus creating a ‘sink’. In the yeast, six genes were identified encoding for acyl-CoA synthases required for activation of imported exogenous fatty acids (*FAA1-4, FAT1* and *FAT2*) (Blobel and Erdmann, 1996; Watkins *et al.*, 1998; Choi and Martin, 1999; Black and DiRusso, 2007). A possible role of either of these genes in fatty acid uptake into the cell or into the peroxisome has been discussed but is at present not completely clarified. It remains elusive how fatty acids enter the cell when exogenously supplied to the growth medium and what their primary fate is within the cell. Accumulation of free fatty acid and especially of oleic acid (C18:1 Δ9) in the yeast has been claimed to lead to a lipotoxic effect (Kohlwein and Petschnigg, 2007). This aspect including the fate of exogenously supplied fatty acids in the yeast cell, lipid homeostasis and cellular function of organelles involved in fatty acid metabolic processes (peroxisomes and lipid particles) was addressed in this Thesis.

Neutral lipids

Excess of free fatty acids in the yeast is stored in the form of neutral lipids either as triacylglycerols (TAG) or steryl esters (SE). Four enzymes are responsible for the formation of these components, namely Dga1p and Lro1p forming TAG and Are1p and Are2p for the formation of SE (Sorger and Daum, 2002). All enzymes are located to the endoplasmic reticulum (ER) with only Dga1p being dually localized also to the LP. Additionally, Are1p and Are2p harbor some minor activity for TAG synthesis. Neutral lipids are stored in the LP with TAG creating the hydrophobic core surrounded by several more or less ordered layers of SE and finally a monolayer of phospholipids (Czabany *et al.*, 2008). LP is, however, not only a storage but also a dynamic organelle harboring several enzymes for the mobilization of neutral lipids upon requirement. Three TAG lipases, Tgl3p, Tgl4p and Tgl5p, are know and have been characterized in the yeast cell, all of them located on the LP (Athenstaedt and Daum, 2003; Athenstaedt and Daum, 2005). Additionally, a subset of hydrolases exists in the cell which enables the mobilization of SE. Tgl1p and Yeh1p are located to the LP and Yeh2p to the plasma membrane (PM) (Koffel *et al.*, 2005; Wagner and Daum, 2005). The mechanism of neutral lipid mobilization still remains elusive because it is not clear how these enzymes gain excess to their substrates in the LP core. This lack of knowledge may be related to our incomplete information about the LP proteome and lipidome. Although the LP has been investigated in the recent years in some detail (Athenstaedt *et al.*, 1999; Zweytick *et al.*, 2000; Daum *et al.*, 2007; Czabany *et al.*, 2007) a complete list of LP proteins and also of distinct LP lipid species is missing. It has been shown in previous studies with *Yarrowia lipolytica* that LP adapt quite efficiently to different growth conditions (Athenstaedt *et al.*, 2006). Assuming that this may also be true for *Saccharomyces cerevisiae* the effects of exogenous oleic acid supply on the formation and composition of LP proteome and lipidome were included into this Thesis. Novel technologies of Mass spectrometry were applied to this study.

Phospholipids

Phospholipids are major lipid compounds in the cell responsible for the formation of cellular membranes and thus compartmentalization of the cell. Phospholipid synthesis occurs mainly in the ER and the mitochondria but is also found to some extent in the Golgi apparatus and the lipid particles (Zinser *et al.*, 1991; Trotter and Voelker, 1995; Leber *et al.*, 1995; Daum and Vance, 1997). This subcellular distribution of lipid biosynthetic steps implies that regulated traffic mechanisms must exist facilitating the shuttle of compounds to the respective destination for assembly or further conversion.

The two most abundant phospholipids in cellular membranes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) which are essential for cell survival. PE can be synthesized via three different pathways: (i) by decarboxylation of PS either in the mitochondria by the enzyme encoded by *PSD1* (Kuchler *et al.*, 1986) or (ii) in the Golgi apparatus by its homologue *PSD2* (Trotter *et al.*, 1995; Trotter and Voelker, 1995). In a third so-called Kennedy pathway (iii) located in the ER, PE is formed from exogenous ethanolamine (Etn) which is first converted to CDP-Etn by Cki1p or Eki1p and subsequently to PE by Ect1p and Ept1p (Kennedy and Weiss, 1956). A similar pathway exists for the synthesis of PC, but not only for exogenous choline (Cho) via CDP-Cho but also as recycling process of PC degradation products (Cleves *et al.*, 1991). When no exogenous Cho is available, PC is synthesized from PE by a three step methylation route catalyzed by two independent methyltransferases Cho2p/Pem1p and Opi3p/Pem2p which are both localized to the ER (Greenberg *et al.*, 1983; Kodaki and Yamashita, 1987; Kodaki and Yamashita, 1989; McGraw and Henry, 1989). Deletion of *CHO2* as well as deletion of both genes lead to choline auxotrophy, whereas Opi3p was found to be also capable of catalyzing the first methylation step from PE to PMME.

Although the underlying mechanisms of PE and PC synthesis are well established it remains largely unclear how these lipids are transported within the cell. A possible route to non-synthesizing organelles like the PM is the vesicular transport (Kornberg and McConnell, 1971) Since blocking of the secretory pathway did not completely abolish protein and lipid transport to the plasma membrane, additional pathways must exist for lipid traffic to the cell periphery (Schnabl *et al.*, 2003).

One alternative route for phospholipid traffic is direct membrane contact which has been found between ER and plasma membrane (PAM, plasma membrane associated membranes) (Pichler *et al.*, 2001) and also between ER and mitochondria (MAM, mitochondrial associated membranes) (Gaigg *et al.*, 1995; Achleitner *et al.*, 1999).

The question remains how other organelles devoid of any phospholipid biosynthetic capacity may gain membrane lipids. One example for such a no-lipid-synthesizing organelle is the peroxisome. Its biogenesis is still under debate, but it is widely accepted that its origin lies in a budding process from the ER (Titorenko and Rachubinski, 1998; Titorenko and Mullen, 2006). Since fission and subsequent enlargement have been proven to be involved in peroxisome formation, assembly of further membrane lipids for membrane biogenesis is needed. Mechanisms of phospholipid supply to peroxisomal membranes are still not clear. Therefore, this problem was addressed in this Thesis employing different experimental strategies which were focused on the transport and assembly of aminoglycerophospholipids, especially PE to peroxisomal membranes.

The following chapters of this Thesis deal with traffic of some of the major lipid classes in the yeast, namely fatty acids, neutral lipids and phospholipids. Thereby, possible transport mechanisms will be addressed with emphasis on the role of the two organelles involved in lipid storage and degradation, LP and Px. The underlying processes of lipid transport to both organelles have not yet been elucidated although they seem to be essential for cell integrity and functionality. Results from this study in yeast will enable us to shed more light on these processes and may to some extent also be relevant to the mammalian system. In humans, defects in such processes result in a couple of diseases. Knowledge developed in this work may therefore help to understand and investigate lipid homeostasis defects linked to disturbed lipid storage, turnover and degradation in a more general way.

Reference List

Achleitner,G., Gaigg,B., Krasser,A., Kainersdorfer,E., Kohlwein,S.D., Perktold,A., Zellnig,G., and Daum,G. (1999). Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. Eur J Biochem *264*, 545-53.

Al-Feel,W., Chirala,S.S., and Wakil,S.J. (1992). Cloning of the yeast FAS3 gene and primary structure of yeast acetyl-CoA carboxylase. Proc. Natl. Acad. Sci. U. S. A *89*, 4534-4538.

Athenstaedt,K. and Daum,G. (2003). YMR313c/TGL3 encodes a novel triacylglycerol lipase located in lipid particles of Saccharomyces cerevisiae. J. Biol. Chem. *278*, 23317-23323.

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

Athenstaedt,K., Jolivet,P., Boulard,C., Zivy,M., Negroni,L., Nicaud,J.M., and Chardot,T. (2006). Lipid particle composition of the yeast Yarrowia lipolytica depends on the carbon sources. Proteomics. *6*, 1450-1459.

Athenstaedt,K., Zweytick,D., Jandrositz,A., Kohlwein,S.D., and Daum,G. (1999). Identification and characterization of major lipid particle proteins of the yeast Saccharomyces cerevisiae. J. Bacteriol. *181*, 6441-6448.

Black,P.N. and DiRusso,C.C. (2007). Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. Biochim. Biophys. Acta *1771*, 286-298.

Blobel,F. and Erdmann,R. (1996). Identification of a yeast peroxisomal member of the family of AMP-binding proteins. Eur. J. Biochem. *240*, 468-476.

Chirala,S.S., Kuziora,M.A., Spector,D.M., and Wakil,S.J. (1987). Complementation of mutations and nucleotide sequence of FAS1 gene encoding beta subunit of yeast fatty acid synthase. J. Biol. Chem. *262*, 4231-4240.

Choi,J.Y. and Martin,C.E. (1999). The Saccharomyces cerevisiae FAT1 gene encodes an acyl-CoA synthetase that is required for maintenance of very long chain fatty acid levels. J. Biol. Chem. *274*, 4671-4683.

Cleves,A.E., McGee,T.P., Whitters,E.A., Champion,K.M., Aitken,J.R., Dowhan,W., Goebl,M., and Bankaitis,V.A. (1991). Mutations in the CDP-choline pathway for phospholipid biosynthesis bypass the requirement for an essential phospholipid transfer protein. Cell *64*, 789-800.

Czabany,T., Athenstaedt,K., and Daum,G. (2007). Synthesis, storage and degradation of neutral lipids in yeast. Biochim. Biophys. Acta *1771*, 299-309.

Czabany,T., Wagner,A., Zweytick,D., Lohner,K., Leitner,E., Ingolic,E., and Daum,G. (2008). Structural and Biochemical Properties of Lipid Particles from the Yeast Saccharomyces cerevisiae. J. Biol. Chem. *283*, 17065-17074.

Daum,G. and Vance,J.E. (1997). Import of lipids into mitochondria. Prog Lipid Res *36*, 103-30.

Daum,G., Wagner,A., Czabany,T., Grillitsch,K., and Athenstaedt,K. (2007). Lipid storage and mobilization pathways in yeast. Novartis. Found. Symp. *286*, 142-151.

Dittrich,F. *et al.* (1998). Fatty acid elongation in yeast--biochemical characteristics of the enzyme system and isolation of elongation-defective mutants. Eur. J. Biochem. *252*, 477-485.

Gaigg,B., Simbeni,R., Hrastnik,C., Paltauf,F., and Daum,G. (1995). Characterization of a microsomal subfraction associated with mitochondria of the yeast, Saccharomyces cerevisiae. Involvement in synthesis and import of phospholipids into mitochondria. Biochim. Biophys. Acta *1234*, 214-220.

Greenberg,M.L., Klig,L.S., Letts,V.A., Loewy,B.S., and Henry,S.A. (1983). Yeast mutant defective in phosphatidylcholine synthesis. J. Bacteriol. *153*, 791-799.

Hamilton,J.A. (1998). Fatty acid transport: difficult or easy? J. Lipid Res. *39*, 467-481.

Hasslacher,M., Ivessa,A.S., Paltauf,F., and Kohlwein,S.D. (1993). Acetyl-CoA carboxylase from yeast is an essential enzyme and is regulated by factors that control phospholipid metabolism. J. Biol. Chem. *268*, 10946-10952.

Kennedy,E.P. and Weiss,S.B. (1956). The function of cytidine coenzymes in the biosynthesis of phospholipides. J. Biol. Chem. *222*, 193-214.

Klein,K., Steinberg,R., Fiethen,B., and Overath,P. (1971). Fatty acid degradation in Escherichia coli. An inducible system for the uptake of fatty acids and further characterization of old mutants. Eur. J. Biochem. *19*, 442-450.

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

Kodaki,T. and Yamashita,S. (1989). Characterization of the methyltransferases in the yeast phosphatidylethanolamine methylation pathway by selective gene disruption. Eur. J. Biochem. *185*, 243-251.

Koffel,R., Tiwari,R., Falquet,L., and Schneiter,R. (2005). The Saccharomyces cerevisiae YLL012/YEH1, YLR020/YEH2, and TGL1 genes encode a novel family of membrane-

anchored lipases that are required for steryl ester hydrolysis. Mol. Cell Biol. *25*, 1655-1668.

Kohlwein,S.D. and Petschnigg,J. (2007). SLipid-induced cell dysfunction and cell death: lessons from yeast. Curr. Hypertens. Rep. *9*, 455-461.

Kornberg,R.D. and McConnell,H.M. (1971). Lateral diffusion of phospholipids in a vesicle membrane. Proc Natl Acad Sci U S A *68*, 2564-8.

Kuchler,K., Daum,G., and Paltauf,F. (1986). Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in Saccharomyces cerevisiae. J Bacteriol *165*, 901-10.

Kunau,W.H., Dommes,V., and Schulz,H. (1995). beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. Prog. Lipid Res. *34*, 267-342.

Leber,A., Hrastnik,C., and Daum,G. (1995). Phospholipid-synthesizing enzymes in Golgi membranes of the yeast, Saccharomyces cerevisiae. FEBS Lett. *377*, 271-274.

Maloy,S.R., Ginsburgh,C.L., Simons,R.W., and Nunn,W.D. (1981). Transport of long and medium chain fatty acids by Escherichia coli K12. J. Biol. Chem. *256*, 3735-3742.

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

Mohamed,A.H., Chirala,S.S., Mody,N.H., Huang,W.Y., and Wakil,S.J. (1988). Primary structure of the multifunctional alpha subunit protein of yeast fatty acid synthase derived from FAS2 gene sequence. J. Biol. Chem. *263*, 12315-12325.

Oh,C.S., Toke,D.A., Mandala,S., and Martin,C.E. (1997). ELO2 and ELO3, homologues of the Saccharomyces cerevisiae ELO1 gene, function in fatty acid elongation and are required for sphingolipid formation. J. Biol. Chem. *272*, 17376-17384.

Pichler,H., Gaigg,B., Hrastnik,C., Achleitner,G., Kohlwein,S.D., Zellnig,G., Perktold,A., and Daum,G. (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.

Schaffer,J.E. and Lodish,H.F. (1994). Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. Cell *79*, 427-436.

Schnabl,M., Oskolkova,O.V., Holic,R., Brezna,B., Pichler,H., Zagorsek,M., Kohlwein,S.D., Paltauf,F., Daum,G., and Griac,P. (2003). Subcellular localization of yeast Sec14

homologues and their involvement in regulation of phospholipid turnover. Eur. J. Biochem. *270*, 3133-3145.

Schweizer,M., Lebert,C., Holtke,J., Roberts,L.M., and Schweizer,E. (1984). Molecular cloning of the yeast fatty acid synthetase genes, FAS1 and FAS2: illustrating the structure of the FAS1 cluster gene by transcript mapping and transformation studies. Mol. Gen. Genet. *194*, 457-465.

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

Tehlivets,O., Scheuringer,K., and Kohlwein,S.D. (2007). Fatty acid synthesis and elongation in yeast. Biochim. Biophys. Acta *1771*, 255-270.

Thoms,S., Gronborg,S., and Gartner,J. (2009). Organelle interplay in peroxisomal disorders. Trends Mol. Med. *15*, 293-302.

Titorenko,V.I. and Mullen,R.T. (2006). Peroxisome biogenesis: the peroxisomal endomembrane system and the role of the ER. J Cell Biol *174*, 11-7.

Titorenko,V.I. and Rachubinski,R.A. (1998). The endoplasmic reticulum plays an essential role in peroxisome biogenesis. Trends Biochem Sci *23*, 231-3.

Toke,D.A. and Martin,C.E. (1996). Isolation and characterization of a gene affecting fatty acid elongation in Saccharomyces cerevisiae. J. Biol. Chem. *271*, 18413-18422.

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

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

Van Nieuwenhoven,F.A., van,d., V, and Glatz,J.F. (1996). Membrane-associated and cytoplasmic fatty acid-binding proteins. Lipids *31 Suppl*, S223-S227.

Wagner,A. and Daum,G. (2005). Formation and mobilization of neutral lipids in the yeast Saccharomyces cerevisiae. Biochem. Soc. Trans. *33*, 1174-1177.

Watkins,P.A., Lu,J.F., Steinberg,S.J., Gould,S.J., Smith,K.D., and Braiterman,L.T. (1998). Disruption of the Saccharomyces cerevisiae FAT1 gene decreases very long-chain fatty acyl-CoA synthetase activity and elevates intracellular very long-chain fatty acid concentrations. J. Biol. Chem. *273*, 18210-18219.

Zinser,E., Sperka-Gottlieb,C.D., Fasch,E.V., Kohlwein,S.D., Paltauf,F., and Daum,G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. J Bacteriol *173*, 2026-34.

Zweytick,D., Athenstaedt,K., and Daum,G. (2000). Intracellular lipid particles of eukaryotic cells. Biochim. Biophys. Acta *1469*, 101-120.

CHAPTER 1

PHOSPHATIDYLETHANOLAMINE SYNTHESIZED BY THREE DIFFERENT PATHWAYS IS SUPPLIED TO PEROXISOMES OF THE YEAST *SACCHAROMYCES CEREVISIAE*

Sabine Rosenberger[a], Melanie Connerth[a], Günther Zellnig[b] and Günther Daum[a*]

[a]Institute of Biochemistry, Graz University of Technology, Graz, Austria;
[b]Institute of Plant Science, Karl Franzens University Graz, Austria

Keywords: phosphatidylethanolamine, peroxisomes, phospholipids, membrane contact, yeast

Abbreviations: CL, cardiolipin; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

published in: Biochimica et Biophysica Acta (Molecular and Cell Biology of Lipids), 2009 May;1791(5):379-87.

Abstract

In the yeast *Saccharomyces cerevisiae* three pathways lead to the formation of phosphatidylethanolamine (PE), namely decarboxylation of phosphatidylserine (PS) (i) by Psd1p in mitochondria, and (ii) by Psd2p in a Golgi/vacuolar compartment; and (iii) synthesis via CDP-ethanolamine pathway in the endoplasmic reticulum. To determine the contribution of these pathways to the supply of PE to peroxisomes, we subjected mutants bearing defects in the respective metabolic routes to biochemical and cell biological analysis. Despite these defects in PE formation mutants were able to grow on oleic acid indicating induction of peroxisome proliferation. Biochemical analysis revealed that PE formed through all three pathways was supplied to peroxisomes. These analyses also demonstrated that selective as well as equilibrium interorganelle flux of PE appear to be equally important for cellular homeostasis of this phospholipid. Electron microscopic inspection confirmed that defects in PE synthesis still allowed formation of peroxisomes, although these organelles from strains lacking *PSD1* were significantly smaller than wild type. The fact that peroxisomes were always found in close vicinity to mitochondria, ER and lipid particles supported the view that membrane contact may play a role in lipid traffic between these organelles.

Introduction

In the yeast *Saccharomyces cerevisiae* the major phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). Within a cell, however, not all membranes have the capacity to synthesize their own lipids. It is well accepted that the endoplasmic reticulum (ER) and mitochondria are the subcellular compartments which contribute most to yeast phospholipid biosynthesis(Zinser *et al.*, 1991; Daum and Vance, 1997). Other subcellular compartments such as the Golgi and lipid particles also harbor certain steps or even branches of phospholipid synthesis (Zinser *et al.*, 1991; Leber *et al.*, 1994; Trotter and Voelker, 1995).

A yeast organelle which cannot synthesize its own lipids is the peroxisome. Peroxisomes are versatile, single membrane-bound organelles occurring ubiquitously in eukaryotic cells. Number, size and functions of these organelles are strongly determined by cell type and physiology (Veenhuis *et al.*, 2000; van der Klei and Veenhuis, 2002). A characteristic feature of peroxisomes is their large amount of matrix enzymes which catalyze various oxidative and biosynthetic reactions involved in hydrogen peroxide metabolism, β-oxidation and the glyoxylate cycle. In yeast and plant cells, β-oxidation has been exclusively localized to peroxisomes, whereas in mammalian cells mitochondria also harbor enzymes of this pathway. Yeast peroxisome proliferation can be induced by growth on media containing alkanes and fatty acids. Lipid analysis of peroxisomal membranes (Zinser *et al.*, 1991; Schneiter *et al.*, 1999) showed that PC and PE are the major phospholipids of peroxisomal membranes. Since peroxisomes lack the capacity to synthesize these glycerophospholipids, whereas the ER, mitochondria and the Golgi had been identified to contribute to the synthesis of these lipids, interorganelle transport of PC and PE is required to keep the lipid composition of peroxisomes balanced and to guarantee functionality of this compartment.

Mechanisms of lipid translocation between subcellular membranes are still a matter of dispute. Presently, there is no evidence that phospholipid transfer proteins (Wirtz, 1991) contribute to the supply of lipids to peroxisomes. Purdue and Lazarow (Purdue and Lazarow, 2001) suggested that specialized vesicles may translocate phospholipids from the ER to peroxisomes, and that such vesicles may contain proteins which facilitate targeting to peroxisomes. There is, however, little experimental evidence supporting these data. The third intracellular mechanism of lipid transport under discussion is membrane contact.

It has been suggested that membrane constituents migrate from the ER to mitochondrial membranes at sites of close apposition (Ardail *et al.*, 1993; Shiao *et al.*, 1998; Achleitner *et al.*, 1999).

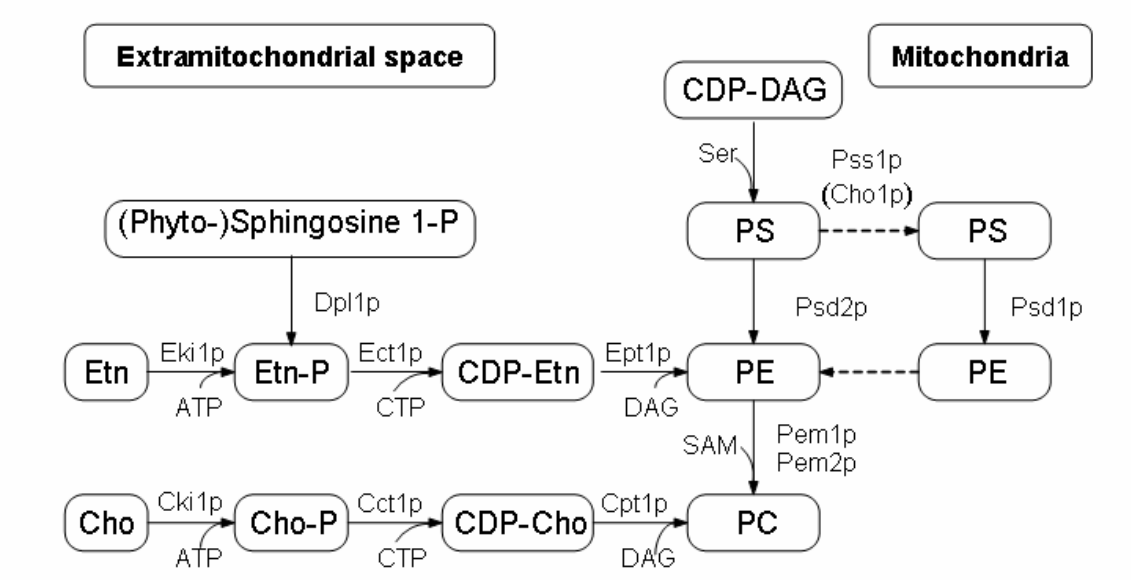


Figure 1: **Biosynthesis of phosphatidylethanolamine in yeast**

Biosynthesis of PE is accomplished by decarboxylation of PS by either Psd1p in the IMM or by Psd2p in the Golgi/vacuole. Alternatively, exogenous ethanolamine can be incorporated into PE via the CDP-ethanolamine branch of the Kennedy pathway. PC is formed by either methylation of PE or from exogenous choline through the CDP-choline branch of the Kennedy pathway.

Although not proven such a mechanism has also to be taken into account for the supply of lipids to peroxisomes. The aim of the work present here was to investigate transport routes and assembly of PE into peroxisomal membranes of the yeast *Saccharomyces cerevisiae*. This process was considered complex because three pathways contribute to yeast PE biosynthesis (Fig. 1), namely (i) decarboxylation of PS through catalysis of phosphatidylserine decarboxylase 1 (Psd1p) in the inner mitochondrial membrane (IMM) (Zinser *et al.*, 1991), (ii) decarboxylation of PS by phosphatidylserine decarboxylase 2 (Psd2p) in the Golgi (Trotter and Voelker, 1995), and (iii) incorporation of ethanolamine through the CDP-ethanolamine branch of the so-called Kennedy pathway (Kennedy and Weiss, 1956) which is located to microsomes (Daum *et al.*, 1998; Nebauer R. *et al.*, 2003).

In the yeast, PS required as a substrate for both PE forming PS decarboxylases is synthesized from cytidinediphosphate diacylglycerol (CDP-DAG) and serine by PS synthase (Pss1p/Cho1p), which is associated with the ER and related membranes (Kuchler *et al.*, 1986; Gaigg *et al.*, 1995). Ethanolamine utilized as a substrate for enzymes of the CDP-ethanolamine route can be taken up by yeast cells from the medium or set free within the cell by phospholipases. Ethanolamine phosphate serving as an intermediate in the Kennedy pathway can also be provided through sphingolipid degradation in a reaction catalyzed by the dihydrosphingosine-1-phosphate lyase Dpl1p (Saba *et al.*, 1997; Gottlieb *et al.*, 1999).

In this study, we present biochemical and cell biological evidence that all three pathways of PE synthesis contribute to the supply of this aminoglycerophospholipid to peroxisomal membranes. To demonstrate these translocation routes, we analyzed peroxisome preparations from mutants bearing defects in PE formation, namely *psd1Δ* and *psd2Δ* single mutants, a *psd1Δpsd2Δ* double mutant and a *cki1Δeki1Δdpl1Δ* triple mutant. These mutations markedly affected the cellular and peroxisomal phospholipid composition and also caused changes in the fatty acid composition of cellular and peroxisomal PE. Electron microscopy of wild type and mutant strains showed some difference in the size of peroxisomes. A computer aided method employed for three-dimensional reconstructions of yeast cells revealed that yeast peroxisomes similar to other organelles have a strong tendency to associate. Based on these findings we discuss contact of peroxisomes with the ER, mitochondria and lipid particles as possible mechanisms of lipid translocation.

Materials and Methods

Strains and culture conditions

The wild type yeast strain *Saccharomyces cerevisiae* FY1679, the *psd1Δ* and *psd2Δ* single mutants, a *psd1Δpsd2Δ* double mutant and a *cki1Δeki1Δdpl1Δ* triple mutant (Birner *et al.*, 2001) (Table1) were used throughout this study. For induction of peroxisomes, cells were grown first in YPGal medium (1% yeast extract, 2% peptone and 2% galactose) to the stationary phase, inoculated to an OD_{600} of 0.1 in YPO containing 0.3% yeast extract, 0.5% peptone, 0.5% potassium phosphate, pH 6, 0.1% oleic acid (herbal oleic acid pure; Merck, Darmstadt, Germany), 0.2% Tween 80 and 0.05% galactose, and grown to the late logarithmic phase. It has to be noted that YPGal and YPO media contained low amounts of ethanolamine and choline. Furthermore, oleic acid preparations used routinely as carbon source contained impurities of margaric acid, myristic acid, stearic acid, palmitic acid, palmitoleic acid, linoleic acid and linolenic acid. Growth of the different strains on liquid media was followed by measuring the OD_{600}.

Growth tests for phenotype analysis were performed on solid YPO media with 2% bactoagar (Difco, Detroit, MI). Cells were incubated for 3 days at 30°C or 37°C, and on media containing 0.5-2 % EtOH, 1-2 mM H_2O_2, 1-30 µg Terbinafine, 2-15 µg Nystatin, 0.0025 % SDS and 1-8 mM sorbitol.

Table 1: Strains

Strain	Genotype	Reference
YRB1 (wt; FY1679)	*MATa his3Δ200 leu 2Δ1 trp1Δ63 ura3-52*	(Birner *et al.*, 2001)
YRB2	*MATa his3Δ200 leu 2Δ1 trp1Δ63 ura3-52 psd1Δ::KanMX4*	(Birner *et al.*, 2001)
YRB3	*MATa his3Δ200 leu 2Δ1 trp1Δ63 ura3-52 psd2Δ::KanMX4*	(Birner *et al.*, 2001)
YRB5	*MATa his3Δ200 leu 2Δ1 trp1Δ63 ura3-52 psd1Δ::KanMX4 psd2Δ::KanMX4*	(Birner *et al.*, 2001)
YIS16	*Mat a his3Δ200 leu 2Δ1 trp1Δ63 ura3-52 cki1Δ::HIS3 dpl1Δ::LEU2 eki1Δ:: TRP1*	Provided by I. Schuiki

Isolation of peroxisomes

For the isolation of peroxisomes and mitochondria, cells grown to the late exponential phase in YPO medium were harvested by centrifugation and converted to spheroplasts as described by Daum *et al.* (Daum *et al.*, 1982). For spheroplasting, 2 mg Zymolyase 20T per g wet weight were used. Cells were lysed in a breakage buffer containing 5 mM MES-KOH, pH 6.0, 0.6 M sorbitol, 1 mM KCl, 0.5 mM EDTA, and 1 mM PMSF (protease inhibitor). Spheroplasts were homogenized on ice using a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 5,000 rpm for 5 min. To enhance the yield of peroxisomes, the resulting pellet was twice resuspended in breakage buffer, re-homogenized and centrifuged as described above (Zinser and Daum, 1995). Combined supernatants were centrifuged at 15,000 rpm in an SS34 rotor (Sorvall) for 15 min. The crude organelle pellet consisting mainly of mitochondria and peroxisomes was gently resuspended in breakage buffer with the aid of a Dounce homogenizer and centrifuged at 5,000 rpm to remove residual cell debris, nuclei and organelle aggregates. The supernatant was centrifuged at 15,000 rpm, the pellet was resuspended in breakage buffer as described above and loaded onto an Accudenz gradient (17-35%; w/v) in 5 mM MES-KOH, pH 6.0, 1 mM KCl, 0.24 M sucrose for further purification. The density gradient centrifugation was carried out in a swing out rotor AH-629 at 26,000 rpm for 90 min. The organelle layer consisting of peroxisomes was withdrawn, diluted with 4 volumes of the breakage buffer and sedimented at 15,000 rpm in an SS34 rotor for 15 min at 4°C. Mitochondria which formed a separate band in the density gradient were also collected and sedimented by centrifugation.

Protein analysis

Proteins from isolated subcellular fractions were precipitated with trichloroacetic acid (TCA) at a final concentration of 10%. For protein quantification the pellet was solubilized in 0.1% SDS, 0.1 M NaOH and analyzed by the method of Lowry *et al.* (Lowry et al., 1951) using bovine serum albumin as a standard. SDS-PAGE (sodium dodecylsulfate polyacrylamide Gel electrophoresis) was carried out as described by Laemmli (Laemmli, 1970) using a BioRad Mini Protean II equipment. Separation of proteins was performed using a 12.5% separation gel. SDS-PAGE was carried out at 45 mA for 1 h.

For subcellular localization of proteins and expression studies, Western blot analysis was performed as described by Haid and Suissa (Haid and Suissa, 1983) using primary rabbit antibodies against porin, Fox1p, Pma1p and GAPDH. Immunoreactive bands were visualized

by ELISA using a peroxidase-linked secondary antibody (Sigma) following the instructions of the manufacturer.

Lipid analysis

Phospholipids were extracted from homogenate and purified peroxisomal membrane fractions (Folch J. *et al.*, 1957), and individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25 % NH_3 (65:35:5, per vol.) as first, and chloroform/acetone/methanol/ acetic acid/water (50:20:10:10:5; per vol.) as second developing solvent. Lipids were stained with iodine vapor, scraped off the plate, and quantified by the method of Broekhuyse (Broekhuyse, 1968).

Fatty acids were analyzed by gas liquid chromatography (GLC). Lipids extracted and separated as described above were subjected to methanolysis using BF_3-methanol and converted to methyl esters (Morrison W.R. and Smith L.M., 1964). Fatty acyl methyl esters were separated by GLC using a Hewlett-Packard Ultra 2 capillary column (5% phenyl methyl silicone) with a temperature gradient (20 min at 200°C, 10 min to 280°C, 15 min at 300°C). Fatty acids were identified by comparison to commercial fatty acyl methyl ester standards (NuCheck).

Electron microscopy of yeast cells

For ultrastructural examination, cells were grown under aerobic conditions at 30°C on YPO as described above. Cells were harvested in the late exponential phase by centrifugation and washed twice with 0.5 % BSA (fatty acid free) and 3 times with H_2O bidest. Then, cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with dist. water and fixed in a 1% aqueous solution of $KMnO_4$ for 20 min again (D. Kolb, personal communication). Fixed cells were washed three times in dist. 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 embedded in acetone:Epon mixture (1:1) for 5 hours, then in acetone:Epon mixture (1:3) over night at room temperature and finally polymerized in pure Epon resin for 48 hours at 60°C. Ultrathin sections of 80 nm were stained with lead citrate and viewed with a Philips CM 10 electron microscope. For 3-dimensional (3D) ultrastructural imaging yeast cells were grown under aerobic conditions at 30°C in YPO to the late logarithmic phase, harvested by centrifugation and subjected to fixation.

Electron microscopic investigations were performed as described by Perktold *et al.* (Perktold *et al.*, 2007). Cells were treated with 4% paraformaldehyde/5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) and 1 mM $CaCl_2$ for 90 min at room temperature. Then, cells were washed in buffer with 1 mM $CaCl_2$ for 1 h and incubated for 1 h with a 2 % aqueous solution of $KMnO_4$. After washing for 30 min, samples were dehydrated in a graded series of ethanol (50-100 %, with en bloc staining in 2 % uranylacetate in 70 % ethanol overnight) and gradually infiltrated with increasing concentrations of Spurr resin (30, 50, 70 and 100 %) mixed with ethanol for a minimum of 3 h for each step. Finally, samples were embedded in pure, fresh Spurr resin and polymerized at 60 °C for 48 h. Series of 80 nm ultrathin sections were prepared with an ultramicrotome (Reichert Ultracut S) and transferred to single slot grids because their transmission area is high enough to show a complete ribbon of serial ultrathin sections without any disturbing bars. Ultrathin sections were stained and viewed as described above. Section thickness restricts resolution in z direction to 80 nm. A computer aided method for 3D reconstruction of yeast cells and determination of surface areas was used as described in detail in Perktold *et al.* (Perktold *et al.*, 2007). In brief, TEM micrographs of the serial sectioned yeast cells were digitized by a scanner (Epson 4990 Photo). Selected organelles structures were traced semi-automatically by a computer program (Corel Trace) or by hand (Corel Draw) to create vectorgraphics. For 3D reconstructions, vectorgraphics of successive sections were aligned by centering specific cell structures. 3D reconstructions were created by the program Carrara Studio (Softline). It has to be mentioned that this method led to images of reconstructed, real existing cell structures and not to models based on statistical possibilities.

The circumference of organelles was measured by the computer program Optimas 6.5 (Bio Scan) and data were exported to the software program Excel. The total surface area of organelles was calculated by the sum of their circumferences multiplied by the section thickness. Organelle associations were determined on the TEM micrographs by counting the number of associations when two selected cell compartments were in a distance of less than 30 nm. Counting was performed on every single section of the section series and resulted in the total number of associations of two compartments within the whole cell. As the number of associations may depend on the abundance and size of the organelles, data were also presented as number of associations per 10μm^2 organelle surface area.

Results

Growth on oleic acid of wild type and mutants bearing defects in PE biosynthesis

To study the relative contribution of the three different pathways of PE synthesis to peroxisomal biogenesis a series of haploid single and multiple deletion strains with defects in the different biosynthetic routes of PE formation (see Introduction) were utilized (Birner *et al.*, 2001). Induction of yeast peroxisome proliferation required cultivation on oleic acid containing medium. As shown in Table 2 all strains tested could grow on YPO which contained small amounts of ethanolamine and choline. The growth rate of strains cultivated on YPO was markedly lower than on YPD, which resulted in ratios of logarithmic growth rates (YPO to YPD) below 1. This ratio is a specific measure for the effect of the respective mutations on peroxisome formation and normalized for general defects of the deletion(s). As can be seen from Table 2, strains bearing mutations in *PSD2* and genes encoding for enzymes of the CDP-ethanolamine pathway showed a reduced growth rate on YPO compared to YPD. This result indicates specific contributions of these two PE biosynthetic routes to the proliferation of peroxisomes.

Table 2: Growth rates of strains bearing defects in PE synthesis on YPD and YPO

Cells were grown on YPD medium and YPO. The optical density OD_{600} was measured at 600 nm. The YPO to YPD ratio of logarithmic growth rates and the ratio of OD values reached in the stationary phase are shown. Values were obtained from 3 different experiments with a mean deviation of ± 10 %.

	Ratio of logarithmic growth rates	Ratio of OD values (stationary phase)
Strains	YPO / YPD	YPO / YPD
FY1679 (wt)	0.65	0.29
psd1Δ	0.73	0.35
psd2Δ	0.44	0.3
psd1Δpsd2Δ	0.5	0.37
cki1Δeki1Δdpl1Δ	0.56	0.27

To study the growth phenotype of strains bearing defects in PE synthesis in more detail we performed growth tests on YPD in the presence of various components or at variable growth conditions (see Methods section). Growth of cells was evaluated at 30°C and 37°C, and on solid media containing 1 % EtOH, 2 mM H_2O_2, 30 µg Terbinafine, 80 µg Nystatin, 0.0025% SDS and 4 mM Sorbitol, respectively. These conditions were chosen because they affected

growth of wild type slightly, but significantly. Under all conditions tested, the *psd1Δ* single mutant, the *psd1Δpsd2Δ* double deletion strain and the *cki1Δeki1Δdpl1Δ* mutant grew worse than wild type (data not shown). Growth of *psd2Δ* was not affected.

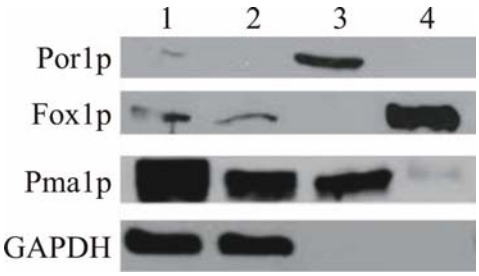


Figure 2: Western blot analysis of subcellular fractions from *Saccharomyces cerevisiae* wild type FY1679 grown on oleic acid

Proteins from homogenate (lane 1), cytosol (lane 2), mitochondria (lane 3) and peroxisomes (lane 4) were separated by SDS-PAGE. Same amounts of protein were loaded. Antibodies against Por1p, Fox1p, Pma1p and GAPDH were used to detect marker proteins as described in the Methods section.

Characterization of peroxisomes from cells defective in PE biosynthesis

Peroxisomes were isolated as described previously (Zinser and Daum, 1995). The quality of peroxisomal preparation was routinely tested by Western blot analysis determining the enrichment of peroxisomes and their contamination with other subcellular fractions (Fig. 2). For this analysis we used antibodies against porin (Por1p) as a marker for the mitochondrial outer membrane, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the cytosol, plasma membrane ATPase (Pma1p) for the plasma membrane, and the multifunctional β-oxidation protein (Fox1p) for peroxisomal membranes. As can be seen from Fig. 2, Fox1p was highly enriched in peroxisomes from wild type cells. The degree of cross-contamination of peroxisomal fractions with other subcellular fractions was low. Impurities caused by co-isolation of mitochondria were marginal as judged from the lack of porin in isolated peroxisomal fractions. Similarly, peroxisomes were practically devoid of cytosolic contamination as shown by the absence of GAPDH. A slight contamination of peroxisomes with the plasma membrane represented by Pma1p had to be taken into account. The strong signal for Pma1p in the homogenate of cells grown on oleic acid suggested the importance of this protein under these conditions. Enrichment of peroxisomes as well as the degree of contamination with other subcellular fractions was similar in wild type and in all mutants bearing defects in PE synthesis (data not shown).

Phospholipid composition of peroxisomes from yeast strains bearing defects in PE formation

To estimate the contribution of Psd1p, Psd2p and the CDP-ethanolamine pathway to the supply of PE to peroxisomes we subjected homogenate and peroxisomal fractions of wild type, *psd1Δ* and *psd2Δ* single mutants, the *psd1Δpsd2Δ* double mutant and the *cki1Δeki1Δdpl1Δ* triple mutant to glycerophospholipid analysis (Table 3). In wild type homogenate, the major phospholipids were PC, PE and PI with PC accounting for approximately 50% of total cellular phospholipids. Other lipids, such as PS, phosphatidic acid (PA) and cardiolipin (CL) were only minor components of total cellular membranes. In peroxisomes from wild type, the amount of PI was slightly increased over the homogenate at the expense of PE.

Table 3: Lipid composition of homogenate and peroxisomes from yeast strains bearing defects in PE formation

The phospholipid composition of homogenate (H) and peroxisomes (P) are expressed as % of total phospholipids in the respective fraction. PI, phosphatidylinositol, PS, phosphatidylserine, PC, phosphatidylcholine, PE, phosphatidylethanolamine, CL, cardiolipin. Values are from four independent measurements with a mean deviation of ± 10 %.

	% of total phospholipids											
	PI		PS		PC		PE		CL		others	
Strains	H	P	H	P	H	P	H	P	H	P	H	P
FY1679 (wt)	14	21	6	5	49	49	22	17	4	1	5	7
psd1Δ	16	18	7	10	60	50	12	12	3	1	2	9
psd2Δ	13	17	6	8	54	50	22	13	3	1	2	11
psd1Δpsd2Δ	18	19	8	9	60	56	7	8	3	0	4	8
cki1Δeki1Δdpl1Δ	21	19	8	9	52	50	11	11	5	1	3	10

In *psd2Δ*, the phospholipid patterns of homogenate and peroxisomes largely resembled wild type confirming that the role of Psd2p in cellular PE formation is minor. In contrast, deletion of *PSD1* alone or in combination with *PSD2* led to a marked decrease of PE in total membranes and peroxisomes. This decrease was compensated by increased amounts of the other phospholipids. The strong decrease of PE in *psd1Δ* resembled data obtained with cells grown on non-fermentable carbon sources (Birner *et al.*, 2001). Surprisingly, the decrease of PE in a mutant lacking enzymes of the CDP-ethanolamine pathway was comparable to *psd1Δ*. No such effect had been observed before with cells grown on other carbon sources.

Whereas in peroxisomes from strains which were well endowed with total cellular PE (wild type, *psd2Δ*) the level of this phospholipid decreased compared to homogenate, cells depleted of PE showed an almost equilibrium level of this phospholipids in all fractions. We interpret these data as a result of a basic equilibrium flux of PE to peroxisomes with some directed specific translocation on top. In summary, however, our results clearly show that all three pathways of PE synthesis contribute to the supply of this lipid to peroxisomes.

Fatty acid composition of peroxisomal membrane phospholipids

Fatty acids of phospholipids are important components with respect to physical properties of a membrane where they are accommodated. For this reason we analyzed the fatty acid composition of peroxisomal lipids and compared them to bulk membranes. As an additional dimension, we compared data from wild type to strains lacking PE-synthesizing enzymes. Since *Saccharomyces cerevisiae* does not synthesize polyunsaturated fatty acids, the acyl chain composition of yeast lipids is rather simple and mainly restricted to 16 and 18 species without or with one double bond.

Table 4: Fatty acid composition of lipid extracts from homogenate and purified peroxisomes of different strains

Values are from at least 3 independent measurements with a mean deviation of ± 10 %. (*) 18:2 fatty acids are constituents from the oleic acid preparation which was used as carbon source to induce formation of peroxisomes. Homogenate (H), purified peroxisomes (P).

	% of total fatty acids									
	FY 1679 (wt)		*psd1Δ*		*psd2Δ*		*psd1Δpsd2Δ*		*cki1Δeki1Δdpl1Δ*	
	H	P	H	P	H	P	H	P	H	P
16:0	8	12	8	12	9	10	8	12	8	13
16:1	2	3	1	3	2	4	1	2	1	3
18:0	1	4	1	3	1	3	1	3	1	3
18:1	74	68	77	70	66	68	72	67	73	68
18:2(*)	14	12	11	9	19	11	15	11	15	10

In Table 4 the fatty acid composition of lipid extracts from homogenate and peroxisomes of wild type and mutants are shown. Growth of *Saccharomyces cerevisiae* on oleic acid dramatically affected the fatty acid pattern of whole cell extracts and peroxisomal membranes.

Under these conditions, oleic acid (18:1) became predominant suggesting that this fatty acid was not only used as a carbon source but was also directly incorporated into complex lipids. Palmitic acid (16:0), palmitoleic acid (16:1) and stearic acid (18:0) were also detected in these lipid extracts, although at minor amounts. Incorporation of linoleic acid (18:2) into complex lipids was due to the fact that this fatty acid is a minor constituent of the oleic acid preparation used as a carbon source in the medium. Differences in the fatty acid compositions of homogenate and peroxisomes from cells grown on oleic acid appeared to be minor at first sight. A closer calculation of our data, however, indicated that the ratio of unsaturated to saturated fatty acids was markedly different in bulk membranes and peroxisomes. Whereas in the homogenate this ratio was approximately 10, it decreased to a value of approximately 5 in peroxisomes. Thus, peroxisomal membranes as a whole have to be considered less fluid than other cellular membranes. This result also indicates that there is at least some selectivity for phospholipid species supplied to peroxisomes.

Analysis of individual phospholipids from total cell extract and peroxisomes isolated from strains bearing defects in PE synthesis are shown in Table 5. Despite the predominant appearance of oleic acid (18:1) in all samples some differences in the species composition of phospholipids were observed. Again, two dimensions of this analysis deserved our attention. First, it is the specific fatty acid composition of individual phospholipids which is result of properties of the enzymes involved. Secondly, differences between bulk membrane (homogenate) and peroxisomal lipid composition have to be taken into account which is indicative of specific translocation and membrane assembly processes for individual lipid species. In combination, these values allow us to estimate the contribution of the different PE biosynthetic pathways to the formation of peroxisomal membranes.

Values obtained with PI (Table 5A) demonstrate that this phospholipid contained higher amounts of 16:0 and lower amounts of 18:1 and 18:2 than total phospholipids (see Table 4). In all strains tested with the exception of *cki1Δeki1Δdpl1Δ* the amount of 18:1 containing PI species was increased in peroxisomes over total cell extracts at the expense of 18:0 and 16:0. Thus, in contrast to total peroxisomal lipids the ratio of unsaturated to saturated fatty acids in peroxisomal PI was increased.

These result suggests that certain PI species were preferentially incorporated into peroxisomal membranes. Mutations in genes encoding PE biosynthetic enzymes did not change this pattern.

Table 5: Fatty acid composition of individual phospholipids of homogenate and purified peroxisomes of different strains

Values are from at least 3 independent measurements with a mean deviation of ± 10 %. (*) 18:2 fatty acids are constituents from the oleic acid preparation used to induce formation of peroxisomes. N.d. not detected. Homogenate (H), purified peroxisomes (P).

A

% of total fatty acids from PI										
	FY 1679		*psd1Δ*		*psd2Δ*		*psd1Δpsd2Δ*		*cki1Δeki1Δdpl1Δ*	
	H	P	H	P	H	P	H	P	H	P
16:0	39	32	39	28	37	28	40	33	40	36
16:1	<1	<1	1	1	2	1	<1	<1	<1	<1
18:0	12	7	14	8	10	4	11	7	9	9
18:1	49	57	47	63	53	63	50	53	51	51
18:2(*)	<1	4	<1	<1	<1	4	<1	6	<1	4

B

% of total fatty acids from PS										
	FY 1679		*psd1Δ*		*psd2Δ*		*psd1Δpsd2Δ*		*cki1Δeki1Δdpl1Δ*	
	H	P	H	P	H	P	H	P	H	P
16:0	25	21	29	18	26	21	20	14	30	21
16:1	4	8	<1	3	9	8	10	8	8	7
18:0	20	5	<1	<1	<1	<1	<1	<1	19	7
18:1	60	66	71	71	62	69	67	70	53	64
18:2(*)	3	6	<1	5	3	<1	<1	7	3	4

C

% of total fatty acids from PE										
	FY 1679		*psd1Δ*		*psd2Δ*		*psd1Δpsd2Δ*		*cki1Δeki1Δdpl1Δ*	
	H	P	H	P	H	P	H	P	H	P
16:0	12	10	9	10	13	11	10	11	19	15
16:1	6	7	6	4	7	7	4	3	12	9
18:0	6	3	n.d.	n.d.	7	1	n.d.	3	7	3
18:1	65	72	73	76	63	66	76	72	62	68
18:2(*)	11	9	11	9	10	15	9	11	7	6

D

% of total fatty acids from PC										
	FY 1679		*psd1Δ*		*psd2Δ*		*psd1Δpsd2Δ*		*cki1Δeki1Δdpl1Δ*	
	H	P	H	P	H	P	H	P	H	P
16:0	14	12	14	13	14	14	15	12	18	15
16:1	2	2	1	2	3	2	2	2	2	2
18:0	1	1	<1	1	1	2	<1	2	4	1
18:1	76	77	79	76	77	71	75	73	68	75
18:2(*)	7	8	6	8	5	10	8	11	7	5

PS (Table 5B), the first phospholipid in the biosynthetic sequence of the aminoglycerophospholipids biosynthetic route, was specific insofar as homogenate samples from wild type contained higher amounts of 18:0 and 16:0 species than total phospholipids. In peroxisomes, however, the level of saturated PS species, especially of 18:0, was dramatically decreased compared to the homogenate, whereas 18:1 and 18:2 species were increased. Consequently, some specificity of PS incorporation into peroxisomes has to be taken into account. A major change in the PS species pattern was observed in mutants lacking *PSD1* and *PSD2*. In these samples, 18:0 species of PS were hardly detectable. This result suggested that these species are not preferred substrates for PS decarboxylases because they are left over in strains where Psd1p and Psd2p are active, namely wild type and *cki1Δeki1Δdpl1Δ*. A decrease of 16:0 in peroxisomes was observed in wild type as well as in all mutants.

Species of PE (Table 5C) being one of the major phospholipids of the yeast were not dramatically different from those of total phospholipids (see Table 4). A slight increase in 16:0, 16:1 and 18:0 species at the expense of 18:1 was observed. Differences between homogenate and peroxisomes were also minor for most species suggesting that PE is distributed among organelles rather through equilibrium than specific transport as has also been suggested before for mitochondria (Burgermeister *et al.*, 2004a). The only PE species that was markedly decreased in peroxisomes was 18:0. This PE species was missing in strains lacking *PSD1*. The fact that the amount of the substrate, PS, in these strains was already low has also to be taken into account. In contrast, a substantial amount of 18:0 PE was formed in *psd2Δ* suggesting a different type of regulation in this strain. In wild type as in *cki1Δeki1Δdpl1Δ* the level of 18:0 PE was lower than the level of 18:0 PS. Taken together, different effects of PE synthesis, regulation and membrane assembly appear to contribute to the PE composition of peroxisomes.

Finally, PC (Fig. 5D) represents a rather constant phospholipid in this scenario. Besides a slight increase of 16:0 species mainly at the expense of 18:2 the pattern was similar to bulk phospholipids. Also in the case of this phospholipid, equilibrium between organelles appears to be predominant. Mutations in the PE biosynthetic pathways practically did not influence the PC patterns of both homogenate and peroxisomes.

Consequences of PE depletion for peroxisomal structure

The fact that mutations in the different PE biosynthetic pathways affected peroxisomal proliferation and lipid composition tempted us to speculate about possible structural consequences caused by these changes. Therefore, we performed electron microscopic investigations to visualize such possible effects. A method of 3D imaging as described previously by Perktold *et al*. (Perktold *et al.*, 2007) was employed to obtain a better insight into the cellular arrangement of yeast cells induced for peroxisome proliferation on oleic acid containing medium.

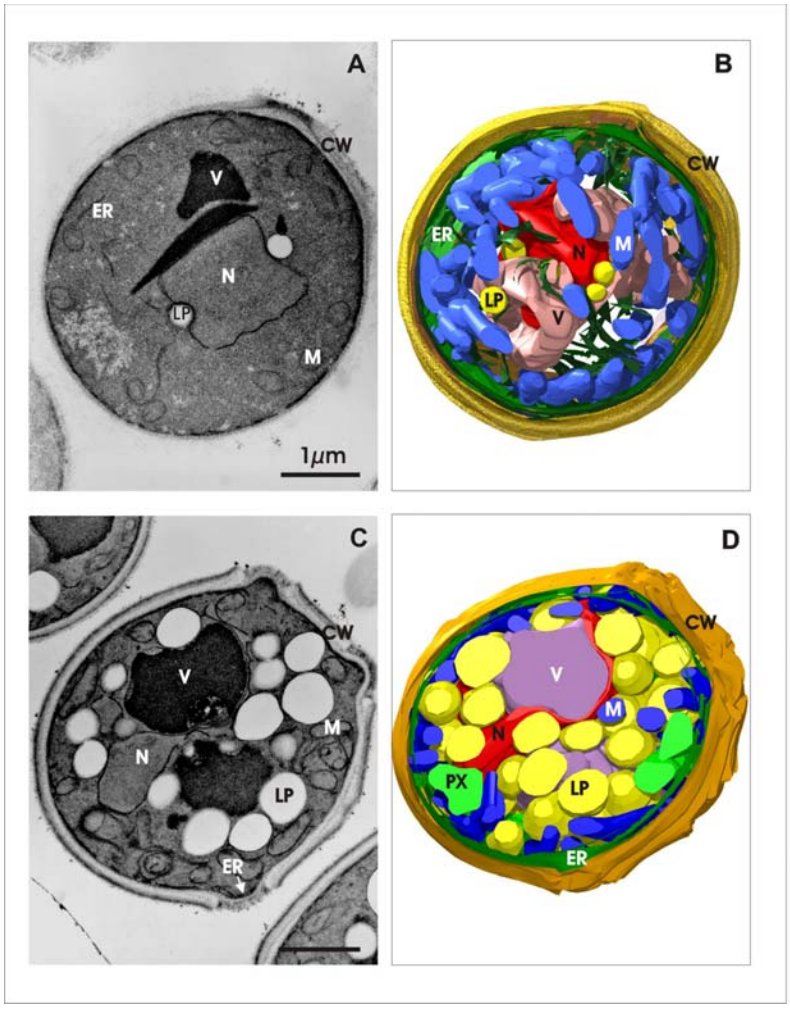


Figure 3: Three-dimensional reconstruction of yeast cells grown on oleic acid

Transmission electron micrographs of ultrathin sections (A, C; bar = 1µm) and corresponding 3D reconstructions of serial sections (B,D) of a chemically fixed wild type cell are shown. Cells shown in A and B were grown on YPD, and cells shown in C and D were grown on oleic acid to induce formation of peroxisomes. CW cell wall; ER endoplasmic reticulum; LP lipid particle; M mitochondrion; N nucleus; V vacuole; PX peroxisomes.

As can be seen from Fig. 3 growth of yeast cells on oleic acid causes massive accumulation of lipid particles within the cell. These lipid bodies mainly consisting of triacylglycerols (our own unpublished results) were markedly larger than those from non-induced cells. Moreover, peroxisomes were detected among the densely packed organelles. To identify possible morphological alterations accompanying defects in PE biosynthesis in cells grown on oleic acid the respective mutants were examined by transmission electron microscopy (Fig. 4). Approximately 30 wild type and mutant cells, each, and 50 peroxisomes from each strain were inspected more closely. All mutants were able to form peroxisomes as expected from growth phenotype analysis (see Table 2). Noteworthy, mutants bearing a defect in the mitochondrial PE formation, namely *psd1Δ* and *psd1Δpsd2Δ*, had smaller peroxisomes than wild type (Table 6). Peroxisomes from *cki1Δeki1Δdpl1Δ* were also slightly smaller than wild type, whereas no changes were observed in the *psd2Δ* mutant. Thus, the size of peroxisomes roughly correlated with the amount of PE present in the organelles (see Table 3). It has to be taken into account, however, that the peroxisomal structure may be rather affected by the total package of changes in the mutants than by the concentration of PE alone.

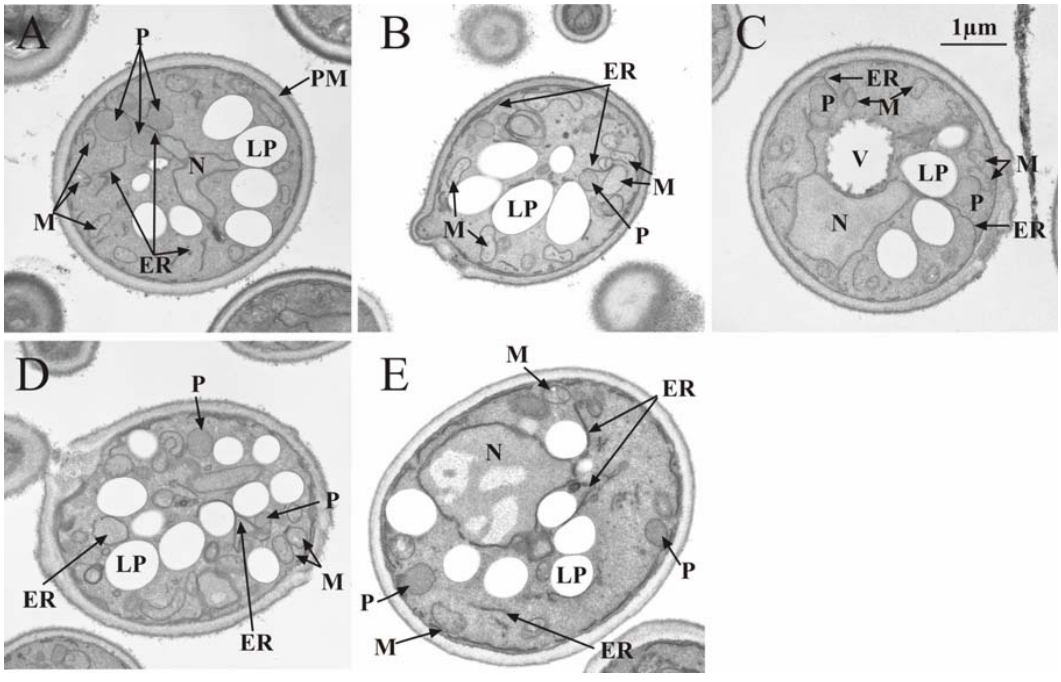


Figure 4: **Ultrastructure of yeast cells**

Cells were grown to the late exponential growth phase in oleate medium and processed for EM. (A) wild type; (B) *psd1Δ;* (C) *psd2Δ*; (D) *psd1Δ psd2Δ*; and (E) *cki1Δ eki1Δ dpl1Δ*. P, peroxisomes; N, nucleus; V, vacuole; M, mitochondria; ER, endoplasmic reticulum; PM, plasma membrane; LP, lipid particle.

Table 6: Average size of peroxisomes from wild type and PtdEtn mutant

The size of peroxisomes from different strains was measured according to their section profiles in the investigated cells on the basis of electron microscopic data. Values of the diameter are means with standard deviations in µm.

	FY1679 (wt)	*psd1Δ*	*psd2Δ*	*psd1Δpsd2Δ*	*cki1Δeki1Δdpl1Δ*
Number of cells counted	24	26	27	28	29
Number of peroxisomes counted	47	51	52	59	42
Diameter of peroxisomes (µm)	0.39 ± 0.075	0.3 ± 0.070	0.36 ± 0.076	0.24 ± 0.058	0.33 ± 0.073

Association between peroxisomes and other organelles

During electron microscopic inspections it became evident that peroxisomes similar to other organelles from the yeast have a high tendency to associate with other subcellular compartments (Fig. 5). 3D images allowed us to quantify association of peroxisomes with other organelles of the yeast cell. Such calculations may be highly relevant for the evaluation of transport or exchange rates of certain substances between different compartments. We considered a distance less than 30 nm between organelles as an association event due to the expected size of "contact proteins" attached to or associated with the membrane surface in the range of 4-45 nm (Achleitner *et al.*, 1999).

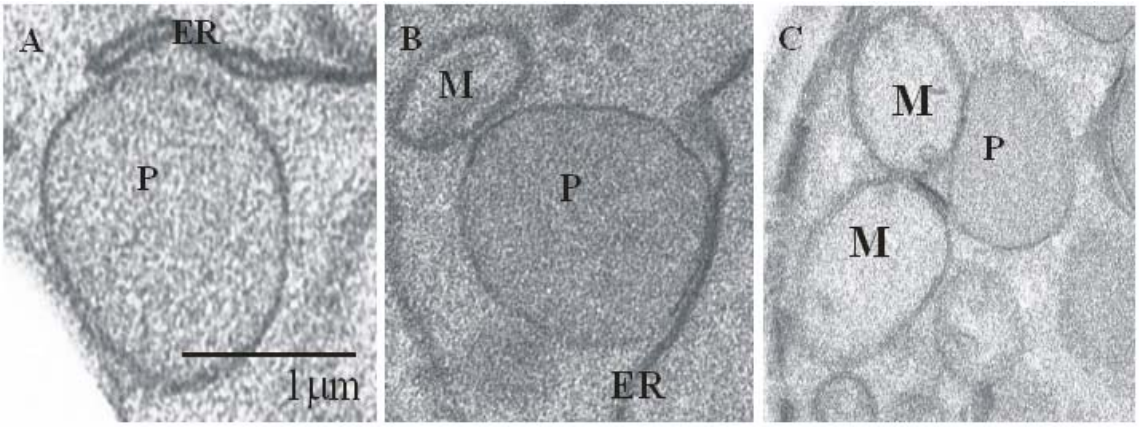


Figure 5: Ultrastructure of peroxisomes and contact with other organelles

Cells were grown in oleate medium and processed for EM. Peroxisomes in contact with ER (A and B) and mitochondria (B and C) are shown. P, peroxisomes; M, mitochondria; ER, endoplasmic reticulum.

Based on 3D images of yeast cells the frequency of association between subcellular compartments was documented by counting membrane contacts per series of cell sections (Table 7). Due to different surface areas of organelles, however, the number of associations per defined organelle surface area was regarded as a more relevant measure for the affinity between two compartments. These calculations demonstrated a high tendency of association between peroxisomes and the ER, mitochondria and lipid particles. Whereas the latter contact may be highly relevant for the supply of fatty acids as a substrate for β-oxidation to peroxisomes, association with ER and mitochondria may be more relevant for the biogenesis of peroxisomes and the supply of structural components, among them phospholipids, to this compartment. It is noteworthy, that association of peroxisomes with the three compartments mentioned above occurred not only in wild type but also in all mutants with defects in PE synthesis (see Fig. 4).

Table 7: Quantification of organelle associations in wild type grown on YPO based on serial sections of the cell

Data were obtained from two sets of cells. The number of total contacts of peroxisomes with other organelles from cells cultivated in the presence of oleic acid (distance between two organelles less than 30 nm) was counted on all serial sections of the cell and is also expressed as number of associations per 10µm^2 organelle surface area. ER, endoplasmic reticulum; LP, lipid particle; M, mitochondria; N, nucleus; pER, peripheral ER; PM, plasma membrane; PX, peroxisomes; V, vacuoles. Data for organelles with the exception of peroxisomes are from ref. (Perktold *et al.*, 2007).

Total organelle surface area								
Fraction	pER	ER	M	N	V	PM	LP	PX
µm^2	73	20	29	19	19	43	56	11

Total number of contacts between organelles								
Fraction	pER	ER	M	N	V	LP	PM	PX
PX	9	14	24	4	7	26	0	1

Number of associations per 10µm^2 organelle surface area								
Fraction	pER	ER	M	N	V	LP	PM	PX
PX	1.1	6.4	7.5	1.9	3.3	4.2	0	0.9

Discussion

In the present study, the involvement of the three biosynthetic routes of PE formation in the supply of PE to peroxisomes of *Saccharomyces cerevisiae* was investigated. To address this question, mutants with defined defects in the PE biosynthetic pathways were used and subjected to biochemical and cell biological analyses.

An important result of this study is that all three pathways of PE biosynthesis in the yeast contribute to the supply of PE to peroxisomes. Deletions of *PSD1* and *CKI1 EKI1 DPL1* had major effects on the cellular and peroxisomal membrane phospholipid composition of cells grown of oleic acid. The effect of *PSD2* deletion was only minor confirming the moderate contribution of this enzyme to cellular PE synthesis (see Table 3). Nevertheless, the growth rate of a *psd2*Δ strain was decreased which cannot be easily attributed to changes in the lipid composition (see Table 2). Noteworthy, Psd2p has been shown to contribute markedly to PC synthesis (Burgermeister *et al.*, 2004b). Surprisingly, deletion of *CKI1 EKI1 DPL1* strongly affected cellular and peroxisomal PE under conditions inducing peroxisome proliferation. This result is in contrast to growth of the triple mutant on YPD where *CKI1 EKI1 DPL1* deletion led only to minor changes in the phospholipid pattern. However, cells lacking both *PSD1* and *PSD2* were able to form sufficient PE through the CDP-ethanolamine pathway to warrant balanced growth on YPO medium and peroxisomal membrane formation. In all mutants, the lack of PE was compensated by increased amounts of PC, PI and/or PS.

Schneiter *et al*. (Schneiter *et al.*, 1999) analyzed the lipid molecular species composition of yeast subcellular compartments involved in the protein secretory pathway and suggested that lipid sorting mechanisms may operate at the level of individual molecular species to maintain the specific lipid composition of a given membrane. Since peroxisomes were isolated from cells cultivated in the presence of oleic acid (18:1), the lipid molecular species profile of the peroxisomal membrane as of other organelles (data not shown) was shifted dramatically towards oleic acid-containing species. These data suggested that this fatty acid was not only used as a carbon source but was also directly incorporated into complex lipids (see Tables 4 and 5). Nevertheless, some specific effects regarding the fatty acid composition of individual peroxisomal phospholipids were observed. As explained in some detail in the Results section changes in the fatty acid pattern of individual phospholipids suggest specificity of biosynthetic steps involved and a non-random mechanism for the assembly of these lipids into

peroxisomal membranes. Accumulation of 16:0 and 18:0 in PI and PS are prominent examples for this selectivity.

The question remains how phospholipids in general and PE in special are transported to peroxisomes. Since peroxisomes are not linked to the classical route of vesicle flux, the secretory pathway, other mechanisms have to govern the transport of lipids to peroxisomal membranes. Purdue and Lazarow (Purdue and Lazarow, 2001) suggested that specialized vesicles different from secretory vesicles might carry phospholipids from the ER to peroxisomes, but the experimental evidence for such a mechanism is weak. Phospholipid exchange protein mediated lipid supply to peroxisomes is not supported by experiments either. Consequently, membrane contact between organelles is left as a mechanism that might be relevant for this transport process. Membrane contact as a possible basis for lipid translocation has been postulated for the supply of lipids from the ER to mitochondrial membranes (Ardail *et al.*, 1993; Shiao *et al.*, 1998; Achleitner *et al.*, 1999; Rosenberger and Daum, 2005). Similarly, close contact between ER and the plasma membrane has been observed and suggested as a possible bridge for lipid migration between these two compartments (Pichler *et al.*, 2001); Schnabl *et al*., manuscript in preparation]. Therefore, membrane contact was also considered as a possible mechanism for lipid supply to peroxisomes. Indeed, large parts of the ER are located in close vicinity to peroxisomes, and a possible role of membrane contact in the supply of lipids from ER to peroxisomes has already been suggested (Titorenko and Rachubinski, 2001; Tabak *et al.*, 2003; Geuze *et al.*, 2003; Schrader, 2006). PE synthesized by Psd1p in mitochondria may be translocated to peroxisomes via direct membrane contact because both organelles were found associated with each other (Schrader, 2006). Alternatively, PE synthesized by Psd1p in mitochondria may be first transported to the ER and then migrate further to peroxisomes. This former translocation step may occur through MAM, the mitochondria associated membrane, whose involvement in lipid import into mitochondria has been suggested before (Gaigg *et al.*, 1995; Achleitner *et al.*, 1999; Voelker, 2004). The PE export process from mitochondria was suggested to occur at reasonable efficiency, because significant amounts of Psd1p generated PE were utilized for PC formation by two-step methylation in the ER. A certain portion of PE reaching the ER on this route may be further transported to other destinations, e.g., the peroxisomes, either by vesicle flux or membrane contact.

Recently, it was also shown that an intimate contact between peroxisomes and lipid bodies exists (Binns *et al.*, 2006). This association may be highly relevant for the supply of fatty acids to peroxisomes because they are required as substrate for β-oxidation. However, also in this case the lipid transport mechanism is presently only ill defined. It has to be taken into account that all mechanisms of lipid transport mentioned above may be relevant and even occur in parallel. Identification of factors involved in these transport processes will be prerequisite for understanding these mechanisms at the molecular level.

Acknowledgments

We thank Dagmar Kolb for providing her protocol for the electron microscopy and Gerhard Graggaber for technical support. We are grateful to Irmgard Schuiki for providing yeast mutant strains. This work was financially supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (projects 17321 and DK Molecular Enzymology W901-305).

Reference List

Achleitner,G., Gaigg,B., Krasser,A., Kainersdorfer,E., Kohlwein,S.D., Perktold,A., Zellnig,G., and Daum,G. (1999). Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. Eur J Biochem *264*, 545-53.

Ardail,D., Gasnier,F., Lerme,F., Simonot,C., Louisot,P., and Gateau-Roesch,O. (1993). Involvement of mitochondrial contact sites in the subcellular compartmentalization of phospholipid biosynthetic enzymes. J. Biol. Chem. *268*, 25985-25992.

Binns,D., Januszewski,T., Chen,Y., Hill,J., Markin,V.S., Zhao,Y., Gilpin,C., Chapman,K.D., Anderson,R.G., and Goodman,J.M. (2006). An intimate collaboration between peroxisomes and lipid bodies. J Cell Biol *173*, 719-31.

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

Broekhuyse,R.M. (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.

Burgermeister,M., Birner-Grunberger,R., Heyn,M., and Daum,G. (2004a). Contribution of different biosynthetic pathways to species selectivity of aminoglycerophospholipids assembled into mitochondrial membranes of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta *1686*, 148-160.

Burgermeister,M., Birner-Grunberger,R., Nebauer,R., and Daum,G. (2004b). 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.

Daum,G., Bohni,P.C., and 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.

Daum,G., Lees,N.D., Bard,M., and Dickson,R. (1998). Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae. Yeast *14*, 1471-510.

Daum,G. and Vance,J.E. (1997). Import of lipids into mitochondria. Prog Lipid Res *36*, 103-30.

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

Gaigg,B., Simbeni,R., Hrastnik,C., Paltauf,F., and Daum,G. (1995). Characterization of a microsomal subfraction associated with mitochondria of the yeast, Saccharomyces cerevisiae. Involvement in synthesis and import of phospholipids into mitochondria. Biochim. Biophys. Acta *1234*, 214-220.

Geuze,H.J., Murk,J.L., Stroobants,A.K., Griffith,J.M., Kleijmeer,M.J., Koster,A.J., Verkleij,A.J., Distel,B., and Tabak,H.F. (2003). Involvement of the endoplasmic reticulum in peroxisome formation. Mol. Biol. Cell *14*, 2900-2907.

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

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

Kennedy,E.P. and Weiss,S.B. (1956). The function of cytidine coenzymes in the biosynthesis of phospholipides. J. Biol. Chem. *222*, 193-214.

Kuchler,K., Daum,G., and Paltauf,F. (1986). Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in Saccharomyces cerevisiae. J Bacteriol *165*, 901-10.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-5.

Leber,R., Zinser,E., Zellnig,G., Paltauf,F., and Daum,G. (1994). Characterization of lipid particles of the yeast, Saccharomyces cerevisiae. Yeast *10*, 1421-1428.

Lowry,O.H., Rosebrough,N.J., Farr A.L., and Randall R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

Morrison W.R. and Smith L.M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride--methanol. J. Lipid Res. *5*, 600-608.

Nebauer R., Birner-Gruenberger,R., and Daum,G. (2003). Biogenesis and cellular dynamics of glycerophospholipids in the yeast Saccharomyces cerevisiae. Top. Curr. Genet. *6*, 125-168.

Perktold,A., Zechmann,B., Daum,G., and Zellnig,G. (2007). Organelle association visualized by three-dimensional ultrastructural imaging of the yeast cell. FEMS Yeast Res. *7*, 629-638.

Pichler,H., Gaigg,B., Hrastnik,C., Achleitner,G., Kohlwein,S.D., Zellnig,G., Perktold,A., and Daum,G. (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.

Purdue,P.E. and Lazarow,P.B. (2001). Peroxisome biogenesis. Annu Rev Cell Dev Biol *17*, 701-52.

Rosenberger,S. and Daum,G. (2005). Lipids of yeast mitochondria. Research signpost *1735*, 111-118.

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

Schneiter,R. *et al.* (1999). Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of the lipid molecular species composition of yeast subcellular membranes reveals acyl chain-based sorting/remodeling of distinct molecular species en route to the plasma membrane. J. Cell Biol. *146*, 741-754.

Schrader,M. (2006). Shared components of mitochondrial and peroxisomal division. Biochim. Biophys. Acta *1763*, 531-541.

Shiao,Y.J., Balcerzak,B., and Vance,J.E. (1998). A mitochondrial membrane protein is required for translocation of phosphatidylserine from mitochondria-associated membranes to mitochondria. Biochem. J. *331 (Pt 1)*, 217-223.

Tabak,H.F., Murk,J.L., Braakman,I., and Geuze,H.J. (2003). Peroxisomes start their life in the endoplasmic reticulum. Traffic *4*, 512-8.

Titorenko,V.I. and Rachubinski,R.A. (2001). The life cycle of the peroxisome. Nat Rev Mol Cell Biol *2*, 357-68.

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

van der Klei,I. and Veenhuis,M. (2002). Peroxisomes: flexible and dynamic organelles. Curr. Opin. Cell Biol. *14*, 500-505.

Veenhuis,M., Salomons,F.A., and van,d.K., I (2000). Peroxisome biogenesis and degradation in yeast: a structure/function analysis. Microsc. Res. Tech. *51*, 584-600.

Voelker,D.R. (2004). Genetic analysis of intracellular aminoglycerophospholipid traffic. Biochem. Cell Biol. *82*, 156-169.

Wirtz,K.W. (1991). Phospholipid transfer proteins. Annu. Rev. Biochem. *60*, 73-99.

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

Zinser,E., Sperka-Gottlieb,C.D., Fasch,E.V., Kohlwein,S.D., Paltauf,F., and Daum,G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. J Bacteriol *173*, 2026-34.

CHAPTER 2

PHOSPHOLIPID TRAFFIC TO PEROXISOMES – Opi3p AS REPORTER ENZYME

Connerth M., Flis V.V. and Daum G.

Institute of Biochemistry, Graz University of Technology, Graz, Austria

Running head: Phosphatidylethanolamine traffic to peroxisomes

Key words: Peroxisomes, phosphatidylethanolamine, phosphatidylcholine, lipid traffic, yeast

Abbreviations: CDP, cytidine diphosphate; Cho, choline; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; ER, endoplasmic reticulum; Etn, ethanolamine; GFP, green fluorescent protein; LP, lipid particle(s); MMPE, monomethylphosphatidyl-ethanolamine; Mt, mitochondria; RT, room temperature; SAM, S-adenosyl-L-methionine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospho-lipid(s); PS, phosphatidylserine; PX, peroxisomes

Abstract

In the yeast *Saccharomyces cerevisiae*, phospholipids can be synthesized via different pathways which are partially connected to each other. One of the major yeast phospholipids, phosphatidylethanolamine (PE), is synthesized either by decarboxylation of phosphatidylserine (PS) catalyzed by the mitochondrial phosphatidylserine decarboxylase Psd1p, by Psd2p in a Golgi/vacuolar compartment, or by the so-called CDP-ethanolamine pathway located to the endoplasmic reticulum (ER). Previous studies from our laboratory suggested that PE formed through all three pathways in different subcellular membranes can be supplied to peroxisomes with comparable efficiency (Rosenberger *et al.*, 2009b). However, mechanisms involved in these translocation processes are still unclear. To address this question, we designed a reporter enzyme driven *in vivo* assay for PE traffic to peroxisomes. For this purpose, the yeast PE methyltransferase Opi3p was fused with a GFP-SKL-tag that directs the originally ER localized Opi3p to peroxisomes. In an *opi3*Δ strain functionality of the hybrid enzyme was verified by compensation of the growth defect caused by the deletion. Opi3p-GFP-SKL was successfully targeted to peroxisomes where it localized to either the peroxisomal matrix or the membrane. Since *in vitro* assays confirmed that Opi3p-GFP-SKL was the only enzyme in this strain capable of producing phosphatidylcholine (PC) from PE in the absence of exogenous choline, the formation of PC was used as marker and measure for PE transport to peroxisomes. A *semi in vitro* assay using permeabilized cells was designed to monitor PE traffic to peroxisomes. In this assay, PC was successfully formed as the end product of the aminoglycerophospholipid biosynthetic cascade confirming translocation of PE or its monomethylated derivative to peroxisomes. This strategy in combination with mutations in the three different PE biosynthetic pathways will enable us to investigate traffic routes and especially mechanisms of PE supply to peroxisomes as well as to elucidate PC distribution formed in peroxisomes to other organelles.

Introduction

Processes involved in phospholipid (PL) biosynthesis are well known and characterized from yeast to mammals. Only certain organelles are capable of glycerophospholipid synthesis including the endoplasmic reticulum, mitochondria and the Golgi apparatus. As many organelles are therefore not able to form phospholipids on their own, lipid transport becomes a fundamental process in membrane biogenesis which is, however, poorly understood. The most abundant PL in the yeast is phosphatidylcholine (PC) which is formed by two different pathways both being located to the ER (Zinser *et al.*, 1991). The first pathway involves three methylation steps of phosphatidylethanolamine (PE) using S-adenosyl-L-methionine (SAM) to form monomethylphosphatidylethanolamine (MMPE) and dimethylphosphatidyl-ethanolamine (DMPE) leading subsequently to the formation of PC (Waechter *et al.*, 1969; Waechter and Lester, 1971; Waechter and Lester, 1973; Carman and Henry, 1999). Enzymes involved in this process are the two PE methyltransferases (PEMT) Pem1p/Cho2p and Pem2p/Opi3p with Pem2p/Opi3p being responsible for the two last methylation steps (Kodaki and Yamashita, 1987a; Summers *et al.*, 1988; McGraw and Henry, 1989). Opi3p, however, has also some activity for the first methylation step forming MMPE (Kodaki and Yamashita, 1987d). Additionally, PC can be formed via the so-called Kennedy pathway using exogenous choline to form PC from CDP-choline (Kennedy and Weiss, 1956; McMaster and Bell, 1994) which in yeast only plays a minor role for *de novo* PL biosynthesis. Similarly, PE, another abundant PL in yeast membranes can be synthesized via the CDP-Etn branch of the Kennedy pathway using exogenous ethanolamine instead of choline as a substrate. Furthermore, two decarboxylation pathways including Psd1p localized to the inner mitochondrial membrane (Zinser *et al.*, 1991) and Psd2p localized to the Golgi compartment (Trotter and Voelker, 1995), respectively, contribute to PE formation (compare Figure 1A)

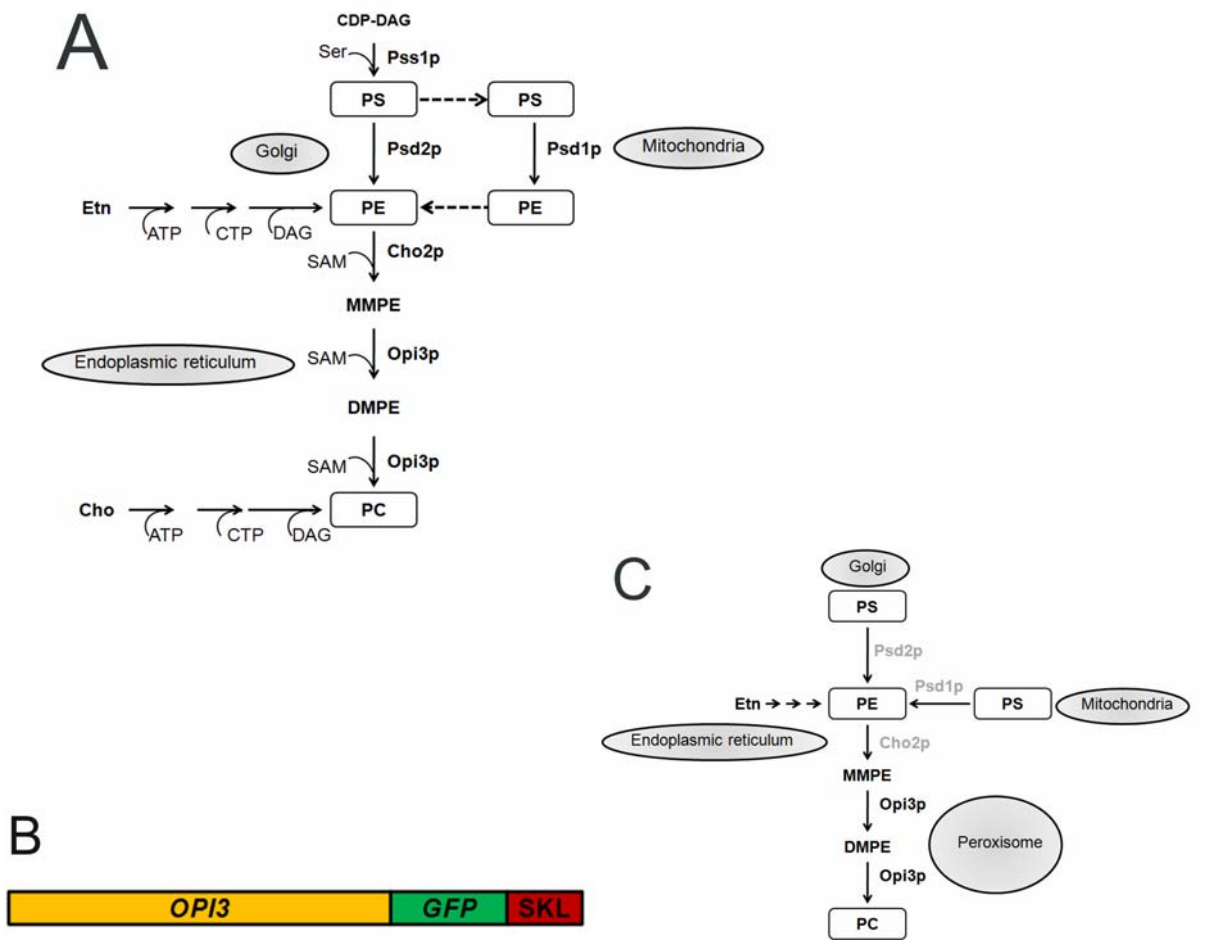


Figure 1: Scheme for phospholipid traffic to and from peroxisomes

(A) Overview of phospholipid synthesis pathways in the yeast. Major phospholipid species are shown in round boxes. Enzymes responsible for the different pathways are indicated and the respective cellular localization is shown.

(B) Schematic view of the OPI3-GFP-SKL construct; ORF of *OPI3* was cloned into a plasmid under the control of the MLS-promoter which enables protein expression under peroxisome inducible conditions. The GFP-tag was fused to the C-terminus of the protein for detection via Western blotting or fluorescence microscopy followed by the three amino acid peroxisomal targeting sequence 1 (PTS1) SKL, which directs proteins to peroxisomes.

(C) Overview over the reporter enzyme assay. The construct Opi3p-GFP-SKL localized to peroxisomes enables to monitor PE traffic from its sites of synthesis (ER, Golgi and mitochondria) to peroxisomes. In a further step, distribution of peroxisomal PC to other cellular compartments can be monitored.

Peroxisomes are small ubiquitous organelles incapable of phospholipid biosynthesis (Bishop and Bell, 1988; Zinser *et al.*, 1991). In the past twenty years, this organelle has been studied in some detail but its biogenesis and proliferation is still a matter of dispute (for recent review see: (Smith and Aitchison, 2009; Nagotu *et al.*, 2010).

In the yeast, peroxisomes are the only organelle harboring enzymes of β-oxidation (Poirier *et al.*, 2006) thus making them essential for cellular growth on oleic acid as carbon source (Erdmann *et al.*, 1989). This feature makes the yeast a perfect model organism for studying peroxisome biogenesis. Proteins essential for peroxisome formation are the so-called peroxines (PEX). So far over 30 of these genes have been identified of which many are found to be conserved from yeast to mammals. Yeast deficient in one of the essential peroxines *PEX3* or *PEX19* is not able to generate functional peroxisomes. Re-introduction of these gene into the respective knock-out strain allowed formation of fully functional peroxisomes in mammals and yeast (South and Gould, 1999; Hettema *et al.*, 2000). In addition, fission of peroxisomes was observed in yeast (Erdmann and Blobel, 1995; Hoepfner *et al.*, 2001; Kuravi *et al.*, 2006; Motley and Hettema, 2007; Guo *et al.*, 2007a; Motley *et al.*, 2008; Nagotu *et al.*, 2008a; Nagotu *et al.*, 2008b) and mammals (Li and Gould, 2003; Koch *et al.*, 2003; Koch *et al.*, 2005; Kobayashi *et al.*, 2007; Matsuzaki and Fujiki, 2008; Huybrechts *et al.*, 2009).

To date a combination of *de novo* synthesis and fission is widely accepted for peroxisome biogenesis. The so-called growth and division model describes a budding process of pre-peroxisomal membranes from the ER followed by introduction of peroxines forming a machinery for importing matrix proteins and thus proliferating into mature peroxisomes (Titorenko and Mullen, 2006). These peroxisomes are then able to grow and divide. It has been shown for the yeast (Tam *et al.*, 2005; Kragt *et al.*, 2005; Hoepfner *et al.*, 2005; Haan *et al.*, 2006; Yan *et al.*, 2008) as well as for mammalian cells (Geuze *et al.*, 2003; Tabak *et al.*, 2003; Kim *et al.*, 2006; Toro *et al.*, 2009) that peroxisomes derive from the ER. The essential genes for peroxisome formation have been largely investigated hence the underlying mechanism of lipid traffic to peroxisomes has not been elucidated. Previous studies were already aimed at examining possible pathways for lipid transport in the cell. A role for putative lipid transfer proteins (Wirtz, 1991; Wirtz, 2006) as well as vesicular transport (Purdue and Lazarow, 2001; Raychaudhuri and Prinz, 2008a) have been discussed. However, none of these mechanisms were found to be essential for peroxisomal biogenesis. In addition, the possibility of direct membrane contact has been discussed for yeast organelles such as mitochondria and the ER (Ardail *et al.*, 1993a; Shiao *et al.*, 1998; Achleitner *et al.*, 1999) but has so far not been investigated for peroxisomal lipid supply.

In recent studies investigating the supply of PE to peroxisomes we found that all pathways of PE synthesis contribute to the supply of PE to peroxisomes (Rosenberger *et al.*, 2009d). We also suggested direct membrane contact as possible mechanism for this process, although only based on evidence obtained by electron microscopy. The present study aimed at the elucidation of phospholipid traffic to peroxisomes in more detail. For this purpose, we set up a reporter enzyme assay introducing Opi3p fused to a GFP-SKL-tag into peroxisomes from an *opi3*Δ strain. This experimental setup allowed us to test the efficiency of substrate (PE and MMPE) supply to peroxisomes by appearance of PC *in vitro* and *in vivo* under various conditions.

Material and Methods

Strains used and growth conditions

Strains used in this study are described in Table 1. Yeast cells were cultivated in liquid minimal medium containing either 2 % glucose (SD) or 0.1 % oleic acid and 0.1 % glucose (SO) as carbon source and 0.67 % Yeast Nitrogen Base. For selection of auxotrophy markers an amino acid mix lacking the respective marker was used. If not otherwise stated, cells were pregrown in a liquid SD culture for 48 h and then used for inoculation to OD_{600} of 0.1 and grown until early stationary phase.

Table 1: Strains used in this study

Name	Genotype	Origin	
BY4741 (wt)	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf, Germany	Frankfurt,
*opi3*Δ	BY4741 opi3Δ::KanMX4	Euroscarf, Germany	Frankfurt,
yMC2	BY4741 opi3::KanMX4 pJR233	This study	
yMC3	BY4741 opi3Δ::KanMX4 pMC13	This study	

Cloning of OPI3

The open reading frame (ORF) of *OPI3* was used as template for PCR amplification using primer pair MC42 5’ CGG CGG GGT ACC ATG AAG GAG TCA GTC CAA GAG 3’ and MC43 5’ GCG CGC GGA TCC CAT ATT CTT TTT GGC CTT ATC ACG 3’. This PCR product missing the Stop Codon and flanked by *Kpn*I and *Bam*HI restriction sites was then digested with the respective endonucleases and cloned into plasmid pJR233 (Brocard *et al.*, 1997a) containing a GFP-SKL expression construct under the control of a MLS-promoter. Correct ligation was verified by control restrictions and sequencing yielding the plasmid pMC13 encoding for OPI3-GFP-SKL. This plasmid was transformed into the yeast strain BY4741 *opi3*Δ*::KanMX6* by a standard yeast transformation protocol. Gen deletion strains of *PSD1* and *PSD2* and *OPI3* were prepared as described by Longtine *et al*. (Longtine *et al.*, 1998).

Organelle isolation

Subcellular fractionation was performed with cells grown in liquid SO medium to early stationary phase according to Rosenberger *et al*. (Rosenberger *et al.,* 2009a). In brief, cells were harvested by centrifugation at 5,000 rpm for 5 min in a Sorvall SLC-3000 rotor. Cell pellet was washed in distilled water and resuspended in twice the volume of buffer A (0.1 M Tris/SO_4, pH 9.4). After addition of 0.66 mg dithiothreitol (DTT) per ml, the cell suspension was incubated at 30 °C shaking for at least 10 min. Cells were washed and resuspended in 7 volumes per g cells pre-warmed buffer B (1.2 M sorbitol and 20 mM KH_2PO_4, pH 7.4). Preparation of spheroplasts was performed using 2 mg zymolyase 20T per g wet weight were incubated with cells in buffer B for approximately 1 h at 30 °C shaking.

Spheroplasts were then washed in buffer B and resuspended in twice the cell volume in breaking buffer containing 5 mM-MES-KOH, pH 6.0, 0.6 M sorbitol, 1 mM KCl, 0.5 mM EDTA and 1 mM PMSF. Spheroplasts were homogenized on ice using a Dounze homogenizer with a tight fitting pestle. Unbroken cells and nuclei were removed by centrifugation at 5,000 rpm for 5 min in a Sorvall SLC3000 rotor. In order to enhance the yield of peroxisomes, the resulting pellet was twice resuspended in breakage buffer, re-homogenized and centrifuged as described above. The combined supernatants were centrifuged at 15,000 rpm in a Sorvall SS34 rotor for 15 min. The crude organelle pellet, consisting of mitochondria and peroxisomes, was gently resuspended in breakage buffer plus 1 mM PMSF, and centrifuged at low speed (5,000 rpm) to remove larger aggregates. Then, the supernatant was centrifuged again at 15,000 rpm, the pellet was resuspended in breakage buffer as described above and loaded for further purification on a Nycodenz gradient (17-35%; w/v) in 5 mM-MES-KOH, pH 6.0, 1 mM KCl, 0.24 M sucrose. Centrifugation was carried out in a swing-out rotor (Sorvall AH-629) at 26,000 rpm for 90 min. The white peroxisome layer was withdrawn using a syringe, diluted with 4 volumes of the breaking buffer and sedimented at 15,000 rpm in an SS34 rotor for 15 min at 4 °C. Mitochondria formed a separate band in the density gradient which was also collected and sedimented by centrifugation.

For quality control, proteins of the organelle samples were precipitated with 50 % (final conc.) trichloroacetic acid (TCA) for at least 1 h on ice and washed in ice cold acetone to remove all lipids. Protein determination was performed using the method of Lowry *et al.,* (Lowry *et al.,* 1951a). SDS-PAGE was done using the method of Laemmli (Laemmli, 1970).

Proteins were analyzed by Western blotting according to the method of Haid and Suissa (Haid and Suissa, 1983). A set of antibodies representing typical marker proteins of various cellular organelles was used to check the quality of the isolated organelles as indicated in the figure. Peroxidase conjugated secondary antibody (Sigma) and enhanced chemiluminescent signal detection reagents (SuperSignal^{TM}, Pierce Chemical Company, Rockford, IL, USA) were used to visualize immunoreactive bands.

Lipid analysis

Total cell extracts were prepared from cells grown to stationary phase in minimal medium. Cells were harvested and washed twice in 0.1 % BSA and three times in distilled water. Then, cells were frozen at -20 °C and thawn on ice. Cell pellet was resuspended in 3-4 ml Breaking Buffer (5 mM MES, 1 mM KCl, 0.5 mM EDTA, 0.6 M sorbitol, pH 6.0). Two volumes of glass beads were added and cells were disrupted on a Vortex mixer at about 1,200 rpm for at least 1 h at 4 °C. Cell homogenate was transferred to a fresh tube and the protein amount was determined by the method of Lowry (Lowry *et al.*, 1951b). Then, 2 mg of protein were used for lipid extraction employing the method from Folch *et al*. (Folch J. *et al.*, 1957). In brief, protein sample was added to 3 ml $CHCl_3$:MeOH (2:1; v/v) in a Pyrex glass tube. Lipids were extracted to the organic phase by vortexing at room temperature (RT) for 1 h. Proteins were removed by consecutive washing steps with 0.2 volumes 0.034 % $MgCl_2$, 1 ml 2 N KCl/MeOH (4:1; v/v) and 1 ml of an artificial upper phase ($CHCl_3$:MeOH:H_2O; 3:48:47; per vol.). These solutions were added to the extracts and incubated with shaking for 3 min. After centrifugation for 3 min at 3,000 rpm in a table-top centrifuge the aqueous phase was removed by aspiration. Finally, lipids were dried under a stream of nitrogen and stored at -20 °C. Individual phospholipids were analyzed as described by Connerth *et al*. (Connerth *et al.*, 2009). In brief, phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25%NH_3 (65:35:5, per vol.) as first, and chloroform / acetone / methanol/ acetic acid/water (50:20:10:10:5; per vol.) as second developing solvent. Lipids were stained with iodine vapour, scraped off the plate, and quantified by the method of Broekhuyse (Broekhuyse, 1968). Individual phospholipid spots were visualized by staining with iodine vapor in a saturated chamber for some minutes. Phospholipids were quantified from TLC plates after removal of the iodine staining.

The plate was moistened with deionized water, phospholipid spots were scrapped off and transferred to a phosphate free glass tube with ground neck. Lipid phosphorus of the respective spot was measured by subjecting the sample to hydrolysis. Therefore, 0.2 ml of conc. H_2SO_4/72 % $HClO_4$ (9:1; v/v) was added to each sample. Hydrolysis was performed at 180°C in a heating block for 30 min. Samples were cooled to RT, and 4.8 ml of freshly prepared 0.26 % ammonium molybdate/ANSA (40.0 g $K_2S_2O_5$, 0.63 g 8-anilio-1-naphthalenesulfonic acid and 1.25 g Na_2SO_3 in 250 ml water) (500:22; v/v) was added. Tubes were sealed with phosphate free glass caps and after vigorous vortexing samples were heated to 100 °C for 30 min in a heating chamber. Finally, samples were cooled to RT and shortly centrifuged in a table top centrifuge at 1,000 x g to sediment the silica gel. Samples were measured spectrophotometrically at a wavelength of 830 nm using a blank spot from the TLC plate without phospholipid as a control. Data were calculated from a standard curve using inorganic phosphate at known amounts.

Permeabilization of yeast cells

A cell permeabilization protocol was adapted from Achleitner *et al*. (Achleitner *et al.*, 1995). In brief, cells were grown in SO medium for 24 h and harvested at 5,000 rpm for 5 min in a Hettich table top centrifuge. Cells were washed with 0.1 % fatty acid free BSA and subsequently in distilled water twice. Then, cells were taken up in two volumes per g cell wet weight (CWW) in Buffer A (0.1 M TrisSO_4, pH 9.6) and incubated with dithiothreitol (DTT) at a final concentration of 154.1 mg DTT / ml buffer. Cells were pelleted and resuspended in seven volumes per g CWW Buffer B (1.2 M sorbitol) containing 2 mg zymolyase/ g CWW and incubated at 30 °C shaking for 1h. Spheroplasts were re-activated in an aliquot of YPD (2 % glucose, 1 % yeast extract, 2 % peptone) and 1.2 M sorbitol (1:1) for 20 min at 30 °C shaking. After harvesting at 5,000 rpm for 2 min spheroplasts were resuspended in Breaking Buffer to a final concentration of 0.5 g CWW/ ml. Permeabilized cells were prepared by gently freezing aliquots of 200 µl in a steam from liquid nitrogen for 15 min and subsequent stored at -70 °C. Permeabilization of cells was verified by microscopy after thawing cells slowly on ice for about 30 min.

PE methyltransferase enzyme assay in vitro

A PE methyltransferase (Opi3p) *in vitro* assay was adapted from Boumann *et al.* (Boumann *et al.*, 2004). In brief, yeast subcellular fractions were prepared as described above. 10µl (~10 µg protein) of organelles were used for a 100 µl assay mixture containing 5 µl 1 M TrisCl (pH 8.0), 49.8 µl 1 mM SAM, 0.2 µl [^{3}H]S-adenosyl methionine (SAM) (10 Ci/mmol), 20 µl 1 mM Epicuron and 15 µl distilled water. The reaction was started by addition of the enzyme source. At time points indicated, aliquots were withdrawn, and lipids were extracted using 300 µl $CHCl_3$:MeOH (2:1; v:v) and washed twice in 300 µl aqueous phase ($CHCl_3$:MeOH:H_2O; 3:48:47; per vol.) to remove remaining protein. Lipids were dried in a stream of nitrogen, separated by TLC in a solvent system containing $CHCl_3$:MeOH:NH_3 (65:25:4; per vol.) and visualized by iodine vapor staining. Spots containing PC were scraped off and subjected to scintillation counting using 7 ml liquid scintillation cocktail (Ultima Gold high flash point luminescence scintillation cocktail; Perkin Elmer) containing 5 % distilled water.

Aminoglycerophospholipid synthesis in permeabilized cells

For analyzing biosynthetic routes of PS, PE and PC biosynthesis, a *semi in vitro* assay was used according to the protocol of Achleitner *et al.* (Achleitner *et al.*, 1995). This assay combines three enzymatic steps and requires addition of different cofactors to the assay mixture for individual reactions. Production of the reactive phospholipid species was followed by incorporation of radiolabeled serine into PS and appearance of the label in PE and PC. The assay was performed in an Eppendorf reaction tube using a total mixture of 0.9 ml which contained 90 µl 6 mM $MnCl_2$, 90 µl distilled water, 195 µl 1.2 M sorbitol, 400 µl assay buffer (0.6 M mannitol, 50 mM TrisCl pH 8.0), 45 µl 2 mM serine, 30 µl [^{3}H]serine (29.5 Ci/mmol) and 50 µl permeabilized cells (~ 0.5 mg protein). The reaction was started by addition of permeabilized cells. 100 µl aliquots were withdrawn at time points indicated and added to 300 µl $CHCl_3$:MeOH (2:1; v/v) to stop the reaction. After 15 min shaking, 5.6 µl 500 mM EDTA (pH 8.0) were added to the reaction mixture and aliquots of 100.8 µl were withdrawn at time points indicated. After 30 min 1.2 µl 0.1 M SAM and 4 µl 1 M $MgCl_2$ were added to the mixture and 101.8 µl were withdrawn. Lipids were extracted in 300 µl $CHCl_3$:MeOH (2:1; v/v) and washed once with an aqueous phase consisting of $CHCl_3$: MeOH: H_2O (48:47:2; per vol.). Dried lipids were dissolved in $CHCl_3$:MeOH and subjected to one dimensional TLC using $CHCl_3$:MeOH:NH_3 (25:12.5:3; per vol.) to separate PL species.

Spots containing PS, PC and PE species were scraped off and measured in a scintillation counter using 7 ml of Ultima Gold high flash point luminescence scintillation cocktail (Perkin Elmer) containing 5 % H_2O.

Protein protection assay

Since isolated peroxisomes were found to be too fragile for standard proteins protection assays employing mild Proteinase K treatment, we performed an adapted assay to analyze the cellular localization of Opi3p-GFP-SKL. For this purpose, cells grown to the early stationary phase were harvested and washed twice in 0.1 % BSA and three times in distilled water. Spheroplasts were prepared as described above, and 200 µl (~ 4 mg protein) were used for further assays. 1,400 µl Breaking Buffer (5 mM MES, 1 mM KCl, 0.5 mM EDTA, 0.6 M sorbitol, pH 6.0) were added, and a homogenate was prepared by carefully pipetting up and down 10 times. Cell homogenate was incubated on ice for 5 min. Cell debris were removed by a low speed spin at 500 rpm for 5 min in a table top centrifuge. The supernatant containing the protein rich lysate was used for proteinase K treatment. Aliquots of 125 µl lysate were used for the Proteinase K protection assay. If not stated otherwise, 1.25 µl of 20 mg/ml Proteinase K were used and 5 µl 20 % Triton X-100. One aliquot of the lysate was centrifuged at 13,000 rpm (16,000 x g) for 10 min to separate all cellular membranes from the cytosol (supernatant). The resulting membrane pellet was washed once and resuspended in the same amount (125 µl) of Breaking Buffer. Proteins of all fractions were precipitated with 50 % TCA and analyzed by SDS-PAGE and Western blotting as described above.

Results

Reporter enzyme assay for monitoring PE and PC traffic to and from peroxisomes

The present study was aimed at the investigation of phospholipid supply to peroxisomal membranes. Therefore, we designed a reporter enzyme assay by targeting Opi3p to peroxisomal membranes. In the *opi3*Δ background lacking the original ER PE methyltransferase Opi3p allowed us to monitor PE traffic to peroxisomes, simply by the appearance of PC. For this purpose we cloned the ORF of *OPI3* into the vector system pJR233 under control of the MLS-promoter together with a GFP-SKL-tag (see Material and Methods). Since induction of yeast peroxisomes requires growth of cells on oleic acid, we had to use an expression system inducing hybrid protein expression and peroxisome proliferation at the same time. Therefore, our choice was the MLS-promoter which is suppressed in the presence of glucose but active in the presence of oleate as carbon source (Brocard *et al.*, 1997b). To target Opi3p to the desired environment we fused it to GFP followed by the SKL-peroxisomal targeting signal 1 (PTS1) which should result in peroxisome localization of the original ER protein (Figure 1B). This construct was transformed into a mutant deleted of endogenous *OPI3* resulting in the strain yMC3. As a control, the empty plasmid containing GFP-SKL was introduced into *opi3*Δ strain yielding yMC2 (see also Table 1).

Since PE and PC are essential phospholipids growth retardation is an indication for insufficient biosynthesis of either of these phospholipids. Opi3p catalyzes the last steps in the methylation process of MMPE to DMPE and finally to PC (see Figure 1A). However, it has been reported before that strains deficient in *OPI3* need not to be supplied with exogenous choline (Greenberg *et al.*, 1983) whereas an *cho2*Δ*opi3*Δ double mutant was auxotroph for choline (Kodaki and Yamashita, 1989a). In addition, growth retardation on oleic acid indicates impairment of functional peroxisomes. Thus, the restored growth defect of yMC3 and yMC2 strains would reflect expression of a functional Opi3p in functional peroxisomes. Growth analysis of the respective strains revealed that on glucose (non-induced) the strain carrying Opi3p-GFP-SKL (yMC3) did not show obvious alterations compared to *opi3*Δ (Figure 2). Growth was found to be independent of exogenous choline. On oleic acid (induced conditions), yMC3 was also able to grow and did not require choline supply. Importantly, a strain harboring the empty plasmid with only GFP-SKL (yMC2) was able to grow in the presence of glucose with some delay and to lower cell density, but was not viable on oleic acid supplemented media. These data indicate that proliferation of functional peroxisomes depended on the expression of Opi3p.

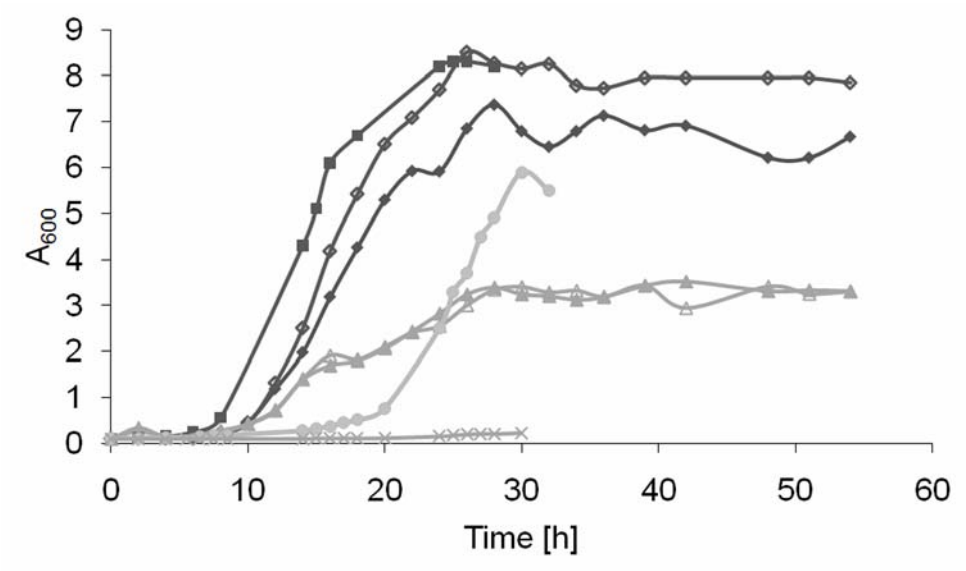


Figure 2: Growth phenotype of strains on different growth medium

Cells from a 48 h pre-culture were used to inoculate a main culture with medium indicated to an OD_{600} of 0.1. Cells were grown at 30 °C shaking and aliquots were taken at time points indicated, washed in 0.1 % BSA and water and optical density was determined. Data shown here are representative from at least three independent experiments.

■ opi3Δ SD; ◊ yMC3 SD; ♦ yMC3 SD + cho; ● yMC2 SD; Δ yMC3 SO; ▲ yMC3 SO +cho; x yMC2 SO

Opi3p-GFP-SKL is localized to peroxisomes

Subcellular localization of Opi3p-GFP-SKL was analyzed by cell fractionation and immunoblotting (Figure 3). Opi3p-GFP-SKL was found to be mainly localized to peroxisomes but some minor amounts were found in mitochondria. It has to be mentioned that Opi3p-GFP-SKL was also detected in vacuoles (data not shown) probably due to overexpression of the protein under the MLS-promoter. It remained unclear whether the vacuolar portion of this protein exhibited activity. Most likely, the polypeptide targeted to this compartment was subjected to degradation, but this has to be proven in future studies. No GFP signal was detected in ER membranes indicating that Opi3p-GFP-SKL was successfully targeted to peroxisomes.

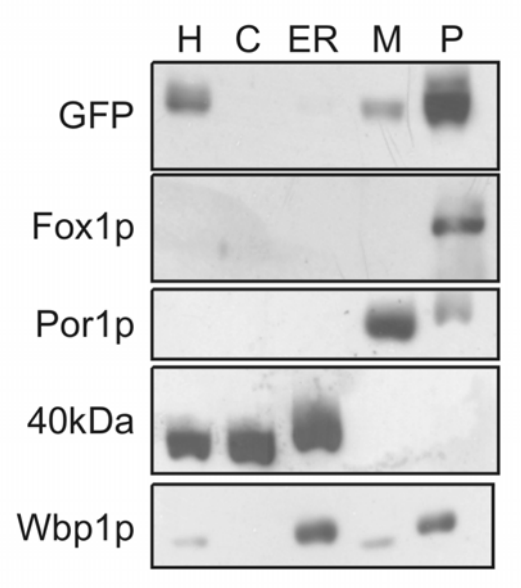


Figure 3: Subcellular localization of Opi3p-GFP-SKL in yeast cells

Yeast cells from a 48 h pre-culture were used to inoculate main culture SO-ura to an OD_{600} of 0.1 and grown for 24 h at 30 °C with shaking. Cells were harvested and cell fractionation was performed as described in Material and Methods. Same amounts of protein were precipitated with TCA and subjected to SDS-PAGE and Western blotting. Marker antibodies were used as indicated. H, homogenate; C, cytosol; ER, endoplasmic reticulum; M, mitochondria; P, peroxisomes.

Since Opi3p has been predicted to be an integral membrane protein according to hydropathy plots (Kodaki and Yamashita, 1987b) we further investigated the topology of Opi3p-GFP-SKL in the host membrane system. Generally, proteins with an SKL-signal sequence are localized to the peroxisomal matrix. To address this question, we performed protease protection assays as described in Material and Method section. Initial experiments with isolated peroxisomes showed that the organelle was too fragile for limited proteolysis with Proteinase K as described in standard protocols. Therefore, we adapted an assay using freshly prepared cell lysate where cell organelles should be largely intact (see Material and Methods). Surprisingly we found that under non-inductive conditions (SD) Opi3p-GFP-SKL was already expressed at low amount indicating a leaky MLS-promoter. Interestingly, the protein was not protected by any membrane resulting in similar degradation in the presence or absence of the detergent (Figure 4A). However, under oleic acid (induced) conditions Opi3p-GFP-SKL localized to peroxisomes and Proteinase K was not able to digest the protein in the absence of Triton X-100 indicating insertion of the protein into the peroxisomal membrane or matrix.

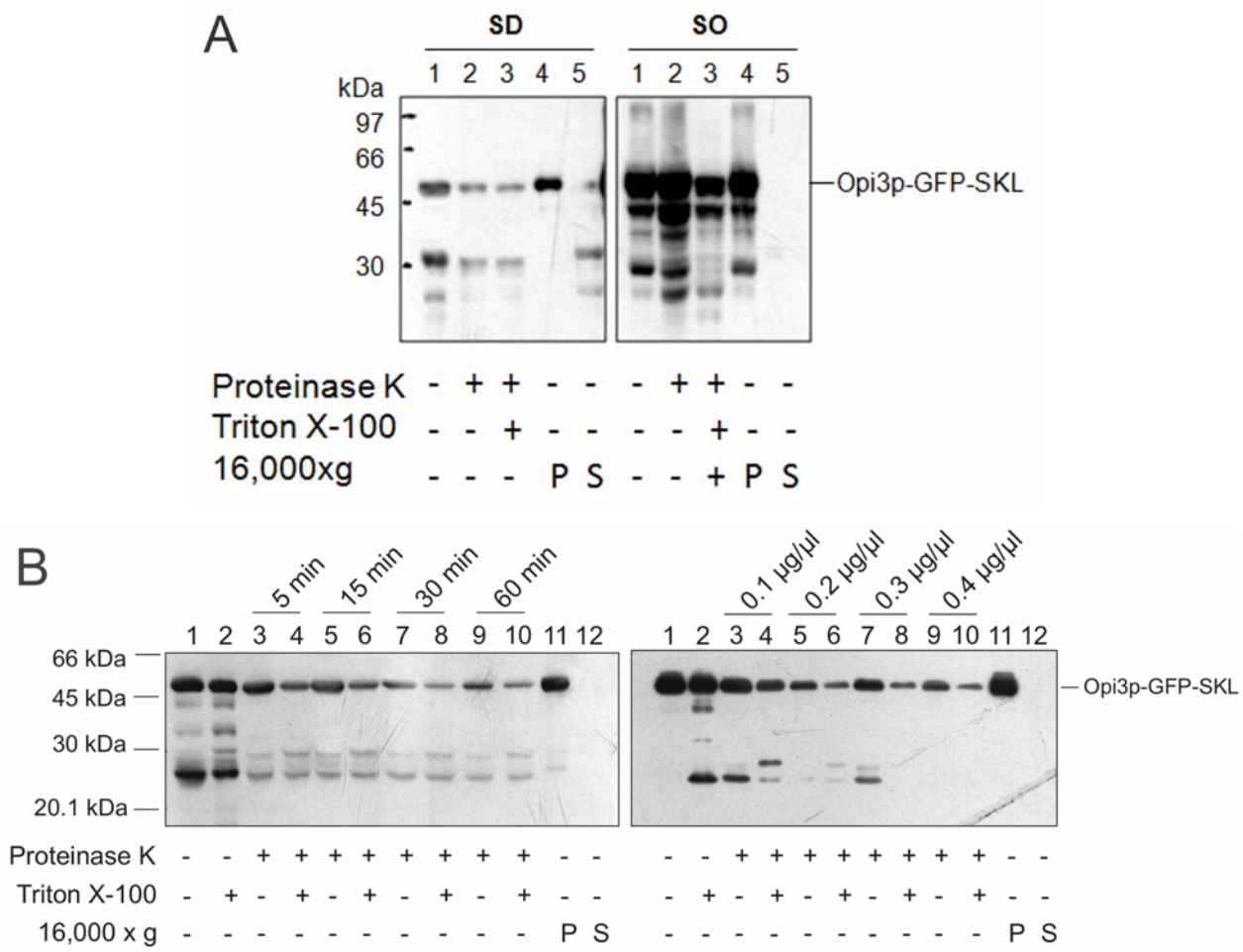


Figure 4: Proteinase K protection assay

Yeast cells from the strain yMC3 were pre-grown on minimal medium for 48 h and used to inoculate a main culture to an OD_{600} of 0.1. Cells were grown for 24 h at 30°C with shaking and harvested. Spheroplasts were prepared as described in Material and Methods, and aliquots were shock frozen in liquid nitrogen and stored at -70 °C until use. Same amounts of lysate were used for Proteinase K protection assay which was performed as described in Material and Methods.

(A) Cells grown on either SD or SO were used for lysate preparation. 1.25 µl 20 mg/ml Proteinase K was used for incubation. When indicated, 5 µl 20 % Triton X-100 were added for solubilization of the membranes. Samples were incubated for 10 min on ice, proteins were then precipitated by addition of 10 % (final) TCA and subjected to SDS-PAGE and Western blotting. Anti-GFP antibody was used to detect the protein. Fractions 1-3: lysate samples with or without Proteinase K and detergent as indicated; fraction 4: 16,000 x g pellet (P; membranes); fraction 5: 16,000 x g supernatant (S; cytosol)

(B) Cells from yMC3 grown on SO medium were used for cell lysate preparation and Proteinase K digestion. Different incubation times were used studying the effect of 1.25 µl 20 mg/ml proteinase K on OPI3-GFP-SKL (left panel). In addition various amounts of Proteinase K were tested treating the samples for 10 min on ice. Fractions 1 and 2: no addition of Proteinase K; fractions 3-10: different incubation times or different amounts of Proteinase K as indicated; fraction 11: 16,000 x g pellet; fraction 12: 16,000 x g supernatant

Since the assay was performed with cell lysate and not isolated organelles, assay conditions are difficult to control. The weak point in preliminary experiments shown here was that the protein was not completely digested after addition of detergent. Therefore, we tried to set assay conditions more precisely. We performed time dependent digestion as well as incubations with increasing amounts of Proteinase K. These analyses revealed that most of the protein was digested over a time period of 60 min even in the absence of detergent (Figure 4B; left panel, fraction 9 and 10) indicating that over longer time of incubation peroxisomes became leaky and Proteinase K was able to penetrate peroxisomal membrane.

However, increasing amounts of Proteinase K revealed that most of the protein seemed to be protected (Figure 4B; right panel, fractions 9 and 10) Together these preliminary data suggest that Opi3p-GFP-SKL was imported into peroxisomal matrix or at least protected in the membrane. A 16,000 x g membrane pellets contained all Opi3p-GFP-SKL (Figure 4A fractions 4 and Figure 4B fraction 11) whereas no signal was found in the cytosol (Figure 4A fraction 5 and Figure 4B fraction 12). This result confirmed organelle association of Opi3p-GFP-SKL.

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

After verification of the localization of Opi3p-GFP-SKL to the peroxisome we investigated its ability to produce PC in the host organelle. Therefore, cells from yMC3 grown on SD and SO medium as well as yMC2 grown on SD were analyzed for the formation of PC *in vivo* (Figure 5A). Apparently only minor amounts of PC were detected in cells harboring the empty GFP-SKL plasmid under non-induced (SD) conditions. Slightly higher amounts were detected in yMC3 under non-induced conditions indicating once again that the promoter was not completely tight. As expected, in cells grown on oleic acid supplemented media high amounts of PC were detected indicating that under these conditions Opi3p-GFP-SKL was able to produce PC *in vivo*. To investigate the PC distribution in the cell we analyzed subcellular fractions from yMC3 cells grown in the presence of oleic acid. The amount of PC was comparable in peroxisomes, mitochondria and microsomes (Figure 5B) indicating that PC produced in peroxisomes was distributed to all other organelles.

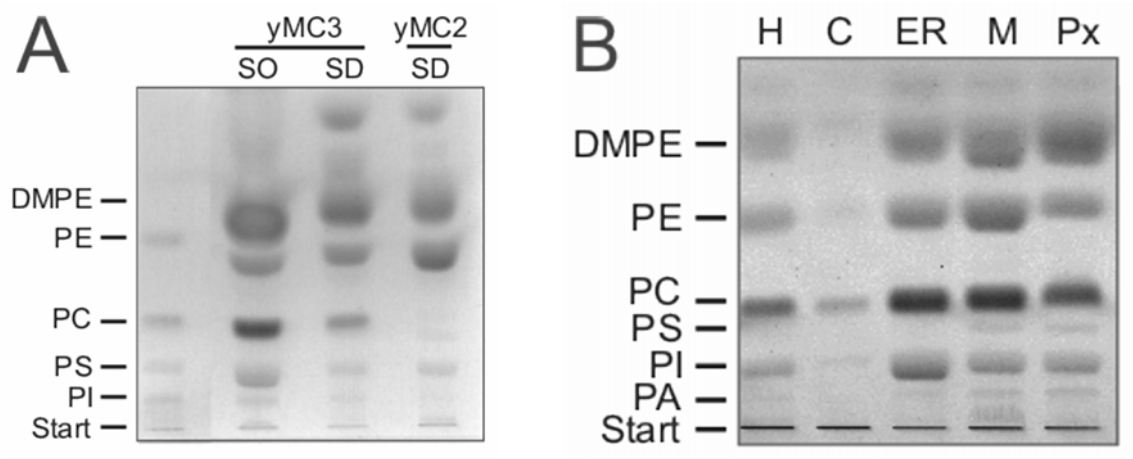


Figure 5: Phospholipid pattern

Lipid extracts were obtained as described in Material and Methods. Same amounts were loaded onto Silica plates and phospholipid species were separated using $CHCl_3$:MeOH:NH_3 (25:12:3; per vol.) solvent system for 50 min in one direction. Phospholipids were visualized by iodine vapor staining and different species are indicated.

(A) Total cell extracts were prepared from yMC3 cells grown on either SD or SO medium for 24 h and from yMC2 cells grown on SD medium.

(B) Subcellular fractions from yMC3 cells grown on SO medium for 24 h were used for lipid extracts. H, homogenate; C, cytosol; ER, endoplasmic reticulum; M, mitochondria; Px, peroxisomes

To test whether PC formed *in vivo* was actually produced exclusively in peroxisomes, we performed *in vitro* Opi3p activity assays with subcellular fractionations. Major activity was detected in peroxisomes and only minor activity was ascribed to mitochondria and ER (Figure 6A). Analyzing the purity of the subcellular fractions revealed that contamination of mitochondria and ER with peroxisomal membranes had occurred which accounted for the remaining Opi3p activity in Mt and ER (Figure 6B). These data indicate that Opi3p-GFP-SKL was active in peroxisomal membranes and exhibited more or less exclusive subcellular PE methyltransferase activity.

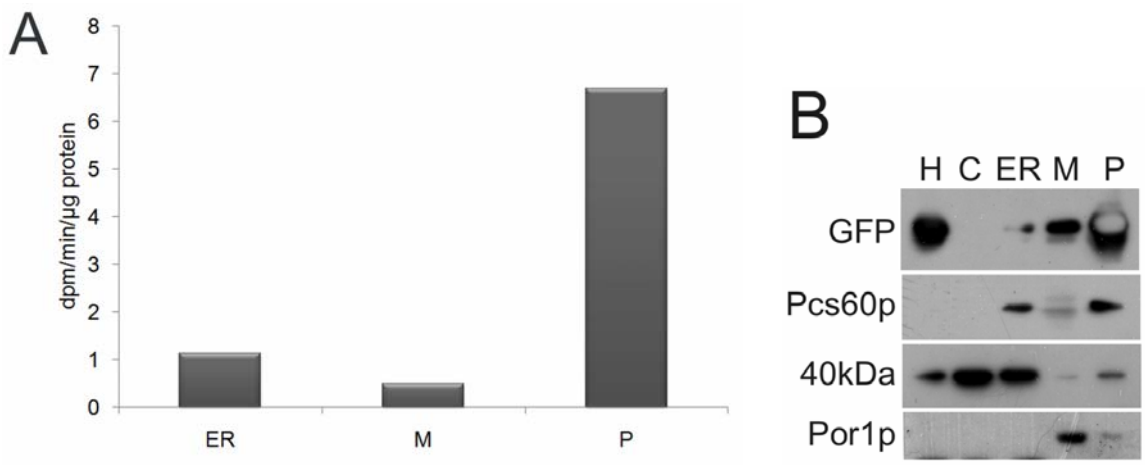


Figure 6: Opi3p-GFP-SKL *in vitro* activity

Cells from yMC3 strain grown on oleic acid medium were used for organelle isolation and methyltransferase activity assays *in vitro*.

(A) Freshly prepared organelles from subcellular fractions were used for *in vitro* PE methylation assay as described in Material and Methods.

(B) Western blotting analysis for quality control of the organelle preparation. Same amount of proteins were loaded, separated by SDS-PAGE and transferred to nitrocellulose membrane by Western blotting. Incubation with first antibodies against GFP, Pcs60p (peroxisomal AMP-binding protein), 40 kDa (endoplasmic protein) and Por1p (mitochondrial porin) indicated the presence of the respective organelle.
H, homogenate; C, cytosol; ER, endoplasmic reticulum; M, mitochondria; P, peroxisomes

Phospholipid traffic to peroxisomes

A *semi in vitro* phospholipid assay was adapted from Achleitner *et al.* (Achleitner *et al.*, 1995) and used to investigate PL traffic to peroxisomes hosting the reporter enzyme Opi3p-GFP-SKL. For this purpose, we prepared permeabilized cells from wt, yMC3 and *opi3*Δ cells to compare the efficiency of PE traffic to peroxisomes used as a substrate for PC synthesis. PC synthesis via PE can be monitored using [^{3}H]serine as precursor for PS and subsequently for PE and PC biosynthesis. The other substrate, CDP-DAG, was found to be present at sufficient levels in permeabilized cells (Achleitner *et al.*, 1995). As Opi3p also possesses activity for MMPE formation, the resulting PC formation indicated the efficiency of PE traffic to peroxisomes.

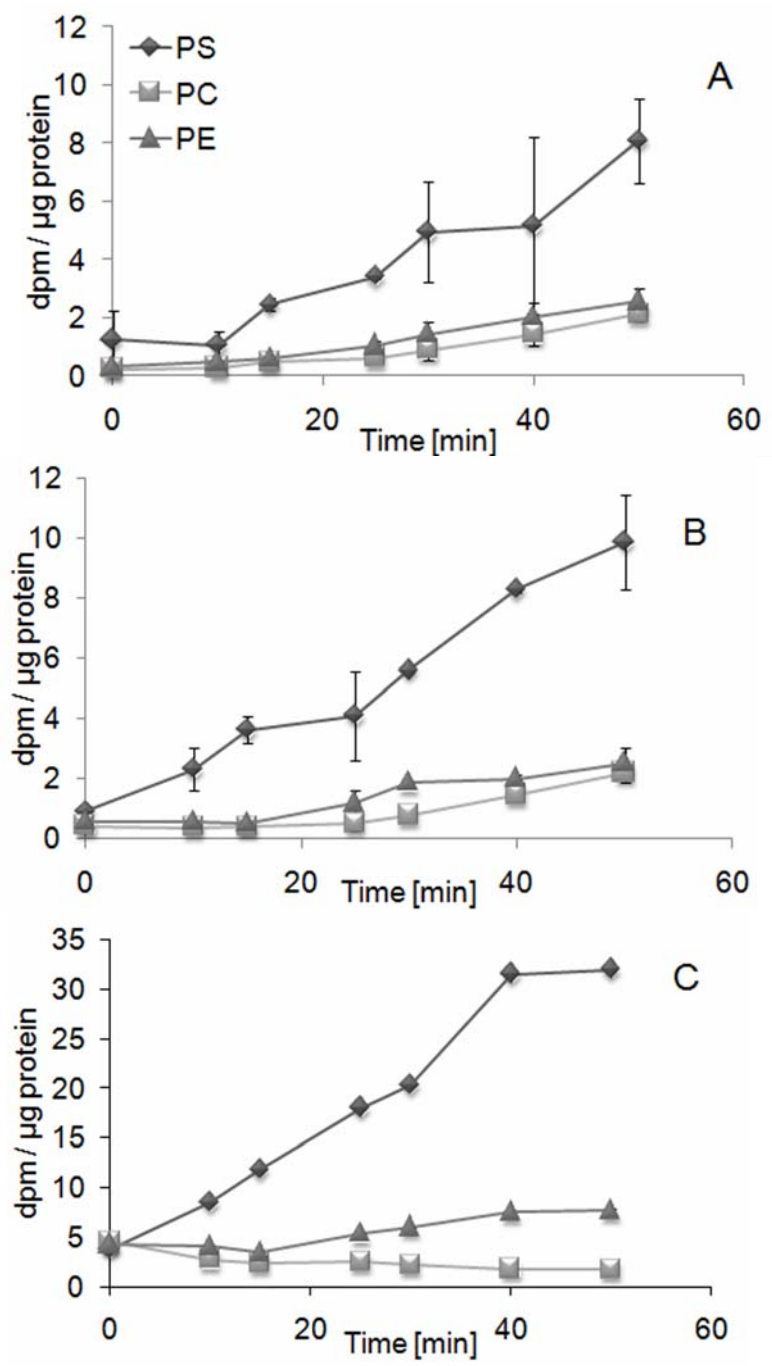


Figure 7: *Semi in vitro* phospholipid synthesis in wt, yMC3 and opi3Δ cells grown on oleic acid medium

Permeabilized cells of the strains BY4741 (wt) (A), yMC3 with Opi3p localized to peroxisomes (B) and *opi3*Δ (C) were prepared and used for *semi in vitro* PL assays as described in detail in Material and Methods.

In a three step *semi in vitro* assay we therefore tested the ability of peroxisomes containing Opi3p-GFP-SKL to produce PC from PE supplied to this organelle in comparison to wild type. For this purpose, we followed up the incorporation of [^{3}H]serine into PS, PE and subsequently into PC (Figure 7). Synthesis of PS requires the presence of bivalent cations such as Mn^{2+} (Bae-Lee and Carman, 1984; Sperka-Gottlieb *et al.*, 1990) which were added to the assay mixture during the first incubation phase. In the following step, decarboxylation of PS by either Psd1p or Psd2p is inhibited by divalent cations but enhanced by EDTA (Lamping *et al.*, 1991). Therefore, an excess of EDTA was added to the reaction mixture for the second step of the reaction cascade. Methylation of PE to PC depends on the presence of Mg^{2+} and SAM as cofactors (Kodaki and Yamashita, 1989b) which were added in a final step to induce formation of PC.

In wild type cells, where Opi3p is localized to the ER (Zinser *et al.*, 1991). Upon incubation with [^{3}H]serine most of the label ended up in PS (Figure 7A). Over the incubation period, however, sufficient amounts of PE and PC were also formed after adding EDTA and SAM/ Mg^{2+}, respectively. Most importantly, amounts of PS, PE and PC formed in yMC3 with Opi3p-GFP-SKL localized to peroxisomes were comparable to wild type (Figure 7B). This result indicated that sufficient supply of PE to peroxisomes had occurred. In a strain lacking Opi3p (Figure 7C), PS and PE were formed but, as expected, formation of PC was not detected.

Discussion

Peroxisome biogenesis has been a matter of debate for the past decades. In general it is believed that peroxisomes are non-autonomous organelles derived from the ER but able to grow and divide. Hence, all studies aiming at elucidation of the origin of peroxisomes were focused on protein supply to peroxisomes. It was found that some of the early and essential peroxines, Pex3p and Pex19p, were localized first to the ER and then shuttled to early peroxisomes (Hoepfner *et al.*, 2005) thus indicating a relationship of ER and peroxisome formation. In addition, fission of peroxisomes and further growth of the daughter organelles have been claimed (Guo *et al.*, 2007b). Since peroxisomes do not harbor enzymes for lipid synthesis they require import of membrane lipids for expansion. A mechanism for lipid import has not yet been identified which could explain lipid supply to already existing peroxisomal membranes.

One possibility of lipid supply to organelles lacking lipid synthesizing enzymes involves vesicular flux. Previous studies, however, excluded vesicle traffic to peroxisomes in plants and *Yarrowia lipolytica* (Chapman and Trelease, 1991; Titorenko *et al.*, 1996) as well as in *Saccharomyces cerevisiae* (Raychaudhuri and Prinz, 2008b). Other models of lipid transport addressing the impact of cytosolic lipid transfer proteins (Lafer *et al.*, 1991; Wirtz, 1991) were restricted to *in vitro* conditions.

One of the most favored models of lipid traffic involves direct membrane contact. Indeed, import of PS from the ER to mitochondria has been shown to act via membrane contact sites (Vance and Shiao, 1996; Daum and Vance, 1997) and has been elucidated with permeabilized cells in mammals (Voelker, 1989b; Vance, 1991) and yeast (Achleitner *et al.*, 1995). It is tempting to speculate that a similar lipid supply mechanism from ER to peroxisome might exist. It has been shown that PS transfer to mitochondria is ATP-independent in permeabilized yeast cells (Achleitner *et al.*, 1995) whereas ATP was required for PS transport from ER to mitochondria in mammalian cells (Voelker, 1989a; Voelker, 1989c; Voelker, 1990; Choi *et al.*, 2005a). Interestingly, studies with isolated ER and mitochondria from mammalian cells were also found to have ATP-independent PS transport. The reasons for the discrepancies are unknown but indicate that direct membrane interaction could account for PS supply in mammals but also in yeast.

In addition, it has been shown that sites of contact between the ER and various other organelles might be important for non-vesicular lipid transfer (Choi *et al.*, 2005b; Levine and Loewen, 2006) supporting the idea that the ER plays a major role in direct membrane contact. In yeast, direct contact sites of the ER were found with mitochondria, the so-called MAM (Vance, 1990; Ardail *et al.*, 1993b; Gaigg *et al.*, 1995; Shiao *et al.*, 1995; Camici and Corazzi, 1995), and the plasma membrane, the so-called PAM (Pichler *et al.*, 2001). Our own studies aiming to isolate peroxisomal associated membranes (PXAM) failed so far and we could neither confirm nor confute a direct contact of ER to peroxisomes (our own unpublished data). It has to be noted that purification of peroxisomes often results in cross-contamination mainly with ER and mitochondria. These findings may support the idea of direct membrane contact resulting in attached membrane fractions during the purification process.

Additionally, contact of peroxisomes has been found to the lipid particle in two independent studies (Binns *et al.*, 2006; Rosenberger *et al.*, 2009c) indicating that membrane association might also plays a role in neutral lipid storage/mobilization. Visualization of direct membrane contact remains difficult. An elegant approach was performed in Arabidopsis by Andersson *et al.*, (Andersson *et al.*, 2007) using optical tweezers to measure direct membrane contact of chloroplasts and the ER. Hence, such an approach was not feasible for yeast organelles due to their small size (our own unpublished results).

Therefore, an approach using a reporter enzyme was used to elucidate lipid traffic to peroxisomes. Peroxisomes are suitable organelles for introducing reporter enzymes since they harbor a specific protein import machinery. Proteins containing specific peroxisomal targeting sequences (PTS) are imported into the peroxisomal matrix or the membrane. In this study, we made use of this system by tagging Opi3p, the major phospholipid methyltransferase, with a GFP-SKL sequence to translocate the original ER protein to peroxisomes. Opi3p was incorporated into the peroxisomal matrix or protected in the peroxisomal membrane which would be in line with previous studies claiming that Opi3p was an integral membrane protein in the ER (Zinser *et al.*, 1991). The protein was found to be fully functional and was able to react with its substrate MMPE or even PE. Although formation of MMPE was thought to be mostly catalyzed by Cho2p some minor activity was also attributed to Opi3p (Kodaki and Yamashita, 1987c). It remains to be elucidated whether the residual Opi3p activity for MMPE formation accounts for the PC formation in Px or whether MMPE is supplied to Px from the ER. In any case, sufficient amounts of MMPE were available in peroxisomes to enable PC synthesis similar to wild type.

Our data shown here support the idea that peroxisomes receive sufficient PE formed in the mitochondria or Golgi (Psd1p or Psd2p, respectively) or MMPE from the ER by Cho2p. Permeabilized yeast cells enabled us to measure PL flux in a *semi in vitro* assay showing that Opi3p-GFP-SKL activity in peroxisomes was comparable to the wild type protein activity in the ER. Further analyses have to elucidate whether the PE traffic to peroxisomes occurs via direct membrane contact or other transport mechanisms such as lipid carrier proteins or vesicle flux may account for this process. Our approach of introducing the reporter enzyme Opi3p-GFP-SKL into peroxisomes opens a new possibility of elucidating lipid traffic to and also from peroxisomes and can be applied to future studies. Further studies with mutant strains deficient in the different PE synthesizing pathways as well as in Cho2p should provide more insight into PE or MMPE supply to peroxisomes. Requirement of additional factors such as cytosol, ATP or several ions should also be tested to elucidate the mechanism of lipid traffic to peroxisomes from their sites of synthesis.

Acknowledgement

We thank Andreas Hartig and Cecil Brocard for providing the plasmid pJR233. This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich Project DK W901-B05 and 21429 (to G. D.).

Reference List

Achleitner,G., Gaigg,B., Krasser,A., Kainersdorfer,E., Kohlwein,S.D., Perktold,A., Zellnig,G., and Daum,G. (1999). Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. Eur J Biochem *264*, 545-53.

Achleitner,G., Zweytick,D., Trotter,P.J., Voelker,D.R., and Daum,G. (1995). Synthesis and intracellular transport of aminoglycerophospholipids in permeabilized cells of the yeast, Saccharomyces cerevisiae. J Biol Chem *270*, 29836-42.

Andersson,M.X., Goksor,M., and Sandelius,A.S. (2007). Optical manipulation reveals strong attracting forces at membrane contact sites between endoplasmic reticulum and chloroplasts
1. J. Biol. Chem. *282*, 1170-1174.

Ardail,D., Gasnier,F., Lerme,F., Simonot,C., Louisot,P., and Gateau-Roesch,O. (1993b). Involvement of mitochondrial contact sites in the subcellular compartmentalization of phospholipid biosynthetic enzymes. J. Biol. Chem. *268*, 25985-25992.

Ardail,D., Gasnier,F., Lerme,F., Simonot,C., Louisot,P., and Gateau-Roesch,O. (1993a). Involvement of mitochondrial contact sites in the subcellular compartmentalization of phospholipid biosynthetic enzymes. J. Biol. Chem. *268*, 25985-25992.

Bae-Lee,M.S. and Carman,G.M. (1984). Phosphatidylserine synthesis in Saccharomyces cerevisiae. Purification and characterization of membrane-associated phosphatidylserine synthase. J. Biol. Chem. *259*, 10857-10862.

Binns,D., Januszewski,T., Chen,Y., Hill,J., Markin,V.S., Zhao,Y., Gilpin,C., Chapman,K.D., Anderson,R.G., and Goodman,J.M. (2006). An intimate collaboration between peroxisomes and lipid bodies. J Cell Biol *173*, 719-31.

Bishop,W.R. and Bell,R.M. (1988). Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement and intracellular translocation. Annu Rev Cell Biol *4*, 579-610.

Boumann,H.A., Chin,P.T., Heck,A.J., De,K.B., and de Kroon,A.I. (2004). The yeast phospholipid N-methyltransferases catalyzing the synthesis of phosphatidylcholine preferentially convert di-C16:1 substrates both in vivo and in vitro. J. Biol. Chem. *279*, 40314-40319.

Brocard,C., Lametschwandtner,G., Koudelka,R., and Hartig,A. (1997a). Pex14p is a member of the protein linkage map of Pex5p. EMBO J. *16*, 5491-5500.

Brocard,C., Lametschwandtner,G., Koudelka,R., and Hartig,A. (1997b). Pex14p is a member of the protein linkage map of Pex5p. EMBO J. *16*, 5491-5500.

Broekhuyse,R.M. (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.

Camici,O. and Corazzi,L. (1995). Import of phosphatidylethanolamine for the assembly of rat brain mitochondrial membranes. J. Membr. Biol. *148*, 169-176.

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

Chapman,K.D. and Trelease,R.N. (1991). Acquisition of membrane lipids by differentiating glyoxysomes: role of lipid bodies. J. Cell Biol. *115*, 995-1007.

Choi,J.Y., Wu,W.I., and Voelker,D.R. (2005b). Phosphatidylserine decarboxylases as genetic and biochemical tools for studying phospholipid traffic. Anal. Biochem. *347*, 165-175.

Choi,J.Y., Wu,W.I., and Voelker,D.R. (2005a). Phosphatidylserine decarboxylases as genetic and biochemical tools for studying phospholipid traffic. Anal. Biochem. *347*, 165-175.

Connerth,M., Grillitsch,K., Kofeler,H., and Daum,G. (2009). Analysis of lipid particles from yeast. Methods Mol. Biol. *579*, 359-374.

Daum,G. and Vance,J.E. (1997). Import of lipids into mitochondria. Prog Lipid Res *36*, 103-30.

Erdmann,R. and Blobel,G. (1995). Giant peroxisomes in oleic acid-induced Saccharomyces cerevisiae lacking the peroxisomal membrane protein Pmp27p. J Cell Biol *128*, 509-23.

Erdmann,R., Veenhuis,M., Mertens,D., and Kunau,W.H. (1989). Isolation of peroxisome-deficient mutants of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *86*, 5419-23.

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

Gaigg,B., Simbeni,R., Hrastnik,C., Paltauf,F., and Daum,G. (1995). Characterization of a microsomal subfraction associated with mitochondria of the yeast, Saccharomyces cerevisiae. Involvement in synthesis and import of phospholipids into mitochondria. Biochim. Biophys. Acta *1234*, 214-220.

Geuze,H.J., Murk,J.L., Stroobants,A.K., Griffith,J.M., Kleijmeer,M.J., Koster,A.J., Verkleij,A.J., Distel,B., and Tabak,H.F. (2003). Involvement of the endoplasmic reticulum in peroxisome formation. Mol. Biol. Cell *14*, 2900-2907.

Greenberg,M.L., Klig,L.S., Letts,V.A., Loewy,B.S., and Henry,S.A. (1983). Yeast mutant defective in phosphatidylcholine synthesis. J. Bacteriol. *153*, 791-799.

Guo,T. *et al.* (2007b). A signal from inside the peroxisome initiates its division by promoting the remodeling of the peroxisomal membrane. J. Cell Biol. *177*, 289-303.

Guo,T. *et al.* (2007a). A signal from inside the peroxisome initiates its division by promoting the remodeling of the peroxisomal membrane. J. Cell Biol. *177*, 289-303.

Haan,G.J., Baerends,R.J., Krikken,A.M., Otzen,M., Veenhuis,M., and van,d.K., I (2006). Reassembly of peroxisomes in Hansenula polymorpha pex3 cells on reintroduction of Pex3p involves the nuclear envelope. FEMS Yeast Res. *6*, 186-194.

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

Hettema,E.H., Girzalsky,W., van den,B.M., Erdmann,R., and Distel,B. (2000). Saccharomyces cerevisiae pex3p and pex19p are required for proper localization and stability of peroxisomal membrane proteins. EMBO J. *19*, 223-233.

Hoepfner,D., Schildknegt,D., Braakman,I., Philippsen,P., and Tabak,H.F. (2005). Contribution of the endoplasmic reticulum to peroxisome formation. Cell *122*, 85-95.

Hoepfner,D., van Den Berg,M., Philippsen,P., Tabak,H.F., and Hettema,E.H. (2001). A role for Vps1p, actin, and the Myo2p motor in peroxisome abundance and inheritance in Saccharomyces cerevisiae. J Cell Biol *155*, 979-90.

Huybrechts,S.J., Van Veldhoven,P.P., Brees,C., Mannaerts,G.P., Los,G.V., and Fransen,M. (2009). Peroxisome dynamics in cultured mammalian cells. Traffic. *10*, 1722-1733.

Kennedy,E.P. and Weiss,S.B. (1956). The function of cytidine coenzymes in the biosynthesis of phospholipides. J. Biol. Chem. *222*, 193-214.

Kim,P.K., Mullen,R.T., Schumann,U., and Lippincott-Schwartz,J. (2006). The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. J Cell Biol *173*, 521-32.

Kobayashi,S., Tanaka,A., and Fujiki,Y. (2007). Fis1, DLP1, and Pex11p coordinately regulate peroxisome morphogenesis. Exp. Cell Res. *313*, 1675-1686.

Koch,A., Thiemann,M., Grabenbauer,M., Yoon,Y., McNiven,M.A., and Schrader,M. (2003). Dynamin-like protein 1 is involved in peroxisomal fission. J. Biol. Chem. *278*, 8597-8605.

Koch,A., Yoon,Y., Bonekamp,N.A., McNiven,M.A., and Schrader,M. (2005). A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. Mol. Biol. Cell *16*, 5077-5086.

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

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

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

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

Kodaki,T. and Yamashita,S. (1989b). Characterization of the methyltransferases in the yeast phosphatidylethanolamine methylation pathway by selective gene disruption. Eur. J. Biochem. *185*, 243-251.

Kodaki,T. and Yamashita,S. (1989a). Characterization of the methyltransferases in the yeast phosphatidylethanolamine methylation pathway by selective gene disruption. Eur. J. Biochem. *185*, 243-251.

Kragt,A., Voorn-Brouwer,T., van Den Berg,M., and Distel,B. (2005). Endoplasmic reticulum-directed Pex3p routes to peroxisomes and restores peroxisome formation in a Saccharomyces cerevisiae pex3Delta strain. J Biol Chem *280*, 34350-7.

Kuravi,K., Nagotu,S., Krikken,A.M., Sjollema,K., Deckers,M., Erdmann,R., Veenhuis,M., and van,d.K., I (2006). Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in Saccharomyces cerevisiae. J. Cell Sci. *119*, 3994-4001.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-5.

Lafer,G., Szolderits,G., Paltauf,F., and Daum,G. (1991). Isolation of a phosphatidylserine transfer protein from yeast cytosol. Biochim. Biophys. Acta *1069*, 139-144.

Lamping,E., Kohlwein,S.D., Henry,S.A., and Paltauf,F. (1991). Coordinate regulation of phosphatidylserine decarboxylase in Saccharomyces cerevisiae. J. Bacteriol. *173*, 6432-6437.

Levine,T. and Loewen,C. (2006). Inter-organelle membrane contact sites: through a glass, darkly. Curr. Opin. Cell Biol. *18*, 371-378.

Li,X. and Gould,S.J. (2003). The dynamin-like GTPase DLP1 is essential for peroxisome division and is recruited to peroxisomes in part by PEX11. J. Biol. Chem. *278*, 17012-17020.

Longtine,M.S., McKenzie,A.3., Demarini,D.J., Shah,N.G., Wach,A., Brachat,A., Philippsen,P., and Pringle,J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-61.

Lowry,O.H., Rosebrough,N.J., Farr A.L., and Randall R.J. (1951a). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

Lowry,O.H., Rosebrough,N.J., Farr A.L., and Randall R.J. (1951b). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

Matsuzaki,T. and Fujiki,Y. (2008). The peroxisomal membrane protein import receptor Pex3p is directly transported to peroxisomes by a novel Pex19p- and Pex16p-dependent pathway. J. Cell Biol. *183*, 1275-1286.

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

McMaster,C.R. and Bell,R.M. (1994). Phosphatidylcholine biosynthesis via the CDP-choline pathway in Saccharomyces cerevisiae. Multiple mechanisms of regulation. J. Biol. Chem. *269*, 14776-14783.

Motley,A.M. and Hettema,E.H. (2007). Yeast peroxisomes multiply by growth and division. J. Cell Biol. *178*, 399-410.

Motley,A.M., Ward,G.P., and Hettema,E.H. (2008). Dnm1p-dependent peroxisome fission requires Caf4p, Mdv1p and Fis1p. J. Cell Sci. *121*, 1633-1640.

Nagotu,S., Krikken,A.M., Otzen,M., Kiel,J.A., Veenhuis,M., and van,d.K., I (2008a). Peroxisome fission in Hansenula polymorpha requires Mdv1 and Fis1, two proteins also involved in mitochondrial fission. Traffic. *9*, 1471-1484.

Nagotu,S., Saraya,R., Otzen,M., Veenhuis,M., and van,d.K., I (2008b). Peroxisome proliferation in Hansenula polymorpha requires Dnm1p which mediates fission but not de novo formation. Biochim. Biophys. Acta *1783*, 760-769.

Nagotu,S., Veenhuis,M., and van,d.K., I (2010). Divide et impera: the dictum of peroxisomes. Traffic. *11*, 175-184.

Pichler,H., Gaigg,B., Hrastnik,C., Achleitner,G., Kohlwein,S.D., Zellnig,G., Perktold,A., and Daum,G. (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.

Poirier,Y., Antonenkov,V.D., Glumoff,T., and Hiltunen,J.K. (2006). Peroxisomal beta-oxidation--a metabolic pathway with multiple functions. Biochim. Biophys. Acta *1763*, 1413-1426.

Purdue,P.E. and Lazarow,P.B. (2001). Peroxisome biogenesis. Annu Rev Cell Dev Biol *17*, 701-52.

Raychaudhuri,S. and Prinz,W.A. (2008b). Nonvesicular phospholipid transfer between peroxisomes and the endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A *105*, 15785-15790.

Raychaudhuri,S. and Prinz,W.A. (2008a). Nonvesicular phospholipid transfer between peroxisomes and the endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A *105*, 15785-15790.

Rosenberger,S., Connerth,M., Zellnig,G., and Daum,G. (2009a). Phosphatidylethanolamine synthesized by three different pathways is supplied to peroxisomes of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta.

Rosenberger,S., Connerth,M., Zellnig,G., and Daum,G. (2009d). Phosphatidylethanolamine synthesized by three different pathways is supplied to peroxisomes of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta.

Rosenberger,S., Connerth,M., Zellnig,G., and Daum,G. (2009b). Phosphatidylethanolamine synthesized by three different pathways is supplied to peroxisomes of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta.

Rosenberger,S., Connerth,M., Zellnig,G., and Daum,G. (2009c). Phosphatidylethanolamine synthesized by three different pathways is supplied to peroxisomes of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta.

Shiao,Y.J., Balcerzak,B., and Vance,J.E. (1998). A mitochondrial membrane protein is required for translocation of phosphatidylserine from mitochondria-associated membranes to mitochondria. Biochem. J. *331 (Pt 1)*, 217-223.

Shiao,Y.J., Lupo,G., and Vance,J.E. (1995). Evidence that phosphatidylserine is imported into mitochondria via a mitochondria-associated membrane and that the majority of mitochondrial phosphatidylethanolamine is derived from decarboxylation of phosphatidylserine. J. Biol. Chem. *270*, 11190-11198.

Smith,J.J. and Aitchison,J.D. (2009). Regulation of peroxisome dynamics. Curr. Opin. Cell Biol. *21*, 119-126.

South,S.T. and Gould,S.J. (1999). Peroxisome synthesis in the absence of preexisting peroxisomes. J. Cell Biol. *144*, 255-266.

Sperka-Gottlieb,C., Fasch,E.V., Kuchler,K., Bailis,A.M., Henry,S.A., Paltauf,F., and Kohlwein,S.D. (1990). The hydrophilic and acidic N-terminus of the integral membrane enzyme phosphatidylserine synthase is required for efficient membrane insertion. Yeast *6*, 331-343.

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

Tabak,H.F., Murk,J.L., Braakman,I., and Geuze,H.J. (2003). Peroxisomes start their life in the endoplasmic reticulum. Traffic *4*, 512-8.

Tam,Y.Y., Fagarasanu,A., Fagarasanu,M., and Rachubinski,R.A. (2005). Pex3p initiates the formation of a preperoxisomal compartment from a subdomain of the endoplasmic reticulum in Saccharomyces cerevisiae. J Biol Chem *280*, 34933-9.

Titorenko,V.I., Eitzen,G.A., and Rachubinski,R.A. (1996). Mutations in the PAY5 gene of the yeast Yarrowia lipolytica cause the accumulation of multiple subpopulations of peroxisomes. J Biol Chem *271*, 20307-14.

Titorenko,V.I. and Mullen,R.T. (2006). Peroxisome biogenesis: the peroxisomal endomembrane system and the role of the ER. J Cell Biol *174*, 11-7.

Toro,A.A. *et al.* (2009). Pex3p-dependent peroxisomal biogenesis initiates in the endoplasmic reticulum of human fibroblasts. J. Cell Biochem. *107*, 1083-1096.

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

Vance,J.E. (1990). Phospholipid synthesis in a membrane fraction associated with mitochondria. J. Biol. Chem. *265*, 7248-7256.

Vance,J.E. (1991). Newly made phosphatidylserine and phosphatidylethanolamine are preferentially translocated between rat liver mitochondria and endoplasmic reticulum. J. Biol. Chem. *266*, 89-97.

Vance,J.E. and Shiao,Y.J. (1996). Intracellular trafficking of phospholipids: import of phosphatidylserine into mitochondria. Anticancer Res. *16*, 1333-1339.

Voelker,D.R. (1989a). Phosphatidylserine translocation to the mitochondrion is an ATP-dependent process in permeabilized animal cells. Proc. Natl. Acad. Sci. U. S. A *86*, 9921-9925.

Voelker,D.R. (1989c). Reconstitution of phosphatidylserine import into rat liver mitochondria. J. Biol. Chem. *264*, 8019-8025.

Voelker,D.R. (1989b). Reconstitution of phosphatidylserine import into rat liver mitochondria. J. Biol. Chem. *264*, 8019-8025.

Voelker,D.R. (1990). Characterization of phosphatidylserine synthesis and translocation in permeabilized animal cells. J. Biol. Chem. *265*, 14340-14346.

Waechter,C.J. and Lester,R.L. (1971). Regulation of phosphatidylcholine biosynthesis in Saccharomyces cerevisiae. J. Bacteriol. *105*, 837-843.

Waechter,C.J. and Lester,R.L. (1973). Differential regulation of the N-methyl transferases responsible for phosphatidylcholine synthesis in Saccharomyces cerevisiae. Arch. Biochem. Biophys. *158*, 401-410.

Waechter,C.J., Steiner,M.R., and Lester,R.L. (1969). Regulation of phosphatidylcholine biosynthesis by the methylation pathway in Saccharomyces cerevisiae. J. Biol. Chem. *244*, 3419-3422.

Wirtz,K.W. (1991). Phospholipid transfer proteins. Annu. Rev. Biochem. *60*, 73-99.

Wirtz,K.W. (2006). Phospholipid transfer proteins in perspective. FEBS Lett *580*, 5436-41.

Yan,M., Rachubinski,D.A., Joshi,S., Rachubinski,R.A., and Subramani,S. (2008). Dysferlin domain-containing proteins, Pex30p and Pex31p, localized to two compartments, control the number and size of oleate-induced peroxisomes in Pichia pastoris. Mol. Biol. Cell *19*, 885-898.

Zinser,E., Sperka-Gottlieb,C.D., Fasch,E.V., Kohlwein,S.D., Paltauf,F., and Daum,G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. J Bacteriol *173*, 2026-34.

CHAPTER 3

ANALYSIS OF LIPID PARTICLES FROM YEAST

Melanie Connerth[1#], Karlheinz Grillitsch[1#], Harald Köfeler[2] and Günther Daum[1*]

[1]Institute of Biochemistry, Graz University of Technology, Graz, Austria
[2]Center for Medical Research, Medical University of Graz, Austria

Key words: Lipid particles/droplets, phospholipid, triacylglycerol, steryl ester, yeast

Abbreviations:

CWW, cell wet weight; ER, endoplasmic reticulum; GLC, gas liquid chromatography; LP, lipid particle(s)/droplet(s); MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; STE, steryl ester; TAG, triacylglycerol; TLC, thin layer chromatography; RT, room temperature.

[#]contributed equally to this manuscript

Published in: Methods in Molecular Biology (2009); 579: 359-374

Abstract

Quantitative analysis of components from different subcellular fractions is a key to the understanding of metabolic function as well as to the origin, the biogenesis and the crosstalk of organelles. The yeast is an excellent model organism to address such questions from the biochemical, molecular biological and cell biological viewpoints. A yeast organelle which gained much interest during the last decade is the lipid particle/droplet (LP), a storage compartment for non-polar lipids but at the same time an organelle actively contributing to cellular metabolism. In this chapter, we describe methods and techniques that are commonly used to analyze lipids from LP at the molecular level by thin-layer chromatography, gas liquid chromatography and mass spectrometry. We provide an easy to follow guideline for the isolation of these organelles, the qualitative and quantitative analysis of lipid components and show results obtained with these methods.

1. Introduction

One of the most important prerequisites to establish a well organized and preserved cellular structure is the formation of biological membranes that protect or separate organelle components from the cellular environment, and shield cells from the exterior. Major constituents of membranes are lipid molecules, especially phospholipids, sterols, sphingolipids and glycolipids. Moreover, all types of cells contain storage lipids which are also referred as neutral lipids or non-polar lipids. In most cases, these neutral lipids are stored in a very specific compartment named lipid particle (LP), lipid droplet, lipid body or oil body (Athenstaedt and Daum, 2005). Contrary to other cellular organelles, the LP does not contain a phospholipid bilayer on its surface but rather a phospholipid monolayer with the polar head groups facing the hydrophilic environment and the hydrophobic part associating with the non-polar lipids of the LP core (Athenstaedt *et al.*, 1999; Zweytick *et al.*, 2000). The current hypothesis of LP biogenesis is based on a budding model suggesting that LP derive from the endoplasmic reticulum (ER) (Scow *et al.*, 1980; Blanchette-Mackie *et al.*, 1995; Robenek *et al.*, 2006; Czabany *et al.*, 2007). It has been proposed that neutral lipids formed in the ER accumulate in certain domains which grow and generate the LP core. Once the size of the non-polar lipid droplet increases and exceeds accommodation in the ER phospholipid bilayer, the LP buds off forming an independent organelle.

In the yeast *Saccharomyces cerevisiae*, steryl esters (STE) and triacylglycerols (TAG) are the major neutral lipid components of LP. Under normal growth conditions of the yeast the existence of LP is important although not essential (Sandager *et al.*, 2002). LP are assumed to store excess amounts of fatty acids and sterols in the biological inert form of STE and TAG and thereby avoid possible lipotoxic effects of these components. At the same time, storage and mobilization of neutral lipids helps the cell to conserve energy and building blocks for membrane biogenesis for conditions of nutritional depletion or cellular stress.

Yeast LP are equipped with a very specific subset of proteins. Many of them being involved in neutral lipid metabolism, e.g., Dga1p, Tgl3p, Tgl4p, Tgl5p, Tgl1p and Yeh2p, or in ergosterol biosynthesis (Erg1p, Erg6p, Erg7p) (Athenstaedt *et al.*, 1999; Rajakumari *et al.*, 2008). Interestingly, some of these proteins such as Erg1p or Dga1p are dually located within the cell, namely in LP and the ER.

How such proteins can be localized to two different types of membrane, a monolayer in the LP and a bilayer in the ER, is not yet understood. This observation also raises the question as to the specificity of the membrane lipid composition in these two compartments which might be important to understand the relationship between the two organelles.

In this chapter, we will describe methods commonly used for the analysis of lipids from yeast LP, which can also be adapted to other cell types. These techniques are versatile and can be easily performed. As a starting point for LP analysis, the isolation procedure of this organelle and the quality control of purified organelle samples will be explained. Then, we will describe lipid extraction and quantification of individual lipid components based on thin layer chromatography (TLC), gas liquid chromatography (GLC), mass spectrometry (MS) and GLC/MS.

2. Material

2.1 Equipment and Supplies

1. Microsyringe (Hamilton, Bonaduz, Switzerland) or sample applicator (CAMAG, Automatic TLC Sampler IV, Muttenz, Switzerland)
2. 12 ml Pyrex glass vials with Teflon liner caps
3. Glass tubes with ground neck
4. Dounce Homogenizer and pestle
5. Silica gel 60 TLC plates (Merck, Darmstadt, Germany)
6. Ultra-Clear Centrifuge Tube (Beckman)
7. TLC chamber (Springfield Mill, UK) with saturation paper (e.g., Whatman filter paper)
8. Iodine vapor chamber
9. Incubator (Heraeus)
10. TLC Scanner (Shimadzu chromatoscanner CS-930)
11. Table-top centrifuge (Hettich Rotina 46 R, Heraeus Fresco17)
12. Sorvall RC5 Plus or RC6 Plus, and SLC3000 or SS34 rotors
13. Ultracentrifuge (Sorvall – combi plus) with AH629 swing out rotor
14. GLC-MS (Hewlett-Packard 5890 Gas-Chromatograph)
15. MS LTQ-FT coupled to and Accela UPLC

2.2 Reagents

1. Medium for yeast cultivation:
   YPD (2 % glucose, 2 % peptone and 1 % yeast extract)
   SD (2 % glucose, 0.67 % yeast nitrogen base and amino acid mixture)
   YPO (0.1 % yeast extract, 0.5 % peptone, 0.5 % KH_2PO_4, 0.1 % glucose, 0.2 % Tween 80, 0.1 % oleic acid)
2. Zymolyase-20 T (Seikagaku corporation, Japan)
3. Ficoll 400 (Sigma)
4. Chemiluminescence solution: SuperSignal™ (Pierce Chemical Company, Rockford, IL, USA)
5. Solvents: chloroform and methanol, analytical grade
6. Washing solutions: 0.034 % $MgCl_2$; 2N KCl/MeOH (4:1; v/v); artificial upper phase (chloroform/methanol/water; 3:48:47; per vol.)

7. Charring solution: 0.63 g $MnCl_2 \cdot 4\ H_2O$, 60 ml water, 60 ml methanol, 4 ml conc. H_2SO_4

3. Methods

3.1 Isolation of lipid particles from the yeast

1. LPs are isolated from 4-5 l of full or selective media (2.2.1; see also section 5.5.). Cells are inoculated from a 48 h pre-culture to an OD_{600} of 0.1 and grown to stationary phase at 30 °C with shaking.
2. After harvesting at 5,000 rpm for 5 min in SLC3000, cells are washed with distilled water.
3. Cells (0.5 g CWW/ml) are incubated with dithiothreitol (DTT; 0.66 mg/ml) in buffer SP-A (0.1 M Tris/SO_4, pH 9.4) for 10 min at 30 °C with shaking.
4. Then, cells (0.15 g CWW/ml) are washed and suspended in pre-warmed SP-B (1.2 M sorbitol, 20 mM KH_2PO_4, pH 7.4). Cell walls are enzymatically digested with Zymolyase-20T (Seikagaku Corporation, Japan) at a concentration of 2 mg enzyme per g CWW for at least 1 h at 30°C with shaking.
5. Resulting spheroplasts are washed twice in buffer SP-B and then resuspended in buffer LP-A (12 % Ficoll, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA \cdot 2\ H_20$) followed by mechanical disruption in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; 1 mM final) with 30 strokes using a 30 ml Dounce Homogenizer with a loose fitting pestle.
6. The homogenate is centrifuged to remove cell debris in a Sorvall SS34 rotor at 7,000 rpm for 5 min.
7. Supernatants are collected, and steps 6 and 7 are repeated.
8. Combined supernatants are carefully overlaid with buffer LP-A in an Ultra-Clear Centrifuge Tube (Beckman). Ultracentrifugation at 28,000 rpm for 45 min using a swing out rotor AH-629 yields a white layer on top (crude LP) that can be transferred with a moistened spatula to a 15 ml Dounce Homogenizer.
9. After homogenizing with 8 strokes using a loose fitting pestle in the presence of 1 mM PMSF the sample is loaded onto a new ultracentrifuge tube and carefully overlaid with buffer LP-B (8 % Ficoll, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA \cdot 2\ H_20$). Ultracentrifugation at 28,000 rpm for 30 min results in a top layer containing LP (see notes 5.6).

10. Prior to the last ultracentrifugation step, buffer LP-D (0.25 M sorbitol, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA \cdot 2\ H_20$) is filled into a fresh ultracentrifuge tube. The homogenized sample is loaded to the bottom of the tube by injection under the buffer using a syringe.
11. Ultracentrifugation at 28,000 rpm for 30 min leads to a top layer consisting of highly purified LP. After homogenizing the isolated LP in a 5 ml Dounce Homogenizer the samples can be stored at -80 °C until required. If desired the pellet from the last centrifugation step which contains vacuoles can be collected as well.

3.2 Lipid particle analysis

3.2.1 Protein analysis

1. When applying ‘delipidation’ to isolated LP fractions, samples are incubated with 2 volumes of diethyl ether with repeated vigorous shaking. After a high speed centrifugation step using a table top centrifuge the extracted non-polar lipids are withdrawn and remaining traces of diethyl ether are removed under a stream of nitrogen.
2. Proteins are precipitated with trichloroacetic acid, and the resulting pellets are either dissolved in one-fold Laemmli buffer for gel electrophoresis (Laemmli, 1970) or solubilized in 0.1 % SDS/0.1 M NaOH for protein quantification.
3. Proteins are quantified using the method of Lowry et al. (Lowry *et al.*, 1951) with bovine serum albumin as a standard. Typical samples of LP fractions contain approximately 0.2-0.02 µg protein/µl depending on strain and growth conditions.
4. Samples must not be dissolved at temperatures higher than 37 °C as such a treatment would result in hydrolysis of proteins or in the formation of aggregates.
5. SDS-polyacrylamide gel electrophoresis is performed as described by Laemmli (Laemmli, 1970).
6. Commassie Blue staining of gels is usually sufficient to visualize protein bands, though more sensitive staining procedures (Silver staining, inverse protein staining) may be required to detect proteins of low abundance.
7. Western blot analysis is performed according to the method of Haid and Suissa (Haid and Suissa, 1983).

8. A set of antibodies representing typical marker proteins of various cellular organelles is used to check the quality of the isolated LP (Table 1 and related results in Figure 1, below)
9. Peroxidase conjugated secondary antibody (Sigma) and enhanced chemiluminescent signal detection reagents (SuperSignal^{TM}, Pierce Chemical Company, Rockford, IL, USA) are used to visualize immunoreactive bands.
10. When LP samples appear to be contaminated with other subcellular compartments a further purification step can be introduced after the standard LP isolation procedure (see Note 5.2.).

Table 1: **Marker antibodies used for the quality control of lipid particles**
LP = lipid particle; ER = endoplasmic reticulum; Mt = mitochondria; Vac = Vacuole

Marker Protein	Subcellular Localization	Function
Erg1p	LP, ER	Squalene epoxidase
Erg6p	LP, ER	Δ(24)-Sterol C-methyltransferase
Erg7p	LP, ER	Lanosterol synthase
Ayr1p	LP, ER	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase
Prc1p	Vac	Vacuolar carboxypeptidase Y
Por1p	Mt	Mitochondrial porin
Wbp1p	ER	β-Subunit of the oligosaccharyl transferase (OST) glycoprotein complex
Sec61p	ER	Integral ER required protein for protein import

3.2.2 Lipid extraction

1. Lipids from LP are extracted using the method of Folch et al. (Folch J. *et al.*, 1957).
2. In brief, an aliquot of the LP sample (~ 0.1 mg protein) is added to 3 ml $CHCl_3$:MeOH (2:1) in a Pyrex glass tube.
3. Lipids are extracted to the polar organic phase by vortexing at room temperature (RT) for 1 h.
4. 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.
5. Proteins and non-polar substances are removed by consecutive washing steps with 0.2 volumes 0.034 % $MgCl_2$, 1 ml 2 N KCl/MeOH (4:1; v/v) and 1 ml of an artificial

upper phase ($CHCl_3$:MeOH:H_2O; 3:48:47; per vol.). These solutions are added to the extracts and incubated with shaking for 3 min.

6. After centrifugation for 3 min at 3,000 rpm in a table-top centrifuge the aqueous phase is removed by aspiration.
7. Washing steps are repeated until no protein layer is formed any more.
8. Finally, lipids are dried under a stream of nitrogen and stored at -20 °C.
9. For lipid analyses, 1D and 2D thin layer chromatography (TLC) can be performed to separate different lipid classes and species according to their properties (see 3.2.3 and 3.2.4).

3.2.3 Thin layer chromatographic analysis of phospholipids

1. Phospholipids are separated by TLC due to different properties of their headgroups. TLC plates can be loaded with a Hamilton syringe or with a sample applicator. For the analysis of PI, PC and PE, lipids are separated by one-dimensional (1D) TLC using chloroform/methanol/25 % ammonia solution (50:25:6; per vol.) as a solvent. Separations usually take 50 min/10 cm of distance on TLC plates.
2. A better separation of phospholipids can be achieved by 2D TLC. The sample is applied as single spot to a TLC plate approximately 1-1.5 cm distant from the corner. For the first dimension, chloroform/methanol/25 % ammonia (65:35:5; per vol.) is used as a solvent, and for the second dimension chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.).
3. Phospholipids are visualized by staining with iodine vapor in a saturated chamber for some minutes. For destaining TLC plates are incubated in a heating chamber for a few minutes.
4. 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.
5. The lipid phosphorus of the respective spot can be measured by subjecting the sample to hydrolysis. Therefore, 0.2 ml of conc. H_2SO_4/72 % $HClO_4$ (9:1; v/v) is 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!
6. Samples are cooled to RT, and 4.8 ml of freshly prepared 0.26 % ammonium molybdate/ANSA (500:22; v/v) is added. ANSA consists of 40.0 g $K_2S_2O_5$, 0.63 g 8-

anilio-1-naphthalenesulfonic acid and 1.25 g Na_2SO_3 in 250 ml water. Tubes are sealed with phosphate free glass caps and after vigorous vortexing samples are heated to 100 °C for 30 min in a heating chamber.

7. Finally, samples are cooled to RT and shortly centrifuged in a table top centrifuge at 1,000 x g to sediment the silica gel. The intensity of the blue color is a measure for the 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. Data are calculated from a standard curve using inorganic phosphate at known amounts.

3.2.4 Thin layer chromatographic analysis of neutral lipids

1. Neutral (non-polar) lipids are extracted as described above (3.2.2). The separation of different classes of neutral lipids can be performed by TLC. For the direct densitometric quantification of lipids on the TLC plate authentic standards are used containing defined amounts of the respective lipids.
2. Dried lipid extracts are dissolved in an appropriate volume of $CHCl_3$/MeOH (2:1; v/v) and spotted onto a TLC plate with a Hamilton syringe or a TLC sample applicator (see above).
3. For a most efficient TLC separation of neutral lipids a two step separation system is recommended. Initially, lipids are separated on a 10 cm TLC plate in an ascending manner using light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) as a solvent until the front has reached two thirds of the plate's height. Then, separation of lipids is continued in the same direction using light petroleum/diethyl ether (49:1; v/v) until the solvent front reaches the top of the plate.
4. After the TLC plate has been dried, sterols can be quantified densitometrically using a TLC scanner (Shimadzu chromatoscanner CS-930) at 275 nm. Longer exposure of the TLC plate to light and oxygen causes oxidation of sterols which should be avoided.
5. Other neutral lipids are irreversibly stained by charring prior to scanning. For this purpose, the TLC plate is incubated approximately 15 sec in a solution of 0.63 g $MnCl_2 \cdot 4H_2O$, 60 ml water, 60 ml methanol, 4 ml conc. sulfuric acid and stained in a heating chamber at 100°C for 30 min. The staining intensity depends on the incubation time. The scanning procedure should be performed directly after charring as the intensity of the spots is fading with time (also see Note 6).

6. Spot of neutral lipids visualized as described above are scanned at a wavelength of 400 nm, and spot areas are quantified relative to standards.

3.2.5 Gas liquid chromatographic analysis of fatty acids

1. Fatty acids are analyzed by gas liquid chromatography after hydrolysis and conversion to methyl esters.
2. For this purpose, lipid extracts (see 3.2.2) are treated with BF_3-methanol as described in the literature (Morrison W.R. and Smith L.M., 1964).
3. In detail, aliquots of lipid extracts (0.1-1 ml) are transferred to glass tubes and dried under a stream of nitrogen. After addition of 1 ml BF_3-methanol, samples are heated to 95°C in a sand bath for 10 min and then cooled to RT. 0.86 ml benzene are added and tubes are heated again in the sand bath at 95 °C for 30 min.
4. After cooling to RT, 1 ml of H_2Odd and 3 ml of light petroleum are added, and samples are vortexed for 30 min. Then, samples are centrifuged at 2,500 rpm for 2 min in a table top centrifuge (Hettich Universal 16), and the upper phase is transferred to a new Pyrex tube. The lower phase is extracted again as described above for 30 min with vortexing using 3 ml of light petroleum. After centrifugation the organic phases are combined and dried under a stream of nitrogen.
5. Then, samples are dissolved in 100 µl light petroleum and transferred to glass tubes. Finally, fatty acid methyl esters are separated by GLC using following parameters:

GLC	HP 6890
Injector mode	split
Injection volume	1 µl
Column	HP-INNOWax Polyethylene Glycol;
	15 m x 0.25 mm i.d. x 0.5 µm film thickness
Carrier	Helium, 5.0
Flow	1.4 ml linear velocity 30 cm/s constant flow
Oven	160 °C (5 min) with 7.5 °C/min to 250 °C (15 min)
Detector	FID mode: constant makeup flow (40.0 ml/ min)
	Air flow: 400.0 ml / min
	Hydrogen flow: 35.0 ml / min
	Makeup gas type: Helium

6. Fatty acids are identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN, USA).

3.2.6 Gas liquid chromatography/mass spectrometry of sterols

1. Sterol analysis is performed as described previously (Quail and Kelly, 1996).
2. A mixture of 0.6 ml methanol (Merck) and 0.4 ml 0.5 % (w/v) pyrogallol (Fluka; 4 °C freshly dissolved in methanol and 0.4 ml 60 % (w/v) aqueous KOH solution) in a Pyrex tube is prepared. Then, 5 µl of a cholesterol solution (2 mg/ml in ethanol) is added as an internal standard.
3. Samples containing approximately 0.3-0.5 mg protein are added to the mixture and tubes are heated in a water bath at 90 °C for 2 h.
4. Lipids are extracted with 1 ml n-heptane. The upper phase is transferred into a fresh tube, and the lower phase is re-extracted twice. Combined upper phases are dried under a stream of nitrogen, and lipids are dissolved in 10 µl pyridine.
5. Samples are treated immediately prior to analysis with 10 µl N'O'-bis (trimethylsilyl)-trifluoracetamide (SIGMA), incubated at RT for 10 min and diluted with 30 µl ethyl acetate.
6. GLC-MS analysis is carried out using the following parameters:

GLC	HP 5890 SeriesII Plus with Electonic Pressure Control and 6890 automated liquid sampler (ALS)
Injector	Split/splitless 270 °C, mode: splitless. Purge on: 2 min
Injector volume	1 µl
Column	HP 5-MS (Crosslinked 5 % Phenyl Methyl Siloxane); 30 m x 0.25 mm i.d. x 0.25 µm film thickness
Carrier	Helium, 5.0
Flow	0.9 ml linear velocity 35.4 cm/s, constant flow
Oven	100 °C (1 min) with 10 °C/min to 250 °C (0 min) and with 3 °C/min to 310 °C (0 min)
Detector	Selective Detector HP 5972 MSD
Ionization	EI, 70 eV
Mode	Scan, scan range: 200-550 amu, 3.27 scans/s
EM Voltage	Tune Voltage
Tune	Auto Tune

7. Sterols are identified according to their retention time and mass fragmentation pattern using MSD ChemStation, D.03.00.552.

3.2.7 Mass spectrometry of neutral lipids and phospholipids

1. Lipids extracts are prepared as described above and diluted 1:100 in acetonitrile/2-propanol (5:2; v/v), 1 % ammonium acetate, 0.1 % formic acid.
2. 5 µM TAG (species 51:0) and PC (species 24:0) are added as internal standards.
3. For chromatographic separation a thermo hypersil GOLD C18, 100 x 1mm, 1.9 µm column is used with solvent A (water with 1 % ammonium acetate, 0.1 % formic acid) and solvent B (acetonitrile/2-propanol, 5:2, v/v; 1 % ammonium acetate; 0.1 % formic acid).
4. Gradients are established from 35 % to 70 % B for 4 min and then to 100 % B in another step of 16 min. These conditions are held for 10 min with a flow rate of 250 µl/min.
5. Mass spectrometry is performed with the following parameters:

MS	LTQ-FT coupled to and Accela UPLC
Data acquisition	FT-MS full scan at a resolution of 100 k and < 2 ppm mass accuracy with external calibration
Spray voltage	5000 V
Capillary voltage	35 V
Tube lens	120 V
Capillary temperature	250 °C

6. Peak areas are calculated by QuanBrowser for all lipid species identified previously by exact mass (< 2 ppm) and retention time. Calculated peak areas for each species are expressed as % of the sum of all peak areas in the respective lipid class.

4. Results

1. Fig. 1A shows a typical SDS-PAGE analysis of a cell fractionation experiment using the yeast wild type strain BY4741. LP proteins occur at low abundance in the cell, but comprise a distinct set of polypeptides (Leber *et al.*, 1994).

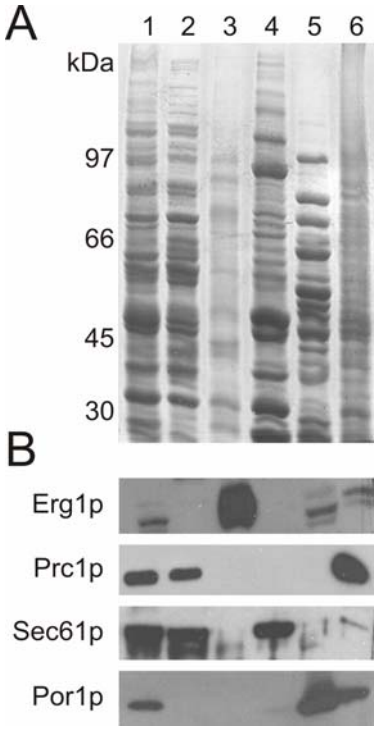


Figure 1: Quality control of lipid particles

(A) Protein pattern of homogenate (lane 1), cytosol (lane 2), lipid particles (lane 3), endoplasmic reticulum (lane 4), mitochondria (lane 5) and vacuoles (lane 6). Proteins were separated on a 12.5 % SDS gel as described in the Methods section.

(B) For Western blot analysis, 15 µg protein from each fraction were separated by electrophoresis, blotted, and probed with the respective antibodies.

2. The purity of LP preparation can be tested by Western blot analysis (Fig. 1B) using marker antibodies as described in Table 1. The enrichment of LP can be verified best with anti-Erg1p antibody (Fig. 1B, line 1). Contamination of LP with other organelles such as mitochondria (Por1p), ER (Sec61p) or vacuole (Prc1p) can be largely excluded since signals with the respective antibodies in the LP fraction are negligible.
3. Phospholipid analysis of LP has been described previously in the literature (Leber *et al.*, 1994). In brief, total phospholipids from LP contained approximately 36 % PC, 20 % PE, 32 % PI, 4 % DMPE and about 3 % PA. To identify species (fatty acid composition) of the major phospholipids from LP, MS analyses can be performed.

Fig. 2 shows an example of PC and PE species analysis from LP isolated from wild type yeast grown on YPD (glucose) or YPO (oleic acid). PC was found to contain primarily C16:1/C16:1 and C16:1/C18:1 fatty acids when cells were grown on glucose as carbon source (Fig. 2A). PC from cells grown on oleate contains mainly C18:1/C18:1 with only minor amounts of C18:1/C16:1. The PE species C16:1/C18:1 and C16:0/C18:1 or C16:1/C18:0 were predominant when cells were grown on YPD (Fig. 2B). PE with C18:1/C18:1 was the major species when cells were cultivated on oleic acid (YPO).

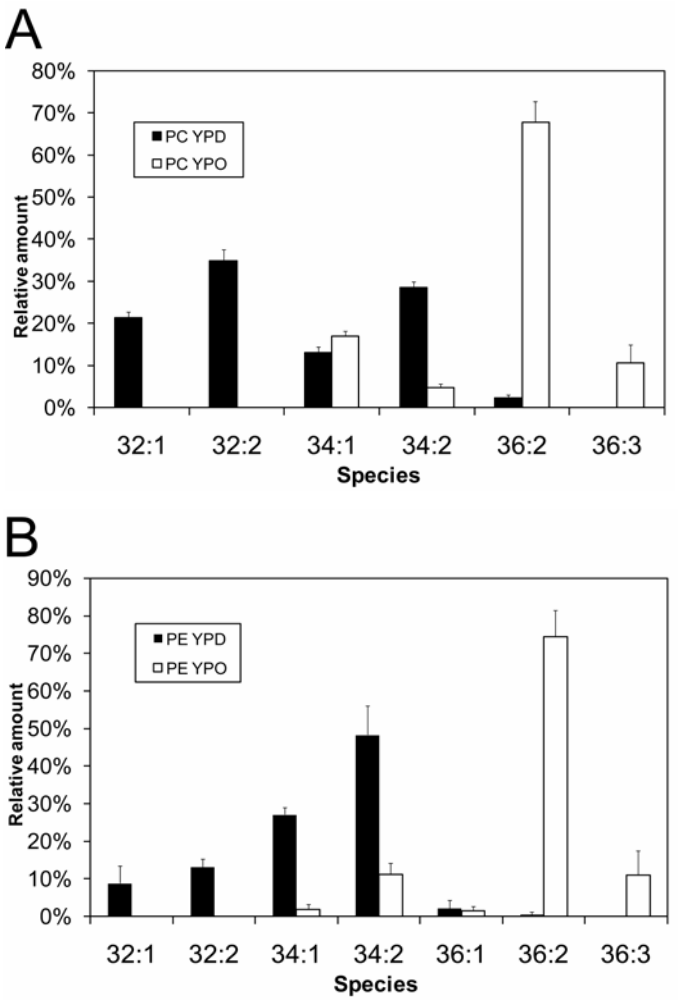


Figure 2: Mass spectrometric analysis of phospholipids from lipid particles

Phosphatidylcholine (A) and phosphatidylethanolamine (B) species were analyzed by MS as described in the Methods section. LP were isolated from cells grown on YPD (black bars) and YPO (white bars). Species are formed as follows: 32:1 (16:1/16:0); 32:2 (16:1/16:1); 34:1 (16:1/18:0) or (16:0/18:1); 34:2 (16:1/18:1); 36:2 (18:1/18:1); 36:3 (18:1/18:2) or (18:0/18:3).

4. Neutral lipids of LP were analyzed by TLC and identified by comparison to standard mixtures (Fig. 3A). As cholesteryl ester was used as standard for steryl esters the R_f-value is slightly higher (Fig. 3A, lanes 1 and 2) than for fatty acyl esters of ergosterol and precursors (lanes 3 and 4). The neutral lipid pattern of LP changes depending on the carbon source used for the cultivation of cells. LP from cells grown on glucose contain large amounts of STE and approximately equal amounts of TAG, whereas LP from cells grown on oleic acid as carbon source mostly accumulate this fatty acid in TAG. Other non-polar lipids are missing in wild type LP when cells are grown under standard conditions. The amounts of the different neutral lipids were quantified by densitometric scanning, and relative amounts (µg lipid/µg protein) were calculated. LP from cells grown on YPD contain approximately 47.2 % STE and 52.8 % TAG, whereas LP from cells grown on YPO have 0.2 % STE and 99.8 % TAG (our own unpublished data).
5. TAG species analysis of the neutral lipid fraction from cells grown on YPD or YPO, respectively, is shown in Fig. 3B. TAG from cells grown on YPD mainly contain C16:1/C16:1/C18:1 (50:3 species) and C18:1/C18:1/C16:1 (52:3 species), whereas the majority of TAG from cells grown on YPO is C18:1/C18:1/C18:1 (54:3 species). In general, most of the yeast TAG contains monounsaturated fatty acids. Polyunsaturated acyl chains were only found in TAG from cells grown on YPO, e.g. C18:1/C18:1/C18:2 (54:4 species) due to the presence of polyunsaturated fatty acids as impurity in the oleic acid used a carbon source.
6. Isolated LP were analyzed for sterol composition as described previously (Czabany *et al.*, 2008). In brief, ergosterol was found to be the major sterol with approximately 70 % of total sterols followed by the precursors zymosterol (10 %), fecosterol (~7 %) and episterol (~7 %).
7. Fatty acid analysis of yeast neutral lipids has also been reported recently (Czabany *et al.*, 2008). It was found that TAG as well as STE contain mostly C18:1 (40-50 %) followed by C16:1 (~35 %) and only minor amounts of C16:0 and C18:0.

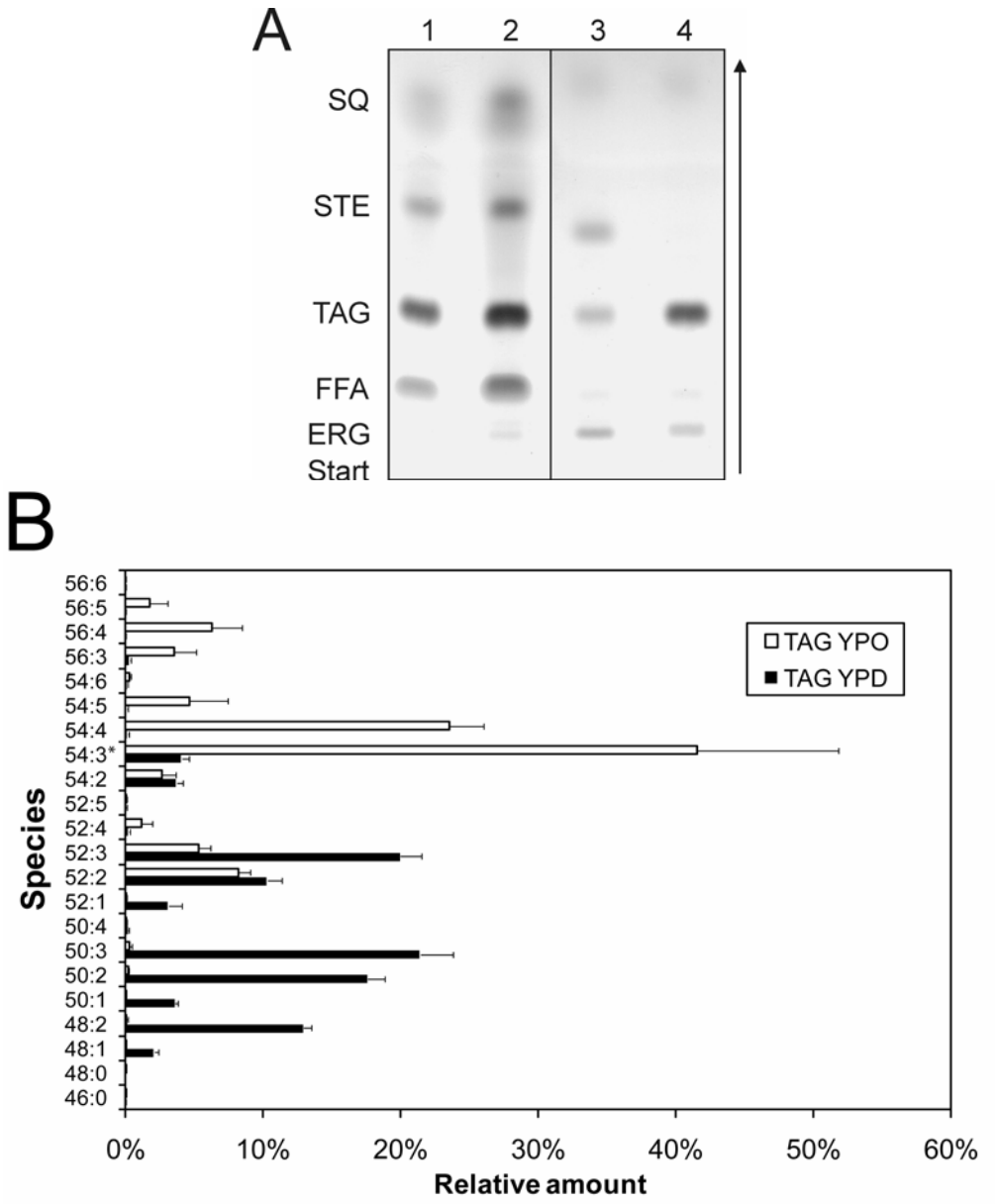


Figure 3: Neutral lipids of lipid particles from wild type yeast cells grown on different carbon sources

(A) Neutral lipids were separated by TLC in a two-step procedure using light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) as the first solvent approximately to one half of the distance on the plate, and light petroleum/diethyl ether (49:1; v/v) as the second solvent (see Methods section). The arrow indicates the ascending mode of TLC. Different neutral lipid classes are indicated at the left. SQ: squalene; STE: steryl esters; TAG: triacylglycerols; FFA: free fatty acids; ERG: ergosterol. 1 and 2: standard mixtures containing the different neutral lipid classes; 3: lipid pattern of LP from cells grown on glucose; 4: lipid pattern of LP from cells grown on oleic acid.

(B) Mass spectrometric analysis of TAG from LP of cells grown on YPD (black bars) or YPO (white bars), respectively. The relative amount of the signal for the different species is shown. The asterisk indicates three C18:1 chains in a TAG molecule.

5. Notes

1. Delipidation of LP samples prior to TCA precipitation of proteins can be omitted, but disturbing effects during SDS-PAGE may be observed. Washing the precipitated protein pellet with cold acetone helps to circumvent this negative effect and is recommended.
2. If the purity of a LP preparation does not show the desired quality due to contamination with other cellular membranes, a further purification step can be added to the standard protocol. For this purpose, LP preparations are treated with 4.5 M urea. After 15 min of incubation at RT, the last floating centrifugation step of the standard procedure is repeated, and highly purified LP can be collected from the top of the gradient.
3. When highly concentrated LP samples are needed, one further floating centrifugation step using a table top centrifuge at maximum speed for app. 15-30 min can be performed. The excess amount of buffer under the LP layer at the top can be removed using a syringe.
4. Induction of LP proliferation with oleic acid increases the yield of LP markedly but at the same time alters the lipid composition dramatically as shown in the previous section.
5. If a yeast strain needs to be cultured on selective minimal media the yield of LP may be extremely low. Large culture volumes may be required to obtain LP at substantial quantities.
6. Neutral lipid analysis by densitometric measurement strongly depends on the intensity of the band color. Therefore, it is necessary to routinely compare bands to a standard loaded onto the same TLC plate. STE are stained more intensively than TAG by the charring method which has to be taken into account when using standards. Moreover, bands should not be too broad to avoid problems during the scanning process.

6. Acknowledgements

Work on yeast non-polar lipid metabolism in our laboratory was recently supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (projects 15141, 18857 and W901-B05 to G.D.).

Reference List

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

Athenstaedt,K., Zweytick,D., Jandrositz,A., Kohlwein,S.D., and Daum,G. (1999). Identification and characterization of major lipid particle proteins of the yeast Saccharomyces cerevisiae. J. Bacteriol. *181*, 6441-6448.

Blanchette-Mackie,E.J., Dwyer,N.K., Barber,T., Coxey,R.A., Takeda,T., Rondinone,C.M., Theodorakis,J.L., Greenberg,A.S., and Londos,C. (1995). Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. J. Lipid Res. *36*, 1211-1226.

Czabany,T., Athenstaedt,K., and Daum,G. (2007). Synthesis, storage and degradation of neutral lipids in yeast. Biochim. Biophys. Acta *1771*, 299-309.

Czabany,T., Wagner,A., Zweytick,D., Lohner,K., Leitner,E., Ingolic,E., and Daum,G. (2008). Structural and Biochemical Properties of Lipid Particles from the Yeast Saccharomyces cerevisiae. J. Biol. Chem. *283*, 17065-17074.

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

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

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-5.

Leber,R., Zinser,E., Zellnig,G., Paltauf,F., and Daum,G. (1994). Characterization of lipid particles of the yeast, Saccharomyces cerevisiae. Yeast *10*, 1421-1428.

Lowry,O.H., Rosebrough,N.J., Farr A.L., and Randall R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

Morrison W.R. and Smith L.M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride--methanol. J. Lipid Res. *5*, 600-608.

Quail,M.A. and Kelly,S.L. (1996). The extraction and analysis of sterols from yeast. Methods Mol. Biol. *53*, 123-131.

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

Robenek,H., Hofnagel,O., Buers,I., Robenek,M.J., Troyer,D., and Severs,N.J. (2006). Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis. J. Cell Sci. *119*, 4215-4224.

Sandager,L., Gustavsson,M.H., Stahl,U., Dahlqvist,A., Wiberg,E., Banas,A., Lenman,M., Ronne,H., and Stymne,S. (2002). Storage Lipid Synthesis Is Non-essential in Yeast. J. Biol. Chem. *277*, 6478-6482.

Scow,R.O., Blanchette-Mackie,E.J., and Smith,L.C. (1980). Transport of lipid across capillary endothelium. Fed. Proc. *39*, 2610-2617.

Zweytick,D., Athenstaedt,K., and Daum,G. (2000). Intracellular lipid particles of eukaryotic cells. Biochim. Biophys. Acta *1469*, 101-120.

CHAPTER 4

OLEATE INHIBITS STERYL ESTER SYNTHESIS AND CAUSES LIPOSENSITIVITY IN THE YEAST

Melanie Connerth[1], Tibor Czabany[1], Andrea Wagner[2], Günther Zellnig[3], Erich Leitner[4], Ernst Steyrer[2] and Günther Daum[1]

[1]Institute of Biochemistry, Graz University of Technology, Graz, Austria
[2]Institute of Molecular Biology and Biochemistry, Medical University of Graz
[3]Institute of Plant Sciences, Karl Franzens University Graz
[4]Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology

Running Title: Liposensitivity in yeast

Keywords: Lipid droplet, steryl ester synthase, oleic acid, ER, triacylglycerol, yeast

Abbreviations: CL, cardiolipin; DAG, diacylglycerol; ER, endoplasmic reticulum; ERG, ergosterol; FFA, free fatty acid; LEM, large ER membrane(s); LP, lipid particle/droplet; LPL, lysophospholipid(s); OAEE, oleic acid ethyl ester; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; RT, room temperature; SE, steryl ester; TAG, triacylglycerol; TM, triple mutant; QM, quadruple mutant

Journal of Biological Chemistry, manuscript under revision

Abstract

In the yeast *Saccharomyces cerevisiae*, neutral lipids can be synthesized by four acyltransferases, namely Dga1p and Lro1p producing triacylglycerols (TAG) and Are1p and Are2p forming steryl esters (SE). TAG and SE are stored in an organelle called lipid particle/droplet. Growth of yeast cells on oleic acid supplemented media strongly induced proliferation of lipid particles and specifically the synthesis of TAG which serve as the major pool for the excess of fatty acids. Surprisingly, SE synthesis was strongly inhibited under these conditions. Here, we show that this effect was not due to decreased expression of *ARE2* encoding the major yeast SE synthase at the transcriptional level, but to competitive enzymatic inhibition of Are2p by free oleic acid. Consequently, a triple mutant *dga1*Δ*lro1*Δ*are1*Δ*ARE2*$^{+}$ grown on oleate did not form substantial amounts of SE and exhibited a growth phenotype similar to the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant including lack of lipid particles. Growth of these mutants on oleate was strongly delayed and cell viability was decreased, but rescued by adaptation. In these strains, oleate stress caused morphological changes of intracellular membranes, altered phospholipid composition and increased formation of ethyl esters as a possible buffer for fatty acids. In summary, our data showed that exposure to oleate led to disturbed lipid and membrane homeostasis along with liposensitivity of the yeast.

Introduction

Fatty acids are essential molecules for all types of cells. They are required as building blocks for the synthesis of complex lipids and thus for membrane assembly. Fatty acids undergo permanent turnover in cellular processes located to different organelle fractions. Exogenous free fatty acids (FFA) are taken up by the yeast cell and activated to acyl-CoA. These activated fatty acids enter metabolic processes such as β-oxidation in the peroxisomes or synthesis of membrane forming and storage lipids. Phospholipids are embedded in cellular membranes whereas neutral lipids such as triacylglycerols (TAG) and steryl esters (SE) are stored in lipid particles/droplets (LP) (Leber *et al.*, 1994; Athenstaedt *et al.*, 1999; Czabany *et al.*, 2008). Storage of non-polar lipids seems to play a major role when cells are exposed to high levels of exogenous FFA. Under these conditions, LP increase in number and size indicating that LP proliferation is enhanced to prevent accumulation of FFA in the cell (Rosenberger *et al.*, 2009). In the yeast *Saccharomyces cerevisiae* as well as in other eukaryotic cells accumulation of FFA was claimed to be toxic for the cell and thus should be avoided (Lockshon *et al.*, 2007; Petschnigg *et al.*, 2009).

Recently, a detailed structural investigation of LP from the yeast revealed that TAG are stored in the core of this organelle being surrounded by several layers of SE (Czabany *et al.*, 2008). In contrast to all other organelles, LP do not possess a phospholipid bilayer but are covered by a surface phospholipid monolayer. The biosynthesis of LP is still a matter of debate but generally a budding model is well accepted (Czabany *et al.*, 2007; Rajakumari *et al.*, 2008; Walther and Farese, Jr., 2009). This model presumes that neutral lipids synthesized in the endoplasmic reticulum (ER) are concentrated in domains between the two leaflets of the bilayer membrane which then leads to formation of a nascent LP.

Enzymes contributing to the synthesis of neutral lipids play a key role in LP biosynthesis (Sandager *et al.*, 2002; Sorger and Daum, 2002; Czabany *et al.*, 2008). Four enzymes are responsible for the synthesis of neutral lipids in the yeast. Dga1p and Lro1p esterify DAG to TAG using either acyl-CoA or phospholipids as acyl donors (Yang *et al.*, 1996; Dahlqvist *et al.*, 2000; Oelkers *et al.*, 2002). SE can be formed by Are1p and Are2p from sterols and acyl-CoA. Additionally, Are-proteins have a minor capacity to form TAG. All four enzymes are localized to the ER supporting the theory that LP biogenesis is initiated in the ER. A substantial portion of Dga1p, however, was also found in LP (Sorger and Daum, 2002) indicating that ER and LP are related organelles.

Little is known about the regulation of neutral lipid synthesis. An obvious requirement is that synthesis and mobilization of these components must not result in a futile cycle. Whereas regulation of Dga1p and Lro1p has not been studied in detail, Valachovic *et al.* (Valachovic *et al.*, 2001) reported that expression of *ARE1* and *ARE2* is heme dependent. Are2p is the major SE synthesizing enzyme under aerobic conditions while Are1p is upregulated in heme-deficient or anaerobic yeast cells. Moreover, Are2p esterifies mainly ergosterol whereas Are1p also accepts ergosterol precursors as adequate substrates (Zweytick *et al.*, 2000). These findings are in line with the fact that ergosterol is a major sterol under aerobic condition whereas heme-deficient cells are unable of ergosterol synthesis and accumulate ergosterol precursors and/or take up sterols from the environment (Valachovic *et al.*, 2001; Valachovic *et al.*, 2002).

A *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant (QM) strain lacking all four enzymes for neutral lipid synthesis does not form LP but is able to grow on glucose (Sandager *et al.*, 2002) thus indicating that LP are dispensable for yeast survival under normal growth conditions. However, it was reported that QM cells die upon exposure to exogenous unsaturated fatty acids, e.g., oleic acid (Lockshon *et al.*, 2007; Petschnigg *et al.*, 2009). This lipotoxic effect was ascribed to increased permeability of the plasma membrane by excess incorporation of oleic acid into phospholipids. Although growth defects of different mutant strains on oleic acid containing medium were shown, a solid proof for the increased membrane permeability is missing.

In this study we provide new evidence for oleate sensitivity of yeast cells. Our data support the view that TAG formation serves as the major buffer for a surplus of oleic acid. In contrast to other studies, however, we found that TAG was not essential for cell survival. We report that strains deficient in TAG or SE synthesis were able to tolerate high amounts of exogenous free fatty acid especially when adapted to these growth conditions. Most interestingly, we also show that SE formation was strongly decreased under oleate stress and thus identified a direct link of FFA accumulation and SE synthase activity. Our finding that Are2p was inhibited by oleate at the enzyme level provides novel molecular insight into yeast liposensitivity caused by fatty acids.

Material and Methods

Yeast strains and growth conditions

Strains used in this study are listed in Table 1. Yeast cells were grown in YPD media containing 1% yeast extract, 2% peptone and 2 % glucose. YPO media contained 0.3% yeast extract, 0.5% peptone, 0.5% KH_2PO_4, 0.1% glucose, 0.1% oleic acid and 0.2% Tween 80 if not stated otherwise. Cells were pre-grown in YPD for 48 h at 30 °C with shaking and used for inoculating YPO media to an A_{600} of 0.1.

For cell viability test, cells from a 48 h culture on YPD were inoculated to YPO to an A_{600} of 0.1. At time points indicated, cells were counted in a Bürker chamber, 100 cells were plated on YPD agar and incubated at 30 °C for 2 days. Then, formation of colonies was determined.

Table 1: Strains used in this study

Strain	Genotype	Source
W303 (wild type)	*SUG2 GAL$^+$ mal mel ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3-1*	ScanBi Ltd., Alnarp, Sweden (Sandager *et al.*, 2002)
TM DGA1	*W303; MATα lro1::TRP1 are1::HIS3 are2::LEU2 ADE2 met ura3*	ScanBi Ltd., Alnarp, Sweden (Sandager *et al.*, 2002)
TM LRO1	*W303; MATα dga1::kanMX4 are1::HIS3 are2::LEU2 ADE2 met trp1 ura3*	ScanBi Ltd., Alnarp, Sweden (Sandager *et al.*, 2002)
TM ARE1	*W303; MATα dga1::kanMX4 lro1::TRP1 are2::LEU2 ADE2 met his3 ura3*	ScanBi Ltd., Alnarp, Sweden (Sandager *et al.*, 2002)
TM ARE2	*W303; MATα dga1::kanMX4 lro1::TRP1 are1::HIS3 ADE2 met leu2 ura3*	ScanBi Ltd., Alnarp, Sweden (Sandager *et al.*, 2002)
QM	*W303; MATα dga1::kanMX4 lro1::TRP1 are1::HIS3 are2::LEU2 ADE2 met ura3*	ScanBi Ltd., Alnarp, Sweden (Sandager *et al.*, 2002)

Cell fractionation

Cells were grown to the early stationary phase in liquid media and harvested in a Sorvall SLC3000 rotor at 4,200 x g for 5 min. Cells were washed twice in double dist. water and the cell wet weight (CWW) was determined. Then, cells (0.5 g CWW/ml) were incubated with dithiothreitol (DTT; 0.66 mg/ml) in buffer A (0.1 M Tris/SO_4, pH 9.4) for 20 min at 30 °C with shaking. Cells were washed once in buffer C (1.2 M sorbitol), resuspended in buffer B (1.2 M sorbitol, 20 mM KH_2PO_4, pH 7.4) (0.15 g CWW/ml) and incubated with Zymolyase-20T (Seikagaku Corporation, Japan) at a concentration of 2 mg enzyme per g CWW for at

least 1 h at 30 °C with shaking. The resulting spheroplasts were washed twice in buffer C and resuspended in buffer D (5 mM MES-KOH, pH 6.0, 0.6 M sorbitol, 1 mM KCl, 0.5 mM EDTA) followed by mechanical disruption in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; 1 mM final concentration) with 15 strokes using a 30 ml Dounce Homogenizer with a loose fitting pestle. The homogenate was centrifuged to remove cell debris in a Sorvall SS34 rotor at 3,000 x g for 5 min. Supernatants were collected and the homogenization process was repeated twice with the pellets. Combined supernatants were centrifuged at 27,000 x g for 15 min in an SS34 (Sorvall) rotor to sediment mitochondria and peroxisomes. The supernatant was transferred to fresh tubes and centrifuged at 40,000 x g for 30 min. The pellet contained microsomal fractions which were resuspended in 10 mM TrisCl, pH 7.4.

Isolation of RNA and RT-PCR analyses

Total RNA from cultivated cells was isolated by RNeasy kit (QIAGEN). Reverse transcription was carried out with 1.5-3 µg of RNA after DNAse-I treatment (1 unit RQ1, Promega) in a total volume of 15 µl reverse transcriptase reaction buffer (15 min at 37 °C). Reverse transcriptase from Invitrogen was used, according to the manufacturer's protocol. Primers for cDNA amplification were used as follows: Are2 For 5´ATG GAC AAG AAG AAG GAT CTA CTG G3´, Are2 Rev 5´TTT CCC TAC TGA GTC GAC AGC ATC T3´, Act1 For 5´GGT CCC AAT TGC TCG AGA GAT3´, Act1 Rev 5´GAA GTC CAA GGC GAC GTA ACA3´.

Electron microscopy

Yeast cells were prepared for electron microscopy as described before (Rosenberger *et al.*, 2009). In brief, cells were grown at 30 °C under aerobic conditions on YPO to the early stationary phase. Cells were harvested by centrifugation and washed twice with 0.5 % fatty acid free BSA and 3 times with double dist. water. Then, cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with double dist. water and fixed in a 1 % aqueous solution of $KMnO_4$ for 20 min. Fixed cells were washed three times in dist. water and incubated in 0.5 % aqueous uranylacetate overnight at 4 °C. Then, samples were dehydrated in a graded series of acetone (50, 70, 90 and 100 %) and embedded in an acetone:Agar 100 (Gröpl, Austria) mixture (1:1) for 5 h, then in an acetone:Agar 100 mixture (1:3) over night at room temperature and finally polymerized in pure Agar 100 resin for 48 h

at 60 °C. Ultrathin sections of 80 nm were stained with lead citrate and viewed with a Philips CM 10 electron microscope.

Lipid extraction

Lipids were extracted by the method of Folch *et al.* (Folch J. *et al.*, 1957). In brief, extraction of total lipids from cells was performed in the presence of 3 ml $CHCl_3$:MeOH (2:1; v/v) in a Pyrex glass tube by vortexing at room temperature for 1 h. Proteins and non-polar substances were removed by consecutive washing steps with 0.2 volumes of 0.034 % $MgCl_2$, 1 ml 2 N KCl/MeOH (4:1; v/v) and 1 ml of $CHCl_3$:MeOH:H_2O (3:48:47; per vol.). After centrifugation for 3 min at 3,000 rpm in a table-top centrifuge, the aqueous phase was removed by aspiration. Finally, lipids were dried under a stream of nitrogen and stored at -20 °C.

Analysis of phospholipids

The different classes of phospholipids were separated on a 10x10 cm Silica TLC plate using chloroform/methanol/25 % ammonia (65:35:5; per vol.) as a developing solvent for the first dimension and chloroform/ acetone/ methanol/ acetic acid/ water (50:20:10:10:5; per vol.) for the second dimension. Spots of phospholipids were visualized by iodine vapor, scraped off and transferred to phosphate free glass tubes with ground neck. Lipid phosphorus was quantified after subjecting samples to hydrolysis (Broekhuyse, 1968). For this purpose, 0.2 ml of conc. H_2SO_4/72 % $HClO_4$ (9:1; v/v) were added to each sample. Hydrolysis was performed at 180°C in a heating block for 30 min. 4.8 ml of freshly prepared 0.26 % ammonium molybdate/ANSA (500:22; v/v) were added to cooled samples. ANSA consists of 40.0 g $K_2S_2O_5$, 0.63 g 8-anilio-1-naphthalenesulfonic acid and 1.25 g Na_2SO_3 in 250 ml water. After vigorous vortexing, samples were heated to 100 °C for 30 min in a heating chamber. Finally, after short centrifugation in a table top centrifuge at 1,000 x g for 4 min, cooled to RT samples were measured spectrophotometrically at a wavelength of 830 nm using a blank spot from the TLC plate without phospholipid as a control. For quantification of total phosphorus a standard curve was prepared using inorganic phosphate at known amounts.

Neutral lipid analysis

Dried lipid extracts were dissolved in an appropriate volume of $CHCl_3$/MeOH (2:1; v/v) and spotted onto Silica gel 60 TLC plates. A two step separation system was employed using

light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) as a solvent until the front reached two thirds of the plate's height. Then, separation of lipids was continued in the same direction using light petroleum/diethyl ether (49:1; v/v) until the solvent front reached the top of the plate. Neutral lipids were irreversibly stained by charring prior to scanning. For this purpose, the TLC plate was incubated approximately 15 sec in a solution of 0.63 g $MnCl_2 \cdot 4H_2O$, 60 ml water, 60 ml methanol, 4 ml conc. sulfuric acid and stained in a heating chamber at 100 °C for 30 min.

Fatty acid analysis

Fatty acids were analyzed by gas liquid chromatography (GLC) after hydrolysis and conversion to methyl esters. For this purpose, lipid extracts were treated with BF_3-methanol as described by Morrison and Smith (Morrison W.R. and Smith L.M., 1964). In brief, after addition of 1 ml BF_3-methanol to dried lipid extracts, samples were heated to 95 °C in a sand bath for 10 min and then cooled to room temperature. Then, 0.86 ml benzene were added and tubes were heated again in the sand bath at 95 °C for 30 min. 1 ml of double dist. water and 3 ml of light petroleum were added, and samples were vortexed for 30 min. After centrifugation at 2,500 rpm for 2 min in a table top centrifuge (Hettich Universal 16) the upper phase was transferred to a new Pyrex tube. The lower phase was extracted again as described above using 3 ml of light petroleum. After centrifugation organic phases were combined and dried under a stream of nitrogen. Then, samples were dissolved in light petroleum and transferred to glass tubes. Finally, fatty acid methyl esters were separated by GLC (HP6890) using a HP-INNOWax Polythylene Glycol column (15 m x 0.25 mm i.d. x 0.5 µm film thickness) with a temperature gradient (160 °C for 5 min with 7.5 °C/min to 250 °C for 15 min). Fatty acids were identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN, USA).

The ethyl ester of oleic acid was prepared by boiling 1 ml of oleic acid and 1 ml of pure ethanol at 80 °C in the tightly closed Pyrex tube in the presence of 50 µl H_2SO_4. Identification of the product was carried out by GLC-MS analysis.

Sterol analysis

Sterol analysis was performed as described previously (Quail and Kelly, 1996). In brief, a mixture of 0.6 ml methanol (Merck) and 0.4 ml 0.5 % (w/v) pyrogallol (Fluka) freshly dissolved in methanol and 0.4 ml 60 % (w/v) aqueous KOH solution was mixed in a Pyrex

tube with 5 µl of a cholesterol solution (2 mg/ml in ethanol) as an internal standard. Samples containing 0.3-0.5 mg protein were added to the mixture and incubated in a water bath at 90 °C for 2 h. Lipids were extracted with 1 ml n-heptane. The organic phase was transferred into a fresh tube, and the lower phase was re-extracted twice. Combined upper phases were dried under a stream of nitrogen, and lipids were dissolved in 10 µl pyridine. Samples were treated with 10 µl N'O'-bis (trimethylsilyl)-trifluoracetamide (SIGMA), incubated at room temperature for 10 min and diluted with 30 µl ethyl acetate. GLC-MS (HP 5890 SeriesII Plus) analysis was carried out using an HP 5-MS column (crosslinked 5 % phenylmethyl siloxane; 30 m x 0.25 mm i.d. x 0.25 µm film thickness) with a temperature gradient (100 °C for 1 min with 10 °C/min to 250 °C and with 3 °C/min to 310 °C). Sterols were identified according to their retention time and mass fragmentation patterns using MSD ChemStation, D.03.00.552 and the library Wiley/NIST05.

Enzyme analysis

The acyl-CoA:ergosterol acyltransferase assay was adapted from Yang *et al.* (Yang *et al.*, 1997) and Czabany *et al.* (Czabany *et al.*, 2008). The assay was performed in a final volume of 100 µl containing 6 nmol of [^{14}C]oleoyl-CoA (specific activity 58 mCi/mmol), 0.025 mM ergosterol. 0.5 mM CHAPS, 100 mM KH_2PO_4 (pH 7.4), 1 mM dithiothreitol (DTT) and 20-100 µg ER protein or 200 µg homogenate protein, respectively. To test the effect of additives on the enzyme activity, assays were performed in the presence of 1.1, 2.2 or 5.5 µM oleic acid, or phospholipids up to a maximum concentration of 96 nmol/µl. Incubations were carried out for 30 min at 30 °C or at different time points as indicated. Reactions were terminated by adding 300 µl chloroform:methanol (2:1; v/v). Lipids were extracted twice for 30 min with shaking using 300 µl chloroform:methanol (2:1; v/v), and combined organic phases were washed twice using methanol:water:chloroform (47:48:3; per vol.). The organic phase was taken to dryness under a stream of nitrogen. Then, lipids were dissolved in 30µl chloroform:methanol (2:1; v/v), separated by TLC as described above and visualized on TLC plates by iodine vapor. SE bands were scrapped off and radioactivity was measured by liquid scintillation counting using LSC Safety Cocktail (Baker, Deventer, The Netherlands) with 5 % water in the scintillation mixture.

Results

Mutants bearing defects in neutral lipid biosynthesis show distinct phenotypes on oleic acid supplemented media

In the present study, we investigated the lipotoxic stress of fatty acids on yeast cells in some detail. To create distinct stress situations we used mutant strains deficient in three or all four acyltransferases required for LP biogenesis (see Table 1) and cultivated them on media containing oleic acid as a carbon source. Surprisingly, we found that these mutant strains exhibited different growth characteristics on this media (Figure 1).

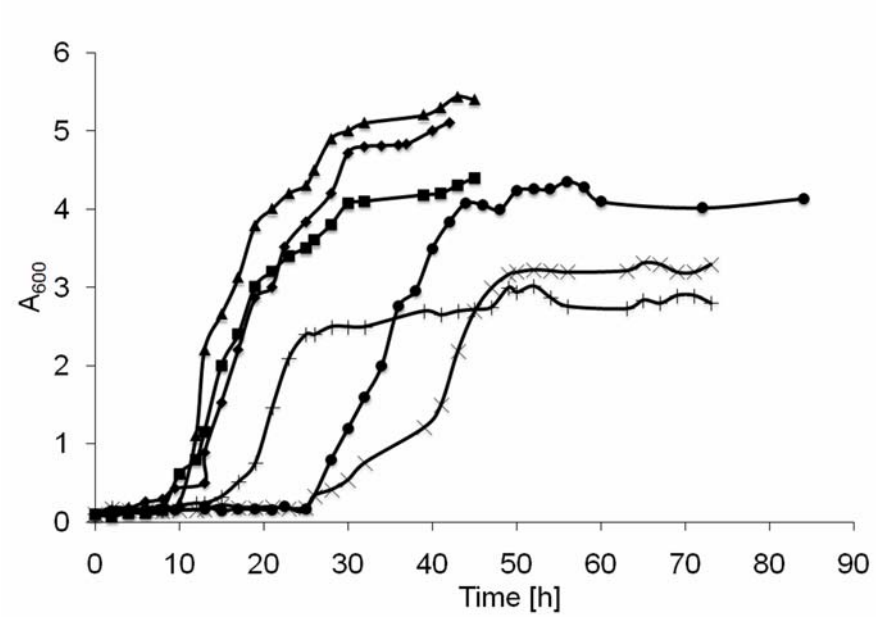


Figure 1: Yeast cells defective in neutral lipid synthesis grown on oleic acid medium

Yeast cells from a preculture grown in YPD for 48 h were used to inoculate fresh YPO media to an OD_{600} of 0.1. Cells were incubated at 30°C shaking. At time points indicated aliquots were withdrawn, cells were washed in 0.5 % fatty acid free BSA and turbidity was measured. Data shown are representative of at least three independent experiments.
♦ Wild type (W303); ▲ TM DGA1; ■ TM LRO1; X TM ARE1; + TM ARE2; ● QM

Growth rates of triple mutants with only Dga1p (TM DGA1) or Lro1p (TM LRO1) active grew similar to wild type. Growth of a strain with only Are2p (TM ARE2) active was delayed which was even more pronounced when only Are1p (TM ARE1) was functional. The latter strains showed extended lag-phases of 12 and 24 h, respectively, and grew to lower density than wild type. In contrast to previous studies (Lockshon *et al.*, 2007) we found that the QM *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ was able to grow on oleic acid containing media although with

marked delay. Recently, also other groups observed growth of QM on oleic acid supplemented media (Petschnigg *et al.*, 2009). To exclude the possibility of oleic acid decomposition during prolonged incubation as possible reason for growth retardation, we tested the fatty acid composition of media over the time period investigated. However, no changes of the fatty acid composition were observed (data not shown).

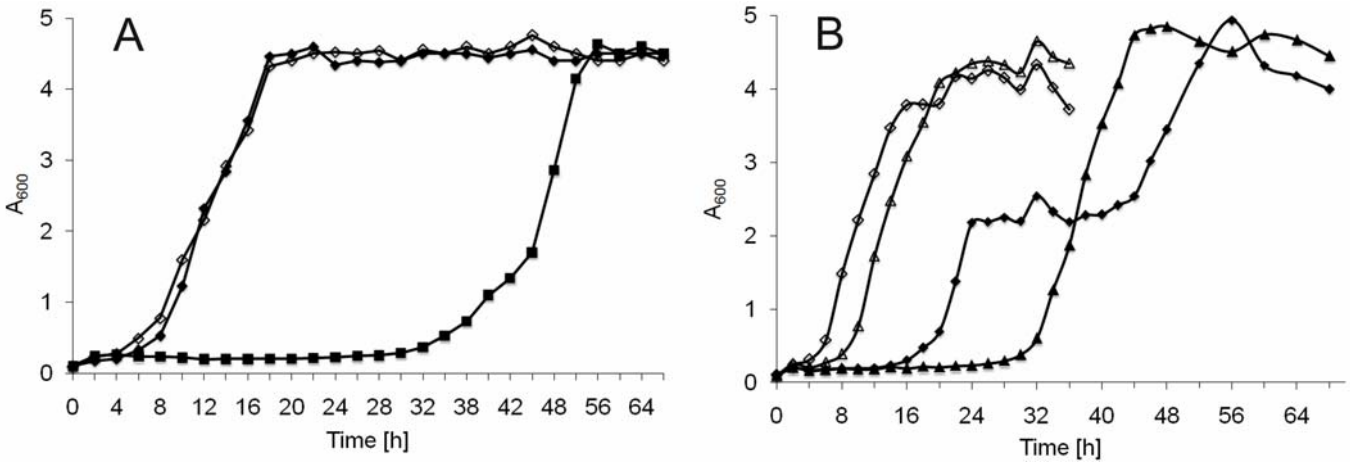


Figure 2: Growth phenotype of TAG deficient strains on oleic acid

Yeast cells from a fresh YPD plate were grown in liquid YPD to the stationary phase. Then cells were used to inoculate a liquid main culture of YPO to an OD_{600} 0.1. At time points indicated, aliquots were withdrawn, cells were washed in 0.1 % BSA and density was measured optically in a spectrophotometer. Data shown are representative of at least three independent experiments.

(A) QM: ■ YPO after growth in YPD for 48 h; ♦ YPO after growth on YPO for 48 h; ◊ YPO from YPD (48h) after pre-incubation on YPO (48h)

(B) TM ARE1 and TM ARE2: ▲ TM ARE1 YPO from YPD (48h); Δ TM ARE1 YPO from YPD after YPO (48h); ♦ TM ARE2 YPO from YPD (48h); ◊ TM ARE2 YPO from YPD after YPO (48h)

We further tested whether growth of oleate sensitive strains on oleic acid containing media might cause a “memory effect”. For this purpose we cultivated cells on oleic acid medium for 48 h and then shifted them to fresh oleic acid medium to an A_{600} 0.1. Interestingly, pre-cultivation of QM on oleic acid containing media abolished the growth delay when cells were further grown on oleate (Figure 2A). We also found that during an intermediate round of cultivation on YPD for 48 h QM did not lose its ability to grow on oleate without delay. Similar results were obtained with TM ARE1 and TM ARE2 (Figure 2B).

Interestingly, both strains recovered almost to wild type growth phenotype when pre-grown on oleic acid although to a slightly reduced A_{600}. TM ARE1 was still growing slower than TM ARE2. These surprising results indicated that an adaptive process had occurred, although the reason for this adaption is presently not known. A reversion of the genetic defect is unlikely because all mutants were deletion strains. Moreover, the neutral lipid profile verified the absence of the respective acyltransferase activity *in vivo.* We can only speculate at present that gain-of-function suppressors may have been activated.

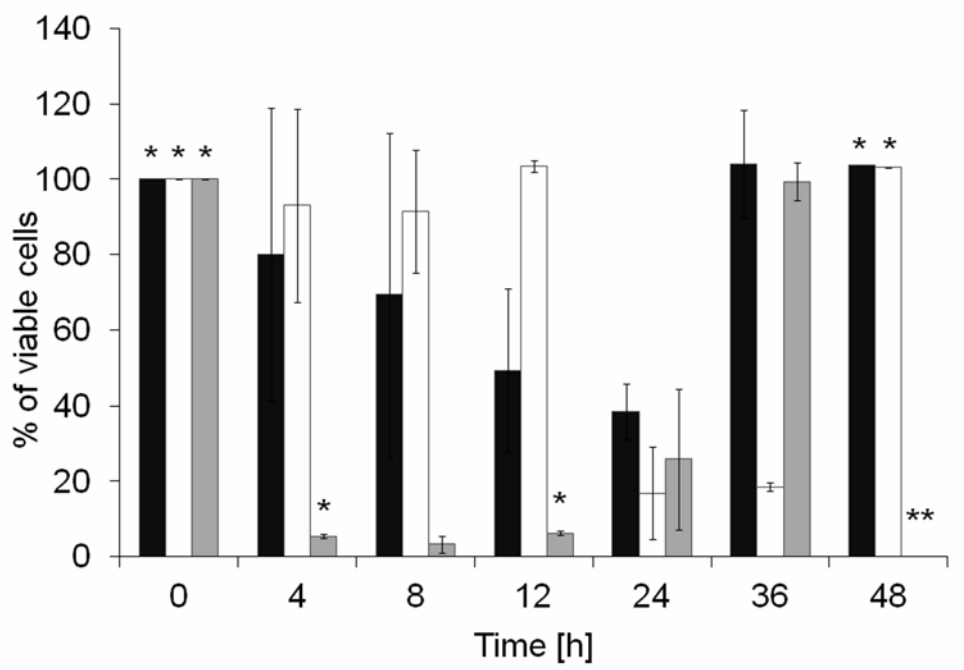


Figure 3: Cell viability on oleic acid supplemented media

Cells were grown in liquid YPO from a YPD preculture. At time points indicated, aliquots were withdrawn and cells were counted in a Bürker cell counter. Same numbers of cells were then plated on fresh YPD plates and incubated for 2 days at 30 °C. The number of colonies formed was determined indicating the rate of cell survival. Data shown was obtained from at least two independent experiments. Black bars, TM ARE1; white bars, TM ARE2; grey bars, QM; * standard deviation lower than 1 %; ** not measured

To shed more light on the survival of QM, TM ARE1 and TM ARE2 on oleate we performed viability tests. These experiments (Figure 3) revealed that most of the cells died within the first 24 hours of oleate exposure. Surprisingly, however, a small number of cells survived and continued growing. TM ARE1 showed a continuously reduced viability to 40 % within the first 24 h of oleic acid exposure. TM ARE2 was found to be stable for 12 h, but then lost viability down to 20% at time points 24 and 36 h.

TM ARE1 cultures fully recovered after 36 h, and TM ARE2 cultures after 48 h of exposure to YPO. QM lost 90 % of its viability within 4 h of growth on oleic acid. After 12 h cells started recovering and were back to 100 % viability after 36 h of growth. The different recovery periods of TMs grown on oleic acid was in line with the different lag-phases of the strains (see Figures 1 and 2).

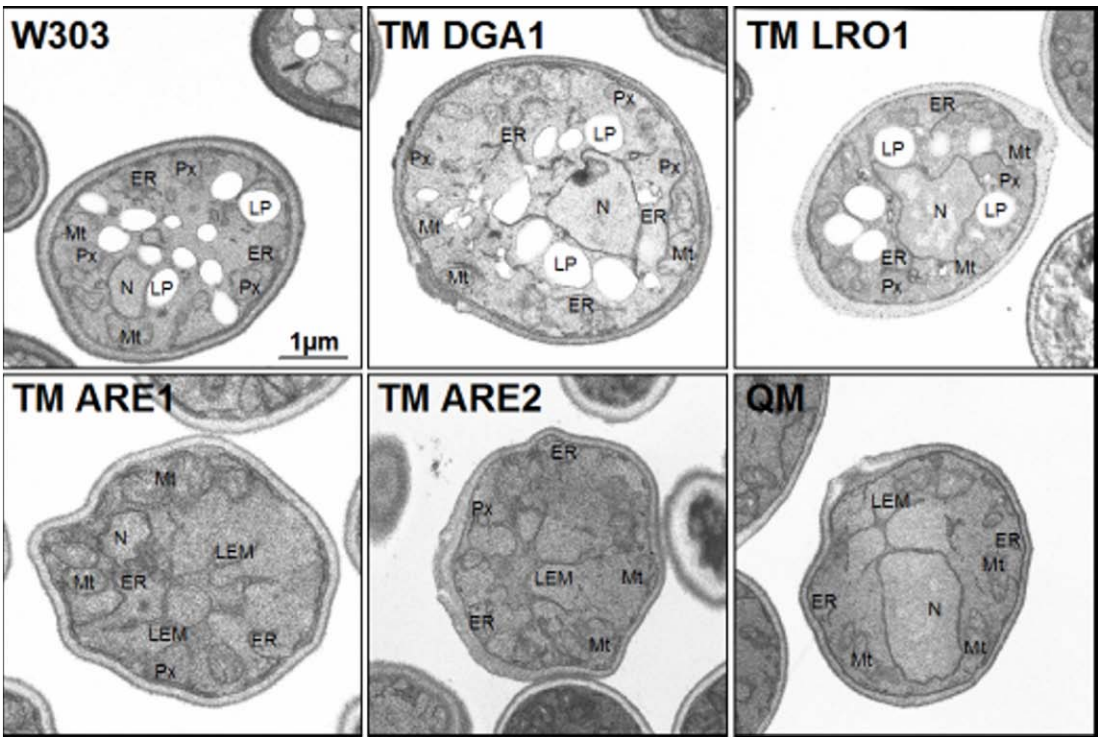


Figure 4: Electron micrographs of yeast cells grown on YPO

Cells were grown to the stationary phase in YPO liquid media. Cell fixation and preparation was performed as described in Material and Methods. Several pictures were taken from each strain and pictures shown here resemble the overall phenotype observed. N, nucleus; Px, peroxisome; Mt, mitochondria; ER, endoplasmic reticulum; LEM, large ER membrane; LP, lipid particle; V, vacuole.

To further elucidate the effect of adaptation of strains to oleic acid we inspected their cellular morphology. Strains were grown on oleate to early stationary phase and then subjected to electron microscopy. Wild type, TM DGA1 and TM LRO1 did not show morphological differences (Figure 4, upper panel). In these cells, TAG was formed and buffered the surplus of fatty acids, large LP accumulated and peroxisomes, ER and mitochondria were normally developed.

In contrast and to our surprise, TM ARE1 and TM ARE2 cells did not contain LP when grown on oleic acid but instead showed abnormal and increased ER structures (Figure 4, lower panel). We named these structures large ER membrane (LEM) which were most prominent in TM ARE2 and QM. These results were in contrast to previous findings with the same set of strains grown on glucose (Czabany *et al.*, 2008). Under these conditions LP were well developed in all TMs and cell morphology was not different from wild type. QM which lacks LP already on glucose media showed the same morphology on oleic acid.

Oleic acid changes the neutral lipid pattern of wild type and mutant strains

The striking morphological changes in mutant strains deficient in TAG biosynthesis (TM ARE1, TM ARE2 and QM) as described above led us to analyze the neutral lipid pattern of these cells grown on oleic acid compared to glucose. For this purpose lipid extracts from cells in the early stationary phase were analyzed (Figure 5). As expected, TM ARE1, TM ARE2 and QM were not able to produce significant amounts of TAG irrespective of the growth medium (Figure 5A). In contrast, massively increased amounts of TAG were found in wild type, TM DGA1 and TM LRO1 when grown on oleate. It has to be mentioned, however, that an increase in TAG was already observed when TM DGA1 and TM LRO1 were grown on glucose as a compensatory effect for the loss of SE formation.

TM ARE2, the strain with the major SE synthase active, accumulated SE when grown in YPD medium (Figure 5 B). Surprisingly, hardly any SE were detected in strains grown on YPO medium. Especially in wild type and TM ARE2 the amount of SE was dramatically decreased when grown on oleic acid compared to glucose media. Wild type grown to early stationary phase on YPD contained TAG and SE at an approx. ratio of 1:1 (see Figure 5 and ref. (Leber *et al.*, 1994). Growth on YPO dramatically shifted the TAG to SE to 40:1 indicating that TAG served as the major buffer for excess fatty acids.

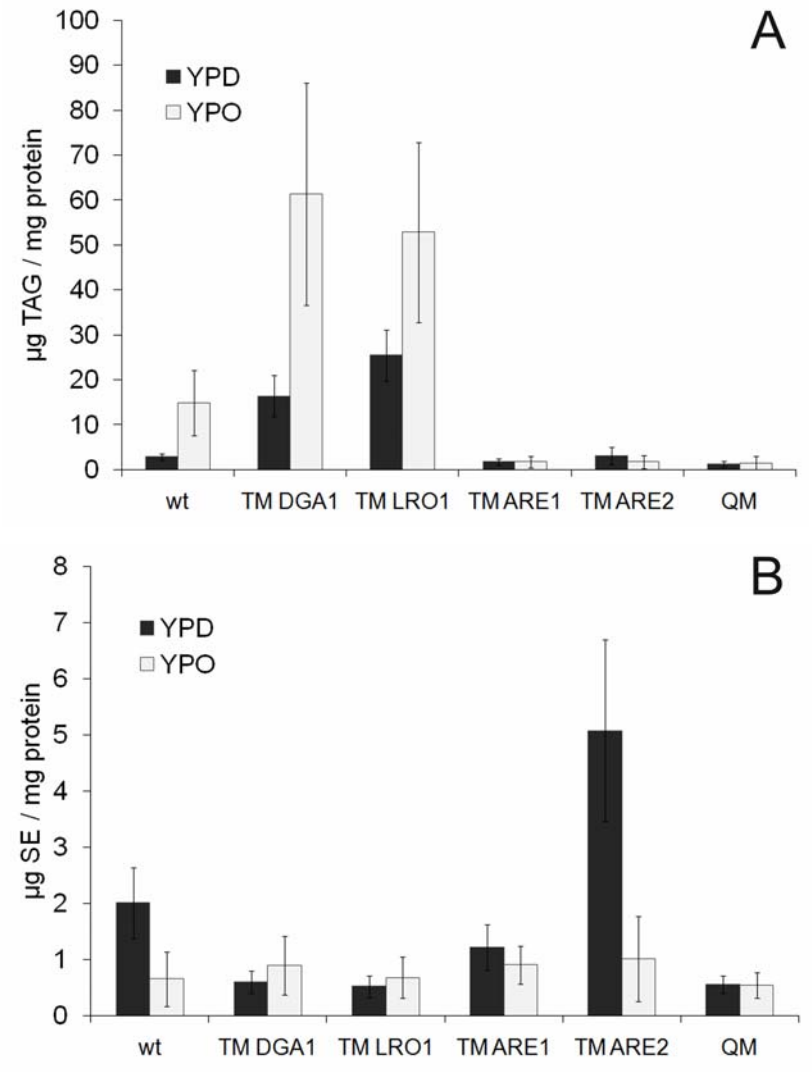


Figure 5: Neutral lipid analyses of strains from YPD and YPO

Cells from different strains were incubated on YPD and YPO until early stationary phase. Total cell extracts were prepared and lipids were extracted as described in Material and Methods. Neutral lipids were then separated by TLC and amounts were analyzed by densitometric scanning. Data show results from at least four independent experiments.

(A) Amount of TAG in strains indicated

(B) Amount of SE in strains indicated.

Formation of fatty acid ethyl esters in strains lacking triacylglycerol formation

Analyses of total lipid extracts from TM ARE1, TM ARE2 and QM grown on oleic acid revealed the occurrence of a new and so far unidentified compound (Figure 6). An additional band was visible on TLC and found to be most prominent in TM ARE1, TM ARE2 and QM grown on YPO. Based on the R_F value and GLC-MS analysis this substance was identified as ethyl esters (EE) of palmitic acid (43 %) and oleic acid (57%).

We assume that this compound although present at minor amount was formed as a consequence of fatty acid overflow in these cells.

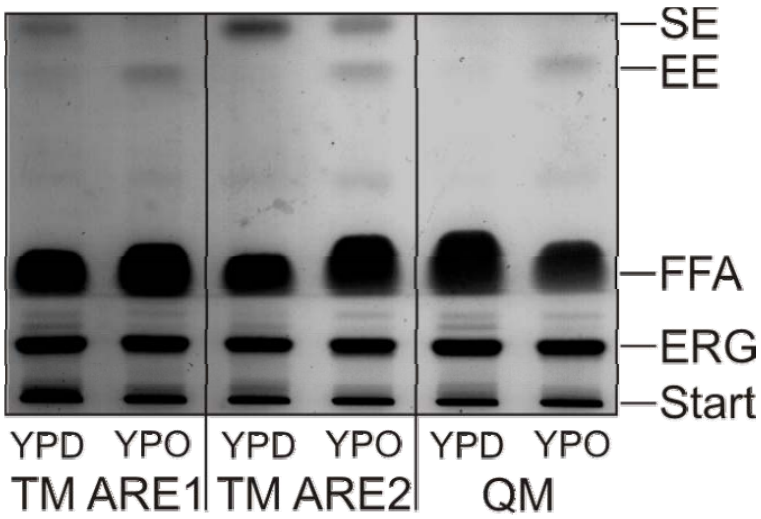


Figure 6: Neutral lipid profile of cells grown on YPD and YPO

Cells from TM ARE1, TM ARE2 and QM were grown to early stationary phase on YPD and YPO liquid media inoculated from the same YPD preculture. Lipids were isolated and same amounts were separated by TLC as described in Material and Methods and visualized with iodine vapor. SE, steryl ester; EE, ethyl ester; FFA, free fatty acid; ERG, ergosterol; Start, loading lane

Alterations of cellular morphology (see Figure 4) and neutral lipid pattern (see Figure 5) led us to speculate that the phospholipid pattern was also altered when cells were shifted to growth on oleic acid. Our analyses showed that in all strains with the exception of TM ARE2 the amount of total phospholipids was indeed increased by growth on oleate (Figure 7). A major increase was found with TM LRO1 and TM ARE1, whereas changes in TM DGA1 and QM were moderate. A more detailed analysis of the phospholipid composition in TM ARE2 cells showed a decrease of phosphatidylethanolamine (PE) and an increase of lysophospholipids (LPL) when cells were grown on oleate compared to glucose (data not shown). Although both PE and LPL are known to disturb the structure of bilayer membranes it is hard to imagine that this effect may become relevant in the complex mixture of phospholipids with large amounts of phosphatidylcholine in organelle membranes.

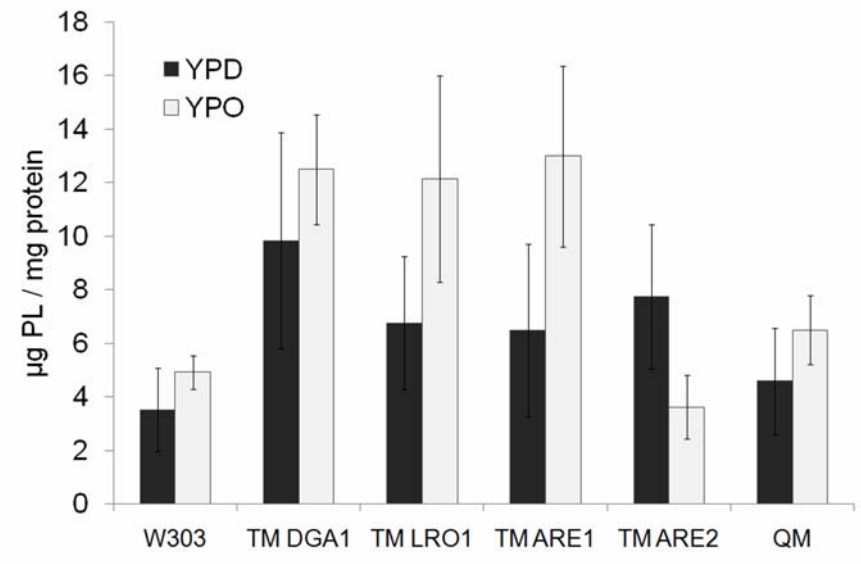


Figure 7: Phospholipid pattern from cells grown on YPD and YPO

Crude lipid extracts were prepared from cells grown on YPD and YPO to stationary phase. Phospholipids were isolated and quantified as described in Material and Methods. Overall amounts of phospholipids in cells grown on YPD (dark bars) and YPO (light bars) were determined in the different mutant strains indicated. Data represent results from at least three independent experiments.

Steryl ester synthase activity is inhibited by oleic acid

The finding that SE levels were dramatically decreased in cells grown on oleic acid was striking and a novel effect of oleic acid in yeast. However, the reason for this down-regulation of sterol esterification was not obvious. Previously, it was reported that genes encoding proteins of ergosterol synthesis (*ERG1*, *ERG3*) were down-regulated by oleic acid (Smith *et al.*, 2002) and knock-out of the acyltransferases decreased expression of genes involved in ergosterol formation (*ERG3*, *ERG4* and *ERG5*) (Garbarino *et al.*, 2009). Therefore, we presumed that lack of substrates, ergosterol and/or free fatty acid, might cause the decrease in sterol esterification. To prove or disprove this hypothesis we analyzed amounts of free ergosterol (unesterified) and FFA in cells grown on oleic acid and or glucose (Figure 8). We found that the level of free ergosterol was increased in all mutant strains compared to wild type (Figure 8A). This effect was observed with cells grown on YPD or YPO. Consequently, free ergosterol was not limiting when cells were grown on oleic acid.

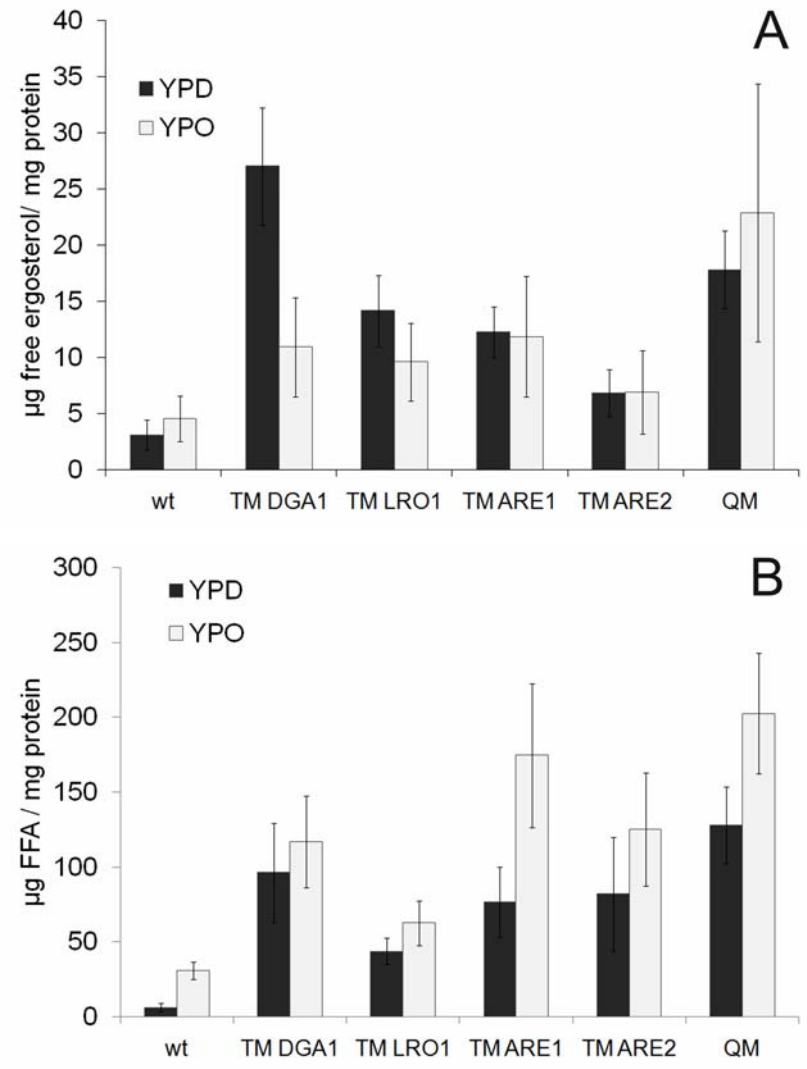


Figure 8: Amounts of free ergosterol and free fatty acids in cells from YPD and YPO

Cells from different strains were incubated on YPD and YPO until early stationary phase. Total cell extracts were prepared and lipids were extracted as described in Material and Methods. Ergosterol and FFA were then separated by TLC and amounts were analyzed by densitometric scanning. Data show results from at least four independent experiments. A) Amount of free ergosterol in strains indicated; B) Amount of FFA in strains indicated.

Although it was very unlikely that the level of FFA in cells grown on oleate would be the bottleneck for SE formation, we also analyzed these components in all strains investigated (Figure 8B). As expected, growth of cells on oleate caused an increase in cellular FFA. The relative increase was most dramatic in wild type because the level of FFA was low in this strain when grown on YPD. In the TMs and in the QM the amount of FFA was already high when grown on glucose due to the defect in neutral lipid formation, and the increase by

growth on oleate was rather moderate. However, also this reaction partner for SE synthases was not limiting.

As mentioned above, it had been shown that oleic acid has a great impact on the expression of a number of genes (Smith *et al.*, 2002; Gurvitz and Rottensteiner, 2006; Smith *et al.*, 2006b; Saleem *et al.*, 2008). Therefore, we wondered whether oleic acid also had an influence on the expression level of *ARE2*, the major yeast SE synthase under aerobic conditions. However, RT-PCR analysis performed with RNA extracted from wild type cells grown on glucose, oleic acid and palmitic acid did not show any significant difference in the expression of *ARE2* (Figure 9).

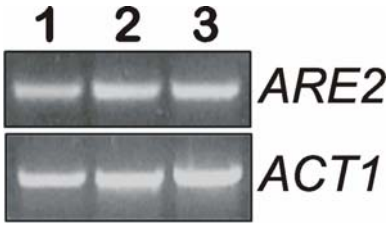


Figure 9: Transcription level of *ARE2*

RNA from yeast wild type cells grown in the presence of glucose (1), palmitic acid (2) or oleic acid (3), respectively, was isolated and subjected to RT-PCR as indicated in Materials and Methods. Similar results were obtained in two independent experiments.

Finally, we investigated whether FFA had an impact on SE synthesis *in vitro*. For this purpose we performed assays with homogenates and purified ER from wild type cells grown on glucose in the absence or presence of oleic acid and palmitic acid, respectively. We found that both FFA decreased the esterification of ergosterol *in vitro* (Figure 10A), but the effect of oleic acid was much stronger than of palmitic acid. This effect was not only seen when radiolabeled oleoyl-CoA was used as a co-substrate for the reaction but also with palmitoyl-CoA (Supplementary Figure 1A). We also ruled out the possibility that oleoyl-CoA instead of free oleic acid caused the decreased SE synthase activity by substrate inhibition. We performed control experiments using increasing amounts of oleoyl-CoA in the assays, but no indication of inhibition was observed (Supplementary Figure 1B). Moreover, we tested PL species containing different fatty acids for their influence on the enzyme activity, but they also did not exhibit an inhibitory effect on the esterification of sterols (Supplementary Figure 1C).

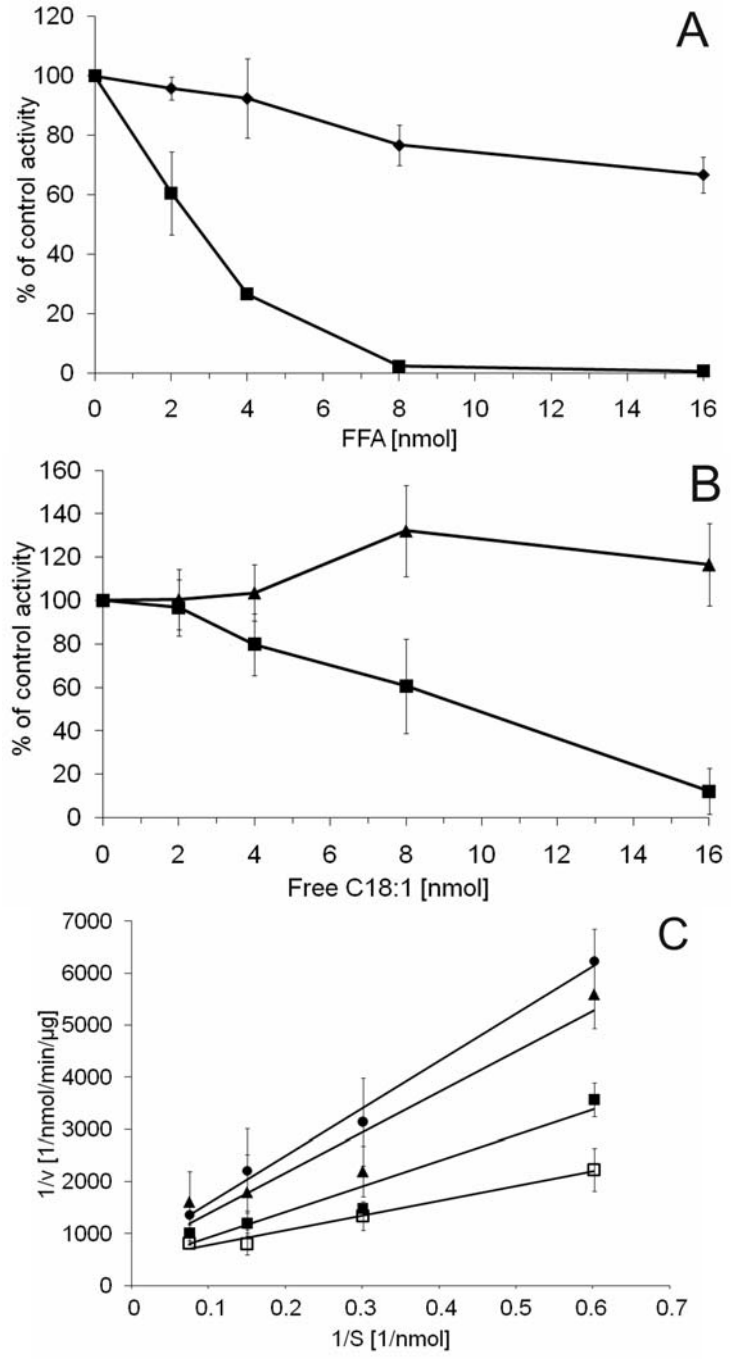


Figure 10: *In vitro* sterol esterification activity

Steryl ester synthase activity was determined as described in Methods section.

(A) Effect of free oleic acid and/or palmitic acid on the esterification of ergosterol. The activity was estimated relative to the activity without addition of FFA to the reaction mixture. ■ addition of oleic acid; ♦ addition of palmitic acid

(B) Comparison of sterol and DAG esterification in the presence of oleic acid: ■ ergosterol esterification; ▲ DAG esterification

(C) Lineweaver Burk plot of steryl ester synthase activity in the presence of different amounts of oleic acid; □ no oleic acid; ■ 1.1 µM oleate; ▲ 2.2µM oleate; ● 5.5 µM oleate

Finally, we wished to exclude an unspecific and more general effect of oleic acid as detergent or inhibitor of acyltransferases. Therefore, we performed DAG acyltransferase (Dga1p) assays as a control. As can bee seen from Figure 10B, free oleic acid did not affect the activity of this enzyme but specifically decreased sterol esterification by Are2p. We concluded from these results that the inhibition of SE synthase by oleic acid was specific and accounted for SE depletion in cells grown on oleate.

To investigate the enzymology of SE synthase inhibition by oleic acid in some more detail we performed enzyme kinetic measurements using increasing amounts of free oleic acid as additive (Figure 10C). These assays showed that v_{max} was constant at value of about 0.002 nmol/min/µg protein in all cases, whereas K_M increased from 5.6 nmol (no oleate) to 11.4, 12.6 and 13.6 nmol when 1.1, 2.2 and 5.5 µM oleate, respectively, were added to the reaction mixture.

Discussion

Cellular effects of oleic acid are diverse. On one hand, oleate plays an essential role in a number of cellular processes, e.g., lipid synthesis (Casey *et al.*, 1993), membrane formation (Wriessnegger *et al.*, 2007; Rosenberger *et al.*, 2009), induction of peroxisome proliferation (Erdmann *et al.*, 1989; Erdmann and Blobel, 1995; Saleem *et al.*, 2008), stimulation of lipid particle biogenesis (Binns *et al.*, 2006; Rosenberger *et al.*, 2009) and gene regulation (Saleem *et al.*, 2008). All these processes appear to be linked and strictly regulated, but so far most of the regulatory mechanisms governing these processes remained elusive. On the other hand, oleic acid has a negative influence on cell survival and causes a so-called lipotoxic effect (Unger and Orci, 2002; Schaffer, 2003; Garbarino and Sturley, 2005).

In contrast to CHO cells which cannot tolerate excess amounts of saturated fatty acids (Listenberger *et al.*, 2003) yeast cells become sensitive to oleate, especially in strains bearing defects in neutral lipid storage (Kohlwein and Petschnigg, 2007; Petschnigg *et al.*, 2009). Whereas exogenous saturated fatty acids were found to be without effect on yeast cells the role of palmitoleate remained controversial. Some strains were found to be sensitive to oleate but tolerate palmitoleate whereas other strains were sensitive to both fatty acids supplemented to the media (Lockshon *et al.*, 2007). Different mechanisms are under discussion which may lead to fatty acid lipotoxicity in different experimental systems. Programmed cell death (Listenberger *et al.*, 2001; Zhang *et al.*, 2003; Low *et al.*, 2008; Petschnigg *et al.*, 2009), JNK-dependent TNF-related apoptosis (Malhi *et al.*, 2007), activation of PPAR (Cury-Boaventura *et al.*, 2006) and activation of the UPR (Kharroubi *et al.*, 2004; Cnop *et al.*, 2007; Cunha *et al.*, 2008; Ota *et al.*, 2008) with the ER may play a role in the observed lipotoxic effects (Lupi *et al.*, 2002; Borradaile *et al.*, 2006). Finally, imbalance in cellular lipid metabolism leading to changes in membrane properties need to be considered (Lockshon *et al.*, 2007; Petschnigg *et al.*, 2009).

Our findings presented here confirm the lipotoxic effect of oleic acid on yeast cells but extend this finding insofar as we demonstrate that the yeast can adapt to this stress situation. We show that strains lacking neutral lipid formation and thus lipid particle proliferation become permanent or at least long-term tolerant to oleate (see Figure 2). This adaptation process, however, is accompanied by several dramatic cellular changes such as increase of the cellular phospholipid level, increase of lysophospholipids and free sterols, formation of ethyl esters of fatty acids and disturbance of the ER membrane network.

Interestingly, lysophospholipids and esters of fatty acids may act as second messengers (Resnick and Tomaska, 1994; McIntyre *et al.*, 2003; Simon *et al.*, 2005; Alhomsi and Laposata, 2006) and their accumulation may lead to disturbed signaling and ultimately to cell death. Ethyl esters of fatty acids are formed by the two acyl-CoA:ethanol O-acyltransferases Eeb1p and Eht1p (Saerens *et al.*, 2006). Upregulation of *EEB1* and *EHT1* expression upon oleate treatment (Smith *et al.*, 2006a) may lead to enhanced formation of fatty acid ethyl esters as a sort of escape mechanism from the excess of FFA.

Exposure of yeast cells to exogenous oleic acid confers dramatic changes of the cell structure. Under these conditions peroxisomes are induced and lipid particles massively proliferate as long as neutral lipid synthesis is active (Rosenberger *et al.*, 2009). In strains bearing defects in non-polar lipid production, however, lipid particles cannot be formed, whereas proliferation of peroxisomes upon induction by oleate appeared to be uneffected (see Figure 4). This result indicates that these two organelles act independently of each other, although functional and structural association of lipid particles and peroxisomes were discussed before (Binns *et al.*, 2006; Rosenberger *et al.*, 2009).

Interestingly, ER membranes strongly proliferate (see Figure 4) in strains lacking lipid particles which may in part be attributed to the enhanced phospholipid production in these mutants (see Figure 7). ER membrane accumulation was also found in yeast cells overproducing PEX15 (Elgersma *et al.*, 1997) or by overexpression of ER proteins such as cytochrome P450 cytochrome b_2 and HMG-CoA reductase (Jones and Fawcett, 1966; Wright *et al.*, 1988; Vergeres *et al.*, 1993). These ER structures were called karmellae which appeared as membrane stacks surrounding the nucleus and were therefore considered to be different from LEM structures (Figure 4) described in this study. Currently, we can only speculate that ER membrane accumulation may be consequence of enhanced lipid to protein ratio in the ER under conditions when lipid particles cannot be formed. This hypothesis is in line with the view that lipid particles and ER are related organelles. However, it was found that under ER stress lipid particle biosynthesis was not essential for cell survival (Fei *et al.*, 2009).

Wild type and the lipid particle deficient quadruple mutant *dga1Δlro1Δare1Δare2Δ* represent black and white situations in the neutral lipid storage scenario and also in oleate sensitivity. In contrast, triple mutants (TM) with only one of the neutral lipid forming acyltransferases active, enabled us to obtain a more precise picture of fatty acid lipotoxicity.

Using these mutants we were able to pinpoint some novel details of lipid storage under the stress of exposure to fatty acids. In brief, major effects observed with these strains were (i) huge accumulation of TAG in TM DGA1 and TM LRO1 confirming that TAG is paramount for fatty acid storage but in contrast to previous findings not essential for cellular survival; and (ii) strong oleate sensitivity of TM ARE1 and TM ARE2 strains similar to QM. Moreover, growth of yeast on oleate changes the phospholipid and protein patterns of lipid particles (our own unpublished results). Finally, we discovered that exposure of yeast cells to oleate led to a strong decrease of the cellular SE level due to inhibition of the major SE synthesizing enzyme. The latter observation we considered especially important because of a potentially new regulatory effect. Previous studies by others reported transcriptional alterations as an oleate response (Duplus *et al.*, 2000; Smith *et al.*, 2002; Gurvitz and Rottensteiner, 2006; Smith *et al.*, 2006a; DeGottardi A. *et al.*, 2007). Morikawa and Yamashita (Morikawa and Yamashita, 1978) showed that oleic acid has a direct inhibitory effect on the activity of yeast glycerolphosphate acyltransferase. Later, the ortholog of this enzyme in rat adipose tissue was also found to be inhibited by oleic acid (Durocher *et al.*, 1990). In bacteria, octanoic acid specifically inhibits glucose-6-phosphate dehydrogenase, phosphofructokinase, pyruvate kinase, fumarase, lactate dehydrogenase and the malic enzyme (Ferdinandus and Clark, 1969). Recent studies on HepG2 cells have shown an inhibitory effect of oleic and linoleic acid on the proteasome (Hamel, 2009). The chain length was essential for the inhibitory effect and not necessarily the degree of saturation.

The reason for reduced production of SE in yeast strains exposed to oleate was not at the transcriptional level of *ARE2* encoding the major SE synthesizing enzyme, but at the enzyme level. Classical enzyme kinetic experiments showed that oleic acid inhibits Are2p in a very specific way. We assume that this effect is also relevant *in vivo* because of the high concentration of free oleic acid more or less in all subcellular fractions of cells grown on oleate (our own unpublished results). Although in TM and QM strains the amount of FFA was already increased when cells were grown on glucose (Figure 8B) no liposensitivity or SE reduction was observed. When analyzing the composition of FFA we discovered that the ratio of oleic to palmitic acid was 1:1 in cells grown on glucose, but 6.5:1 in cells grown on oleate supplemented media. We conclude that this overflow of free oleic acid accounts for Are2p inhibition *in vivo* leading to decreased levels of SE in cells grown on oleate (see Figure 5B). The saturated fatty acid palmitate did neither show any growth defect nor marked inhibition of Are2p *in vitro* (Figure 10A).

As a result the ratio of TAG to SE was hardly altered in cells grown on palmitate whereas oleate grown cells produced 19 times more TAG than SE (data not shown). This finding raised the question as to the physiological relevance of the inhibition of sterol esterification. It was reported before that the toxic effect of oleate in yeast was due to changes in the plasma membrane fluidity (Lockshon *et al.*, 2007). More recently, Petschnigg *et al.* (Petschnigg *et al.*, 2009) also discussed possible oleic acid mediated lipotoxicity due to altered acyl chain distribution in membranes. Previous studies with *Bacillus subtilis* had shown that the ratio of unsaturated to saturated fatty acids was critical for membrane fluidity (Grau and de, 1993; Weber *et al.*, 2001). Therefore, unsaturated fatty acids were regarded as key regulators of cellular membrane fluidity (Aguilar and deMendoza, 2006). This view is in line with our findings that the membrane phospholipid composition was altered when cells were grown in oleic acid supplemented medium (Rosenberger *et al.*, 2009). We suggest that high amounts of oleic acid in phospholipids may lead to increased membrane fluidity and to a challenge of cell viability. Inhibition of Are2p preventing the conversion of free sterols to SE may lead to higher concentrations of free sterols. This effect may contribute to adaptation of membranes towards more rigidity. At this point we can only speculate that this effect might also be true for other unsaturated fatty acids. Indeed growth retardation (Lockshon *et al.*, 2007; Garbarino *et al.*, 2009) and reduced viability (Petschnigg *et al.*, 2009) were found with yeast cells grown on palmitoleate.

In summary, results described in this study are one step forward to the understanding of liposensitivity caused by oleic acid. We hypothesize that oleic acid sensitivity occurs rather through a membrane adaption processes than defects in lipid storage. We postulate that the mechanism of oleic acid induced enzyme inhibition may also be relevant for other enzymes as well as for other organisms such as mammals or plants.

Acknowledgement

We thank Sten Stymne for providing yeast mutant strains. Furthermore, we thank Claudia Hrastnik for technical support and Karin Athenstaedt for fruitful discussion. This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich, Projects 18857 and W901-B05 (to G. D.).

Reference List

Aguilar,P.S. and deMendoza,D. (2006). Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. Mol. Microbiol. *62*, 1507-1514.

Alhomsi,K. and Laposata,M. (2006). Fatty acid ethyl ester effects on interleukin-2 production, cyclic AMP synthesis, and calcium influx in human mononuclear cells. Alcohol Clin. Exp. Res. *30*, 1121-1125.

Athenstaedt,K., Zweytick,D., Jandrositz,A., Kohlwein,S.D., and Daum,G. (1999). Identification and characterization of major lipid particle proteins of the yeast Saccharomyces cerevisiae. J. Bacteriol. *181*, 6441-6448.

Binns,D., Januszewski,T., Chen,Y., Hill,J., Markin,V.S., Zhao,Y., Gilpin,C., Chapman,K.D., Anderson,R.G., and Goodman,J.M. (2006). An intimate collaboration between peroxisomes and lipid bodies. J Cell Biol *173*, 719-31.

Borradaile,N.M., Han,X., Harp,J.D., Gale,S.E., Ory,D.S., and Schaffer,J.E. (2006). Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. J. Lipid Res. *47*, 2726-2737.

Broekhuyse,R.M. (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.

Casey,W.M., Rolph,C.E., Tomeo,M.E., and Parks,L.W. (1993). Effects of unsaturated fatty acid supplementation on phospholipid and triacylglycerol biosynthesis in Saccharomyces cerevisiae
5. Biochem. Biophys. Res. Commun. *193*, 1297-1303.

Cnop,M., Vidal,J., Hull,R.L., Utzschneider,K.M., Carr,D.B., Schraw,T., Scherer,P.E., Boyko,E.J., Fujimoto,W.Y., and Kahn,S.E. (2007). Progressive loss of beta-cell function leads to worsening glucose tolerance in first-degree relatives of subjects with type 2 diabetes. Diabetes Care *30*, 677-682.

Cunha,D.A. *et al.* (2008). Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. J. Cell Sci. *121*, 2308-2318.

Cury-Boaventura,M.F., Gorjao,R., de Lima,T.M., Newsholme,P., and Curi,R. (2006). Comparative toxicity of oleic and linoleic acid on human lymphocytes. Life Sci. *78*, 1448-1456.

Czabany,T., Athenstaedt,K., and Daum,G. (2007). Synthesis, storage and degradation of neutral lipids in yeast. Biochim. Biophys. Acta *1771*, 299-309.

Czabany,T., Wagner,A., Zweytick,D., Lohner,K., Leitner,E., Ingolic,E., and Daum,G. (2008). Structural and Biochemical Properties of Lipid Particles from the Yeast Saccharomyces cerevisiae. J. Biol. Chem. *283*, 17065-17074.

Dahlqvist,A., Stahl,U., Lenman,M., Banas,A., Lee,M., Sandager,L., Ronne,H., and Stymne,S. (2000). Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc. Natl. Acad. Sci. U. S. A *97*, 6487-6492.

DeGottardi A., Vinciguerra,M., Sgroi,A., Moukil,M., Ravier-Dall'Antonia,F., Pazienza,V., Pugnale,P., Foti,M., and Hadengue,A. (2007). Microarray analyses and molecular profiling of steatosis induction in immortalized human hepatocytes. Lab Invest *87*, 792-806.

Duplus,E., Glorian,M., and Forest,C. (2000). Fatty acid regulation of gene transcription. J. Biol. Chem. *275*, 30749-30752.

Durocher,V., Miller,M., and Rodriguez,M.A. (1990). Microsomal glycerolphosphate acyltransferase inactivation by fatty acids. Can. J. Physiol Pharmacol. *68*, 1255-1260.

Elgersma,Y., Kwast,L., van den,B.M., Snyder,W.B., Distel,B., Subramani,S., and Tabak,H.F. (1997). Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in S.cerevisiae, causes proliferation of the endoplasmic reticulum membrane. EMBO J. *16*, 7326-7341.

Erdmann,R. and Blobel,G. (1995). Giant peroxisomes in oleic acid-induced Saccharomyces cerevisiae lacking the peroxisomal membrane protein Pmp27p. J Cell Biol *128*, 509-23.

Erdmann,R., Veenhuis,M., Mertens,D., and Kunau,W.H. (1989). Isolation of peroxisome-deficient mutants of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *86*, 5419-23.

Fei,W., Wang,H., Fu,X., Bielby,C., and Yang,H. (2009). Conditions of endoplasmic reticulum stress stimulate lipid droplet formation in Saccharomyces cerevisiae. Biochemical Journal *424*, 61-67.

Ferdinandus,J. and Clark,J.B. (1969). Selective inhibition of bacterial enzymes by free fatty acids. J. Bacteriol. *98*, 1109-1113.

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

Garbarino,J., Padamsee,M., Wilcox,L., Oelkers,P.M., D',A.D., Ruggles,K., Ramsey,N., Jabado,O., Turkish,A., and Sturley,S.L. (2009). Sterol and diacylglycerol acyltransferase deficiency triggers fatty acid mediated cell death. J. Biol. Chem.

Garbarino,J. and Sturley,S.L. (2005). Homoeostatic systems for sterols and other lipids. Biochem. Soc. Trans. *33*, 1182-1185.

Grau,R. and de,M.D. (1993). Regulation of the synthesis of unsaturated fatty acids by growth temperature in Bacillus subtilis 1. Mol. Microbiol. *8*, 535-542.

Gurvitz,A. and Rottensteiner,H. (2006). The biochemistry of oleate induction: Transcriptional upregulation and peroxisome proliferation. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1763*, 1392-1402.

Hamel,F.G. (2009). Preliminary report: inhibition of cellular proteasome activity by free fatty acids. Metabolism *58*, 1047-1049.

Jones,A.L. and Fawcett,D.W. (1966). Hypertrophy of the agranular endoplasmic reticulum in hamster liver induced by phenobarbital (with a review on the functions of this organelle in liver). J. Histochem. Cytochem. *14*, 215-232.

Kharroubi,I., Ladriere,L., Cardozo,A.K., Dogusan,Z., Cnop,M., and Eizirik,D.L. (2004). Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. Endocrinology *145*, 5087-5096.

Kohlwein,S.D. and Petschnigg,J. (2007). SLipid-induced cell dysfunction and cell death: lessons from yeast. Curr. Hypertens. Rep. *9*, 455-461.

Leber,R., Zinser,E., Zellnig,G., Paltauf,F., and Daum,G. (1994). Characterization of lipid particles of the yeast, Saccharomyces cerevisiae. Yeast *10*, 1421-1428.

Listenberger,L.L., Han,X., Lewis,S.E., Cases,S., Farese,R.V., Jr., Ory,D.S., and Schaffer,J.E. (2003). Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc. Natl. Acad. Sci. U. S. A *100*, 3077-3082.

Listenberger,L.L., Ory,D.S., and Schaffer,J.E. (2001). Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J. Biol. Chem. *276*, 14890-14895.

Lockshon,D., Surface,L.E., Kerr,E.O., Kaeberlein,M., and Kennedy,B.K. (2007). The sensitivity of yeast mutants to oleic acid implicates the peroxisome and other processes in membrane function. Genetics *175*, 77-91.

Low,C.P., Shui,G., Liew,L.P., Buttner,S., Madeo,F., Dawes,I.W., Wenk,M.R., and Yang,H. (2008). Caspase-dependent and -independent lipotoxic cell-death pathways in fission yeast. J. Cell Sci. *121*, 2671-2684.

Lupi,R. *et al.* (2002). Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. Diabetes *51*, 1437-1442.

Malhi,H., Barreyro,F.J., Isomoto,H., Bronk,S.F., and Gores,G.J. (2007). Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. Gut *56*, 1124-1131.

McIntyre,T.M. *et al.* (2003). Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARgamma agonist. Proc. Natl. Acad. Sci. U. S. A *100*, 131-136.

Morikawa,M. and Yamashita,S. (1978). Inhibition of yeast phosphatidic-acid synthesis by free fatty acids. Eur. J. Biochem. *84*, 61-68.

Morrison W.R. and Smith L.M. (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride--methanol. J. Lipid Res. *5*, 600-608.

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

Ota,T., Gayet,C., and Ginsberg,H.N. (2008). Inhibition of apolipoprotein B100 secretion by lipid-induced hepatic endoplasmic reticulum stress in rodents. J. Clin. Invest *118*, 316-332.

Petschnigg,J., Wolinski,H., Kolb,D., Zellnig,G., Kurat,C.F., Natter,K., and Kohlwein,S.D. (2009). Good fat - essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast. J. Biol. Chem.

Quail,M.A. and Kelly,S.L. (1996). The extraction and analysis of sterols from yeast. Methods Mol. Biol. *53*, 123-131.

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

Resnick,R.J. and Tomaska,L. (1994). Stimulation of yeast adenylyl cyclase activity by lysophospholipids and fatty acids. Implications for the regulation of Ras/effector function by lipids. J. Biol. Chem. *269*, 32336-32341.

Rosenberger,S., Connerth,M., Zellnig,G., and Daum,G. (2009). Phosphatidylethanolamine synthesized by three different pathways is supplied to peroxisomes of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta.

Saerens,S.M., Verstrepen,K.J., Van Laere,S.D., Voet,A.R., Van,D.P., Delvaux,F.R., and Thevelein,J.M. (2006). The Saccharomyces cerevisiae EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. J. Biol. Chem. *281*, 4446-4456.

Saleem,R.A., Knoblach,B., Mast,F.D., Smith,J.J., Boyle,J., Dobson,C.M., Long-O'Donnell,R., Rachubinski,R.A., and Aitchison,J.D. (2008). Genome-wide analysis of signaling networks

regulating fatty acid-induced gene expression and organelle biogenesis. J. Cell Biol. *181*, 281-292.

Sandager,L., Gustavsson,M.H., Stahl,U., Dahlqvist,A., Wiberg,E., Banas,A., Lenman,M., Ronne,H., and Stymne,S. (2002). Storage Lipid Synthesis Is Non-essential in Yeast. J. Biol. Chem. *277*, 6478-6482.

Schaffer,J.E. (2003). Lipotoxicity: when tissues overeat. Curr. Opin. Lipidol. *14*, 281-287.

Simon,M.F., Daviaud,D., Pradere,J.P., Gres,S., Guigne,C., Wabitsch,M., Chun,J., Valet,P., and Saulnier-Blache,J.S. (2005). Lysophosphatidic acid inhibits adipocyte differentiation via lysophosphatidic acid 1 receptor-dependent down-regulation of peroxisome proliferator-activated receptor gamma2. J. Biol. Chem. *280*, 14656-14662.

Smith,J.J., Marelli,M., Christmas,R.H., Vizeacoumar,F.J., Dilworth,D.J., Ideker,T., Galitski,T., Dimitrov,K., Rachubinski,R.A., and Aitchison,J.D. (2002). Transcriptome profiling to identify genes involved in peroxisome assembly and function. J Cell Biol *158*, 259-71.

Smith,J.J., Sydorskyy,Y., Marelli,M., Hwang,D., Bolouri,H., Rachubinski,R.A., and Aitchison,J.D. (2006a). Expression and functional profiling reveal distinct gene classes involved in fatty acid metabolism. Mol. Syst. Biol. *2*, 2006.

Smith,J.J., Sydorskyy,Y., Marelli,M., Hwang,D., Bolouri,H., Rachubinski,R.A., and Aitchison,J.D. (2006b). Expression and functional profiling reveal distinct gene classes involved in fatty acid metabolism 3. Mol. Syst. Biol. *2*, 2006.

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

Unger,R.H. and Orci,L. (2002). Lipoapoptosis: its mechanism and its diseases. Biochim. Biophys. Acta *1585*, 202-212.

Valachovic,M., Hronska,L., and Hapala,I. (2001). Anaerobiosis induces complex changes in sterol esterification pattern in the yeast Saccharomyces cerevisiae. FEMS Microbiol. Lett. *197*, 41-45.

Valachovic,M., Klobucnikova,V., Griac,P., and Hapala,I. (2002). Heme-regulated expression of two yeast acyl-CoA:sterol acyltransferases is involved in the specific response of sterol esterification to anaerobiosis. FEMS Microbiol. Lett. *206*, 121-125.

Vergeres,G., Yen,T.S., Aggeler,J., Lausier,J., and Waskell,L. (1993). A model system for studying membrane biogenesis. Overexpression of cytochrome b5 in yeast results in marked proliferation of the intracellular membrane. J. Cell Sci. *106 (Pt 1)*, 249-259.

Walther,T.C. and Farese,R.V., Jr. (2009). The life of lipid droplets. Biochim. Biophys. Acta *1791*, 459-466.

Weber,M.H., Klein,W., Muller,L., Niess,U.M., and Marahiel,M.A. (2001). Role of the Bacillus subtilis fatty acid desaturase in membrane adaptation during cold shock 2. Mol. Microbiol. *39*, 1321-1329.

Wriessnegger,T., Gubitz,G., Leitner,E., Ingolic,E., Cregg,J., de la Cruz,B.J., and Daum,G. (2007). Lipid composition of peroxisomes from the yeast Pichia pastoris grown on different carbon sources. Biochim. Biophys. Acta *1771*, 455-461.

Wright,R., Basson,M., D'Ari,L., and Rine,J. (1988). Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. J. Cell Biol. *107*, 101-114.

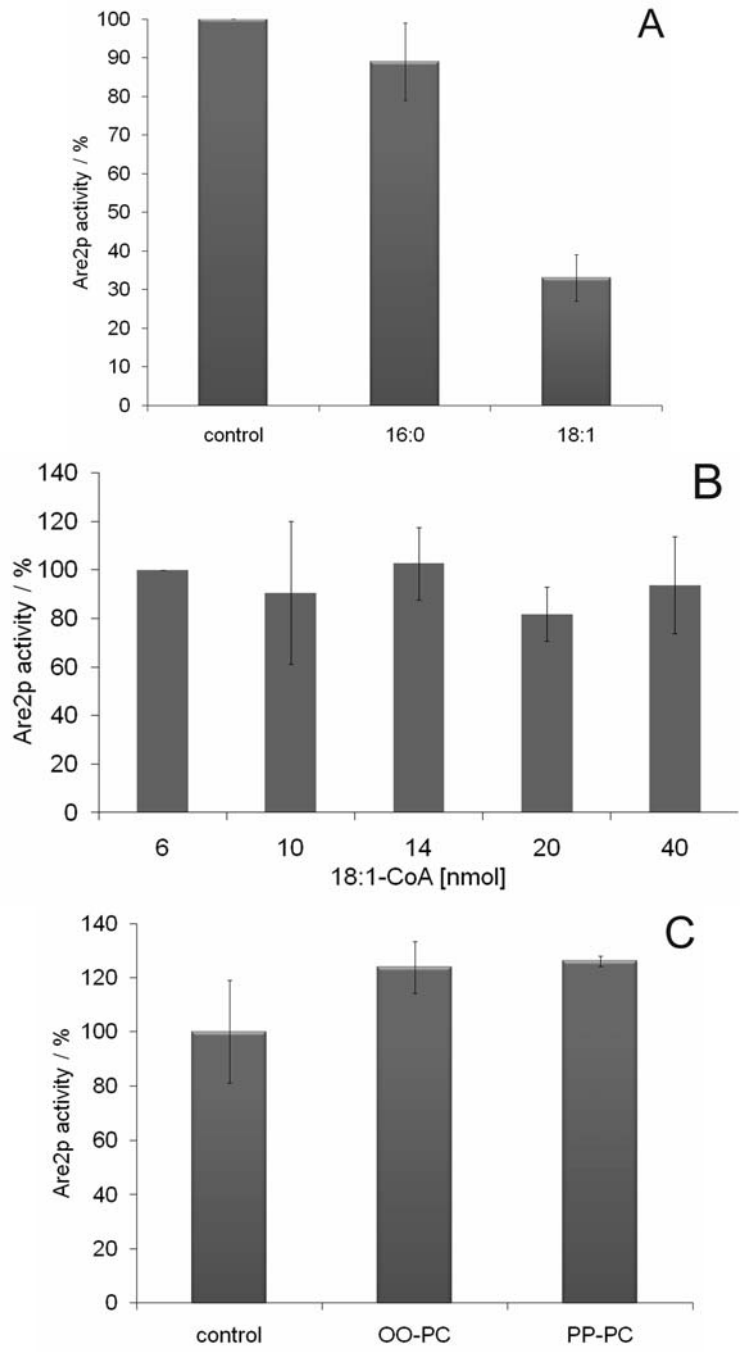
Yang,H., Bard,M., Bruner,D.A., Gleeson,A., Deckelbaum,R.J., Aljinovic,G., Pohl,T.M., Rothstein,R., and Sturley,S.L. (1996). Sterol esterification in yeast: a two-gene process. Science *272*, 1353-1356.

Yang,H., Cromley,D., Wang,H., Billheimer,J.T., and Sturley,S.L. (1997). Functional expression of a cDNA to human acyl-coenzyme A:cholesterol acyltransferase in yeast. Species-dependent substrate specificity and inhibitor sensitivity. J. Biol. Chem. *272*, 3980-3985.

Zhang,Q., Chieu,H.K., Low,C.P., Zhang,S., Heng,C.K., and Yang,H. (2003). Schizosaccharomyces pombe cells deficient in triacylglycerols synthesis undergo apoptosis upon entry into the stationary phase. J. Biol. Chem. *278*, 47145-47155.

Zweytick,D., Leitner,E., Kohlwein,S.D., Yu,C., Rothblatt,J., and Daum,G. (2000). Contribution of Are1p and Are2p to steryl ester synthesis in the yeast Saccharomyces cerevisiae. Eur. J. Biochem. *267*, 1075-1082.

Supplements



Supplementary Figure 1: Are2p *in vitro* activity under different conditions

Crude cell lysates were analyzed for *in vitro* activity of Are2p as described in Material and Method section.

(A) Sterol esterification activity using palmitoyl-CoA as acyl-donor was measured in absence of free fatty acids (control), in presence of palmitic acid (16:0) and in presence of oleic acid (18:1). Activity is described relative to control activity.

(B) Increasing amounts of oleoyl-CoA were added to the reaction mixture as indicated.

(C) Different PL species were added to the *in vitro* assay mixture; OO-PC, di-oleoyl-phosphatidylcholine; PP-PC, di-palmitoyl-phosphatidylcholine

CHAPTER 5

LIPID PARTICLES OF THE YEAST *SACCHAROMYCES CEREVISIAE* LIPIDOME MEETS PROTEOME

Karlheinz Grillitsch [a§], Melanie Connerth [a§], Harald Köfeler [b], Tabi Arrey[c], Benjamin Rietschel[c], Michael Karas [c] and Günther Daum[a]

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

[b)] Core facility for Mass Spectrometry/Lipidomics, Center for Medical Research, Medical University of Graz, Austria

[c)] Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe University of Frankfurt, Germany

Key words: triacylglycerol, phospholipid, mass spectrometry, protein, lipid particles, yeast

Abbreviations: TAG: triacylglycerols; STE: steryl esters; LP: lipid particles

[§] contributed equally and should be considered as co-first authors

Abstract

In the yeast *Saccharomyces cerevisiae* as in other eukaryotes neutral lipids are a reservoir of energy and building blocks for membrane lipid synthesis. The yeast neutral lipids, triacylglycerols (TAG) and steryl esters (STE), are stored in so called lipid particles (LP) as biologically inert form of fatty acids and sterols. Prerequisite for the understanding of LP function and structure is elucidation of their molecular equipment. For this purpose, we performed conventional analysis and mass spectrometric analysis of lipids (TAG, STE, phospholipids), and MS analysis of proteins which are present on the surface of LP. These analyses were carried out with LP from cells grown on the two carbon sources glucose and oleic acid. Results obtained by these methods revealed marked differences in the lipidome, but also in the proteome of LP isolated from yeast cells grown under different conditions. Changes in the cultivation conditions toward oleic acid led to serious intracellular adaptations regarding the lipid composition of neutral lipids and glycerophospholipids. Most notably we found a huge increase in the amount of TAG at the expense of STE with oleic acid being the major fatty acid esterified in TAG. Levels of phospholipids were also strongly influenced by the change of carbon source, with each of the phospholipids responding in a case sui generis. Apparently, the shift of the carbon source caused a strong increase in the degree of unsaturation with a strong tendency toward the mono-unsaturated oleic acid. Moreover, proteome analysis of LP led to identification of several new putative LP proteins. The detailed analysis of the lipid and protein composition of this organelle will help to deepen our knowledge regarding function of LP in the cellular interplay of organelles.

Introduction

A strong increase of adipositas and obesity mainly related to unbalanced diet led scientists to study of lipid uptake, biosynthesis, storage and mobilization with high priority. Lipid (fat) metabolism and especially storage are inevitably linked to one specific organelle named the lipid particle (LP), lipid droplet or oil body. The baker's yeast *Saccharomyces cerevisiae* has a long standing tradition in lipid particle research. Using this experimental system fundamental studies addressing structural, functional and metabolic aspects of lipid particles were performed (Clausen *et al.*, 1974; Zinser *et al.*, 1993; Leber *et al.*, 1994; Czabany *et al.*, 2008). One characteristic property of LP is the surface monolayer of phospholipids which protects the highly hydrophobic interior from the cellular environment (Tauchi-Sato *et al.*, 2002). The polar head groups of these phospholipids face the cytosol, and the hydrophobic side chains (fatty acids) associate with the non-polar core of the LP. This hydrophobic core which represents more than 95 mass % of LP consists of the non-polar lipids triacylglycerols (TAG) and steryl esters (STE) which lack charged groups and are therefore not suited as constituents of membrane bilayers. As shown recently in our laboratory, STE of LP form several ordered shells below the phospholipid monolayer, whereas TAG are more or less randomly packed in the center of LP (Czabany *et al.*, 2008).

The surface phospholipid monolayer membrane of LP contains a small but specific set of proteins embedded. Prominent proteins of mammalian LP are perilipin, adipophilin, hormone-sensitive lipase (HSL), adipose TAG lipase (ATGL), TIP47 (Tail-Interacting Protein 47 kDa), PAT-proteins, S3-12 and OXPAT (Ohsaki *et al.*, 2006b; Goodman, 2008; Olofsson *et al.*, 2008; Thiele and Spandl, 2008; Farese, Jr. and Walther, 2009; Robenek *et al.*, 2009; Bickel *et al.*, 2009; Yamaguchi and Osumi, 2009). A functional link between mammalian lipid droplets to the spliceosome and proteasome was suggested (Ohsaki *et al.*, 2006a; Cho *et al.*, 2007; Guo *et al.*, 2008). The most prominent proteins from plant oil droplets are the oleosins which cover the surface of the droplet and prevent them from coalescence (Huang *et al.*, 2009). In yeast LP, so far homologues of perilipins or oleosines were not detected. However, a number of proteins involved in lipid metabolism are characteristic for this organelle (Athenstaedt *et al.*, 1999; Rajakumari *et al.*, 2008). Besides storing lipids as chemical energy and building blocks of biological membranes, yeast LP were assumed to be important players of lipid homeostasis (Kurat *et al.*, 2009). The mechanism by which LP proteins are targeted to and associated with the LP surface is still a matter of dispute.

A favoured model suggests that TAG and/or STE synthesizing enzymes form large amounts of non-polar lipids between the two leaflets of the ER which finally results in budding of a nascent LP (Athenstaedt and Daum, 2006; Czabany *et al.*, 2007; Rajakumari *et al.*, 2008; Walther and Farese, Jr., 2009; Ohsaki *et al.*, 2009). Some alternative mechanisms have also been discussed (Robenek *et al.*, 2006) but all of them agreed that LP evolve from the ER.

The composition of LP strongly depends on the carbon source used for cell cultivation (Athenstaedt *et al.*, 2006). A helpful tool for making yeast cells "obese" is growth on oleic acid. Under these conditions, peroxisome proliferation is induced because this organelle is the only subcellular fraction in yeast where β-oxidation of fatty acids occurs (Erdmann *et al.*, 1989). Nevertheless, oleic acid grown yeast cells accumulate large amounts of lipids with a substantial portion of oleic acid (Rosenberger *et al.*, 2009). Moreover, fatty acids may act as signalling molecules in gene expression. As an example, oleic acid binds to upstream promoter elements with ORE (oleate response element) being the most prominent (Duplus *et al.*, 2000; Trotter, 2001).

The aim of the present study was to re-visit the yeast LP and to explore molecular details of the molecular composition of this subcellular compartment. Knowledge about lipid and protein components forming LP is an important prerequisite for a broader understanding of its function. Highly improved and sophisticated methods of mass spectrometry were employed to analyse the lipidome of LP on one hand and the proteome on the other hand. Variation of the carbon source (glucose vs. oleate) for growth of yeast cells allowed us to isolate variants of LP which strongly differed in their lipid composition. Here we describe how these changes affected not only the lipidome but also the proteome of LP thereby highlighting general as well as adaptative aspects of LP formation.

Methods and Materials

Yeast strains and culture conditions

Yeast strains used in this study are listed in Table 1. Cells were grown at 30°C in rich medium containing 1% yeast extract, 2% peptone, and 2% glucose (YPD) or 0.3% yeast extract, 0.5% peptone, 0.1% glucose, 0.5% KH_2PO_4 and 0.1% oleic acid (YPO). For solubilizing fatty acids in YPO, 0.2% Tween 80 was added to the media.

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source
BY4741	*Mata lys2*Δ0 *leu2* Δ0 *ura3* Δ0 *his3*Δ1	EUROSCARF
ATCC 201388 (OSH4-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *OSH4-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (VPS66-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *VPS66-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (CPR5-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *CPR5-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (GTT1-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *GTT1-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (UBX2-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *UBX2-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (YPT7-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *YPT7-GFP::HIS3MX*	INVITROGEN

Subcellular fractionation of yeast cells

Subcellular fractions of yeast cells were prepared by published procedures (Serrano, 1988; Leber *et al.*, 1994; Zinser and Daum, 1995) and routinely tested by Western Blot analysis (Haid and Suissa, 1983) using rabbit antibodies against Erg1p, Por1p, Pma1p, Pcs60p and Prc1p/CPY. Peroxidase conjugated secondary antibody and enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce Chemical Company, Rockford, IL, USA) were used to visualize immunoreactive bands.

Protein analysis

Proteins from isolated LP and homogenates were precipitated with trichloroacetic acid (TCA) at a final concentration of 10%. Proteins were quantified by the method of Lowry *et al*. (Lowry *et al.*, 1951) using bovine serum albumin as standard. Prior to protein analysis, samples of LP were delipidated. Non-polar lipids were extracted with two volumes of diethyl ether, the organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were analyzed as described above. SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) was carried out as described by Laemmli (Laemmli, 1970) using 12.5% separation gels.

Lipid analysis

Lipids from yeast cells grown on YPD or YPO were extracted as described by Folch *et al*. (Folch J. *et al.*, 1957). For quantification of neutral lipids extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4), and chromatograms were developed in an ascending manner by using the solvent system light petroleum/diethyl ether/acetic acid (25:25:1; per vol.) for the first third of the distance. Then, plates were briefly dried and further developed to the top of the plate using the solvent system light petroleum/diethyl ether (49:1; v/v). Quantification of ergosteryl esters was carried out by densitometric scanning at 275 nm with a Shimadzu dual wavelength chromatoscanner CS-930 using ergosterol as standard. To further analyze TAG, TLC plates were dipped into a charring solution consisting of 0.63 g of $MnCl_2 \cdot 4H_2O$, 60 ml of water, 60 ml of methanol, and 4 ml of concentrated sulphuric acid, briefly dried and heated at 100 °C for 30 min. Then, lipids were quantified by densitometric scanning at 400 nm using a Shimadzu dual-wave length chromatoscanner CS-930 with triolein as standard.

For phospholipid analysis, lipid extracts from homogenate and lipid particles were loaded manually on silica gel 60 plates (Merck, Darmstadt, Germany). Individual phospholipids were separated by two dimensional TLC using chloroform/methanol/25% NH_3 (65:35:5; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second developing solvent. Lipids were stained with iodine vapour, scraped off the plate and quantified by the method of Broekhyuse (Broekhuyse, 1968). For quantification of total phospholipids the respective band from neutral lipid analysis (see above) was scraped off the plate and analyzed by the method of Broekhuyse (Broekhuyse, 1968).

Mass spectrometry of neutral lipids and phospholipids

Lipid extracts were prepared as described above and diluted 1:100 in acetonitrile/2-propanol (5:2; v/v), 1% ammonium acetate, 0.1% formic acid. As internal standards 5 µM TAG (species 51:0) and PC (species 24:0) were added. For chromatographic separation a thermo hypersil GOLD C18 column (100 x 1 mm, 1.9 mm) was used employing solvent A (water with 1% ammonium acetate, 0.1% formic acid) and solvent B (acetonitrile/2-propanol (5:2; v/v);1% ammonium acetate; 0.1% formic acid). Gradients were established from 35 to 70 % B for 4 min and then to 100% B in another step of 16 min. These conditions were held for 10 min with a flow rate of 250 ml/min. Mass spectrometry was performed by HPLC direct coupling to a FT-ICR-MS hybrid mass spectrometer (LTQ-FT, Thermo Scientific) equipped with an IonMax ESI source. The mass spectrometer was operated at an accuracy of <2 ppm with external calibration and a resolution of 200,000 full width at half height (FWHH) at m/z 400. The spray voltage was set to 5,000 V, capillary voltage to 35 V, and the tube lens was at 120 V. The capillary temperature was at 250 °C. Peak areas are calculated by QuanBrowser for all lipid species identified previously by exact mass (<2 ppm) and retention time. Calculated peak areas for each species were expressed as % of the sum of all peak areas in the respective lipid class.

Mass spectrometry of proteins

Digestion: After TCA precipitation, 100 µg of the protein pellet was dissolved in 1 ml of 25 mM NH_4HCO_3 in an Eppendorf tube. Disulfide bridges were reduced in 45 mM DTT for 1 h at 60°C and 400 rpm shaking in a Thermomixer comfort (Eppendorf). The solution was then allowed to cool to room temperature, and cysteine residues were alkylated in the presence of 100 mM iodoacetamide for 45 min in a dark at room temperature. To avoid subsequent alkylation of trypsin, the reaction was quenched after 45 min by adding additional 12.5 µl of 45 mM DTT and incubating for another 45 min at room temperature. Then, trypsin was added to the reduced and alkylated samples to obtain a protein/enzyme ratio of 1:50 (w/w). The solution was incubated overnight at 37 °C. The digestion was stopped by addition of 1 µl of a 10 % trifluoroacetic acid (TFA) solution.

HPLC separation: Prior to nano-liquid chromatographic (nLC) separation, the samples were vacuum concentrated to approximately 8 µl and Solvent A (8% ACN, 0.1% TFA) was added to a final volume of 15 µl. Separation was performed on a Proxeon Biosystems EASY-

nLC™ system (Odense, Denmark) coupled to a SunCollect MALDI spotting device (Sunchrom, Germany).

The peptides were loaded onto an in-house packed 100 µm x 30 mm pre-column (Waters X-Bridge™ BEH 180 C_{18} 300 Å 3.5 µm,) and desalted with 30 µl solvent A for 15 min and separated on an in-house packed 100 µm x 150 mm column (Waters X-Bridge™ BEH 180 C_{18} 300 Å 3.5 µm) at a flow rate of 400 nl/min. The gradient profile linearly increased from 8 to 45% solvent B (92% ACN, 0.1% TFA) within 100 min, to 90% B within 20 min, 10 min at 90% B, back to 8% B within 5 min and at 8% for another 5 min. The effluent from the LC was pre-mixed with matrix, α-cyano-4-hydroxycinnamic acid, via a tee (Upchurch Scientific, USA) from an auxiliary pump (flow rate, 1.2 µl/min) and spotted every 20 sec on a blank 123 x 81 mm Opti-TOF™ LC/MALDI Insert metal target. This matrix solution contained 3.5 mg/ml α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) dissolved in 70% ACN, 30% H_2O, 0.1% TFA, spiked with 60 fmol [Glu^1]-Fibrinopeptide B (Bachem, Weil, Germany) for internal calibration.

MS and MS/MS: Mass spectra were acquired using an Applied Biosystems/MDS Sciex 4800 TOF/TOF™ Analyzer. The instrument was equipped with a Nd:YAG laser, emitting at 355 nm with a repetition of 200 Hz. All spectra were acquired in the positive reflector mode between 700 and 4,500 *m/z* with fixed laser intensity. A total of 750 laser shots per spot were accumulated. An 8-point plate model External calibration was performed using a Sequazyme™ Peptide Mass Standards Kit (Applied Biosystems). Fragmentation was performed with collision energy of 1 kV using air as collision gas at a pressure of 1 x 10^{-6} Torr. To reduce sample consumption during measurement, stop conditions for MS/MS were defined. A minimal number of 15 peaks above 45 S/N with at least 12 accumulated sub-spectra; a minimum of 1,250 and maximum of 2,500 laser shots were recorded. To avoid unnecessary multiple selections of identical precursor, MS/MS precursor selection was carried out via the instrument's software, job-wide interpretation. A total of 6 precursors per spot with a minimum signal-to-noise-ratio of 80 were selected for fragmentation. Potential matrix signals were removed from precursor selection, by excluding all masses in the range from 700 to 1,400 *m/z* having values of .030 +/- 0 .1 *m/z* as well as internal calibration.

Database searches and interpretation: Mascot Generic Format (MGF) files were retrieved from each MALDI MS/MS spectra using the built-in Peaks2Mascot feature, exporting up to 65 peaks per MS/MS spectrum, each requiring a minimum signal-to-noise of 5. The MGF

files were processed using the Mascot™ database search engine v2.2.03 (Matrix Science Ltd., UK).

The following settings were used: Enzyme, trypsin; allowed miss cleavages for trypsin 3, fixed modification, carboxymethylation of cysteine; variable modification, oxidation of methionine. MS precursor mass tolerance was set to 50 ppm and MS/MS mass tolerance to 0.5 Da. The search was performed with a custom Saccharomyces Genome Database generated from Saccharomyces Genome Database (http://www.yeastgenome.org) containing 6,717 entries as at November 30th 2008. A decoy database consisting of same-length random protein sequences was automatically generated and searched. All statistical analyses were based on peptides having Mascot™ MS/MS ions scores exceeding the "identity or extensive homology threshold" ($p < 0.05$). In the case of multiple fragmentations of identical precursors, due to recurrence in repetitive runs, only data from the highest scoring peptide were kept.

Results

Exogenous oleic acid is preferentially incorporated into triacylglycerols

Growth of yeast cells on oleic acid dramatically affects the lipid metabolism and has a major impact on the lipid storage organelle, the lipid particle (LP). Whereas wild type cells cultivated on glucose (YPD) contain roughly same amounts of storage lipids TAG and STE, growth on oleate dramatically increased the amount of TAG in LP at the expense of STE (Table 2). To address the massive formation of TAG in more detail, molecular species of this lipid class from LP were analyzed by mass spectrometry. TAG patterns from cells grown on YPD or YPO, respectively, showed marked differences (Figure 1). When cells were grown on glucose, 52:1, 52:2, 52:3 and 54:2 constituted the major TAG species. The 52 species contain one C16 and two C18 fatty acids. Either one, two or all three fatty acids can be unsaturated. Stereospecific positions could not be deduced from this analysis. The four TAG species mentioned above displayed approximately 65% of total TAG. The remaining species contained two or three C16 fatty acids, either saturated or unsaturated. Approximately 5 % of 54:3 TAG was detected indicating that oleic acid was the exclusive fatty acid in these lipids.

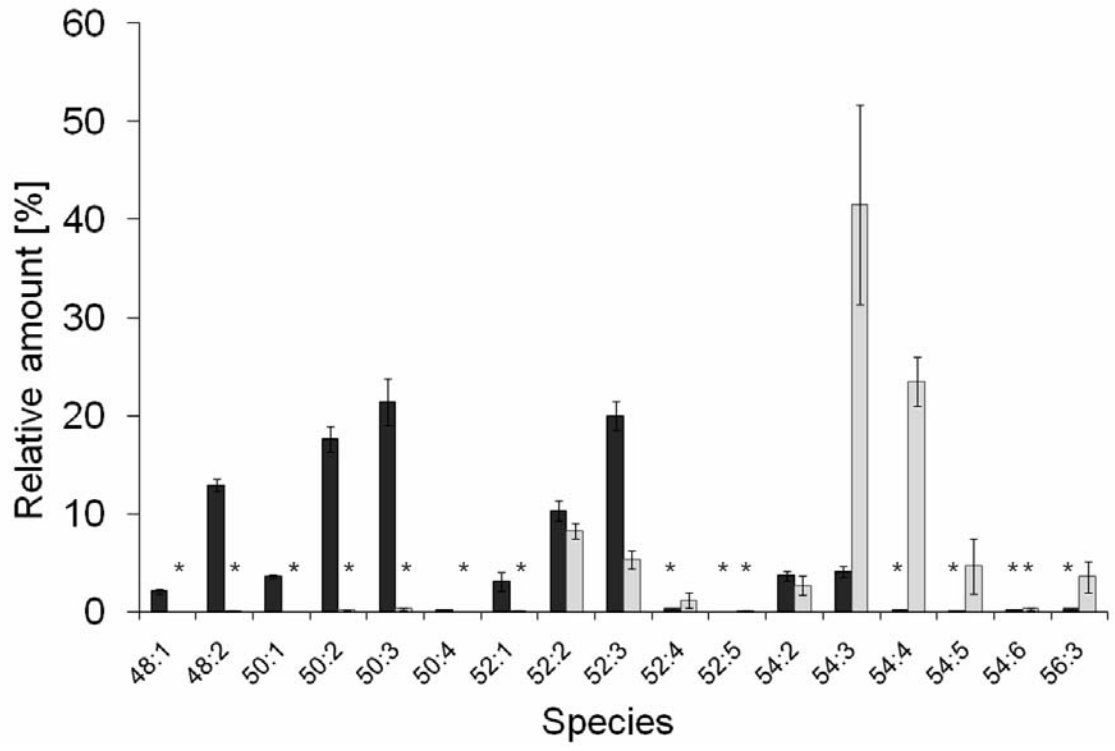


Figure 1: Oleic acid has great impact on the formation of TAG species

TAG of LP from wild type cells grown on glucose (dark bar) or oleate (white bar) were analysed by MS. Data are mean values from at least 2 independent experiments. *= values are ≤ 0.1%

When exogenous oleic acid was supplied to the media as carbon source the fatty acid pattern of the TAG changed tremendously. In contrast to cells grown on glucose, C16 was incorporated into TAG only at a minor percentage when oleic acid was the carbon source. The vast majority of fatty acids in TAG from cells grown on YPO were C18, preferentially C18:1. This result indicated that oleic acid was not only used as a carbon source, but also directly incorporated into complex lipids. Noteworthy, several TAG species containing polyunsaturated fatty acids, e.g. 54:5 species (18:1/18:2/18:2), and species with longer acyl chains, e.g. 56:3 species (C18:1/C18:2/C20:0) were detected in cells grown on YPO. This finding is due to impurities of oleate samples used as carbon source.

Growth of yeast cells on oleate increases the amount of total phospholipids

To investigate the effect of oleate as carbon source on the level and the composition of phospholipids from total cell extracts and LP, respectively, we performed conventional phospholipid analysis and mass spectrometry. As can be seen from Table 2 cultivation of cells on oleic acid resulted in an 1.5 fold increase of total phospholipids in the homogenate. An even higher increase of total phospholipids was seen with LP from YPO grown cells.

Table 2: Lipid composition of homogenate and lipid particles of *Saccharomyces cerevisiae* wild type grown on either YPD or YPO

mg / mg protein		
	Glucose	**Oleate**
Triacylglycerol	32.0 ± 4.0	97.3 ± 8.9
Steryl ester	36.7 ± 4.1	1.0 ± 0.3

mg phospholipid / mg protein		
	Glucose	**Oleate**
Homogenate	0.047 ± 0.003	0.071 ± 0.004
Lipid particle	0.423 ± 0.048	0.889 ± 0.054

To investigate variations in distribution and abundance of individual phospholipids we subjected homogenate and LP from wild type to glycerophospholipid analysis (Table 3). In glucose grown cells, the major phospholipids from the homogenate were phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) with PC being the most abundant. These major phospholipids constituted approximately 90 % of total cellular

phospholipids. Only minor amounts of phosphatidic acid (PA), phosphatidylserine (PS) and cardiolipin (CL) were detected.

Table 3: Pospholipid composition of homogenate and LP isolated from cells grown on glucose (YPD) or oleate (YPO)

	% of total phospholipids			
	Homogenate		Lipid particle	
	YPD	YPO	YPD	YPO
PA	2.8 ± 0.4	0.7 ± 0.7	1.8 ± 1.3	1.3 ± 2.7
PI	14.5 ± 5.9	16.9 ± 3.8	21.5 ± 3.4	21.5 ± 3.4
PS	3.8 ± 0.4	3.3 ± 0.9	2.1 ± 2.6	0.8 ± 0.9
PC	51.1 ± 5.5	53.0 ± 1.4	57.5 ± 1.7	56.4 ± 2.7
PE	23.6 ± 1.4	20.1 ± 3.7	16.6 ± 1.9	16.9 ± 2.8
CL	2.3 ± 0.3	3.7 ± 0.8	0 ± 0	1.0 ± 1.2
LP	0 ± 0	0.3 ± 0.6	0.3 ± 0.6	0.7 ± 0.5
DMPE	1.9 ± 1.2	1.5 ± 1.4	0 ± 0	1.3 ± 1.9

Homogenates from cells grown on oleate showed the same tendency with slight variations. Again, PC, PE and PI were the major phospholipids with even higher amounts of PC. The level of PE decreased for 3.5 %, whereas PI increased for 2.5 %. The amounts of PS and CL did not change much, but PA dropped to levels close to detection limits. In LP from cells grown on YPD, PC, PE and PI were also the most prominent glycerophospholipids. Interestingly, levels of PC and especially PI were higher and that of PE lower in LP than in the homogenate. Other phospholipids occurred only at negligible amounts in LP. When cells were grown on oleate, the phospholipid pattern of LP was very similar to that of cells grown on YPD. The only marked exception was PS which dropped from ~2 % on YPD to ~0.8 % on YPO.

Mass spectrometric analysis of phospholipids from homogenates and lipid particles

As described above no major differences were observed in the pattern phospholipid classes of LP from cells grown on glucose and oleate. These experiments, however, did not include species analysis. Therefore, we extended our studies to mass spectrometry of phospholipids from homogenates and lipid particles. The fact that major fatty acids of the yeast *Saccharomyces cerevisiae* are of the C16 and C18 types makes the species patterns rather simple. Therefore, as expected, PC, PE, PS and PI from cells grown on glucose showed a strong preference for C16 and C18 in their mono-unsaturated or saturated form (Figure 2).

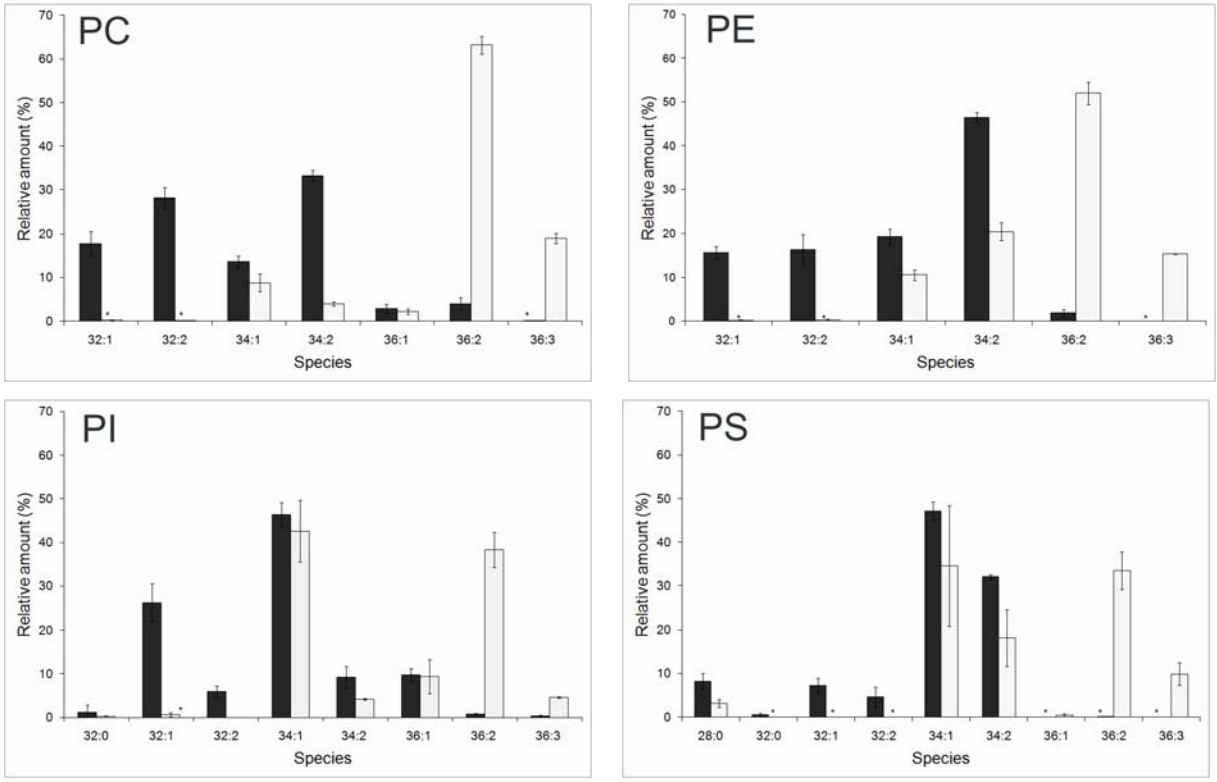


Figure 2: Molecular composition of phospholipid species from homogenates

Lipid extracts of homogenate from wild type cells grown on either glucose (dark bar) or oleate (white bar) were analysed by MS for phospholipid (PE, PC, PI, PS) species. Data are mean values from at least 2 independent experiments. *= values are ≤ 0.1%

Nevertheless, the species patterns of individual phospholipids varied in a most typical way. PE showed a strong preference for 34:2 species (C16:1/C18:1), whereas in PC 32:2 and 34:1 occurred at a similar level. Only small amounts of 36:2 (C18:1/C18:1) were detected in PE and PC. Contrary to PE and PC, PI and PS showed a strong preference for 34:1 species. In PI the second abundant species was 32:1, whereas in PS 34:2 occurred as the other major species. Another interesting observation was the presence of substantial amounts of 36:1 in PI. This species was also found in PC, but not in PS and PE. PS also contained substantial amounts of 28:0 which is most likely composed of C16:0 and C12:0.

Mass spectrometry of phospholipids from homogenates of cells grown on oleate showed a completely different distribution of fatty acyl species. Not surprisingly, the incorporation of oleic acid (C18:1) into phospholipids was high under these cultivation conditions. The major phospholipids behaved more or less in pairs. PC and PE strongly preferred oleic acid as a constituent (see Figure 2) resulting in a strong appearance of 36:2 species.

This effect was most pronounced in PC, whereas in PE C18:1 was combined with C16:1 (34:2 species). Species patterns of PI and PS from cells grown on oleate were different. Although in both phospholipids oleic acid was highly present in the di-unsaturated species 36:2, the 34:1 species (C16:0/C18:1 or C16:1/C18:0) occurred at an almost equal level. In PS and PE 34:2 was another prominent combination of fatty acids. The appearance of 36:3 species in all phospholipids was due the impurities of oleate samples used a carbon source as described above.

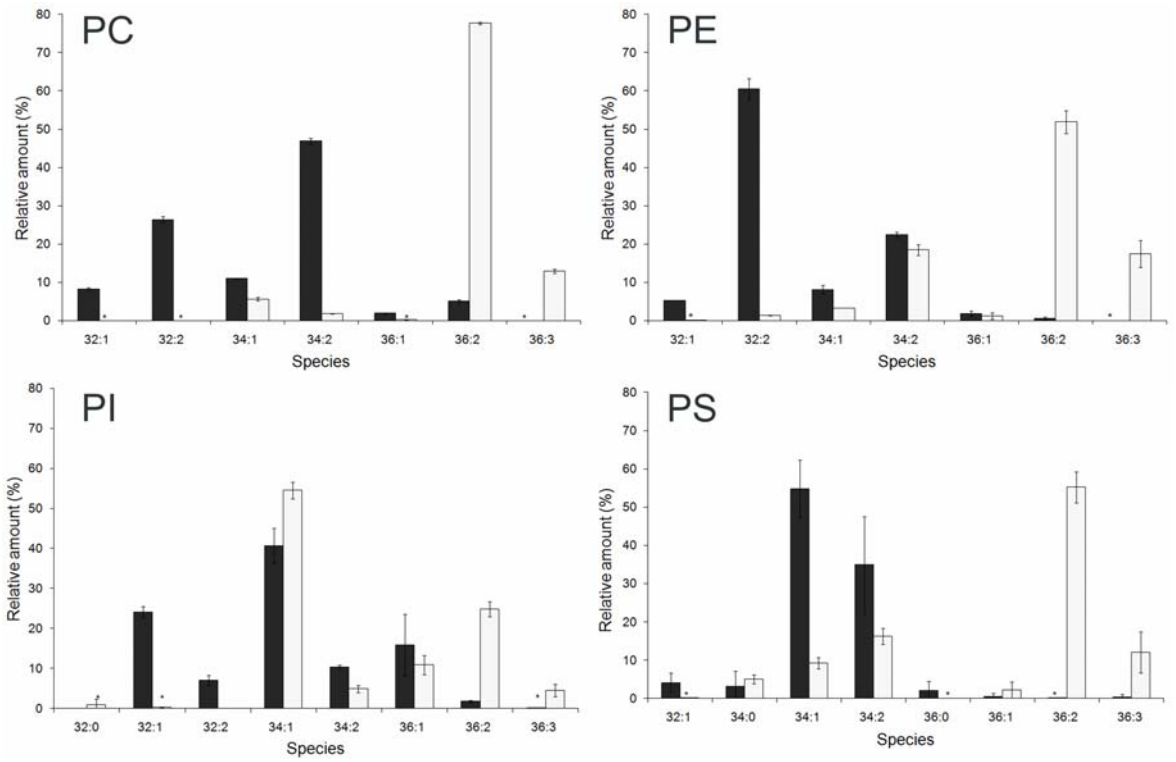


Figure 3: Molecular composition of phospholipid species from lipid particles.

Lipid extracts of LP from wild type cells grown on either glucose (dark bar) or oleate (white bar) were analysed by MS for phospholipid (PE, PC, PI, PS) species. Data are mean values from at least 2 independent experiments. *= values are ≤ 0.1%

When phospholipid species from LP of cells grown on glucose (Figure 3) were analyzed we realized that in most cases the species patterns reflected very much those of phospholipids from total cell extracts (see Figure 2) following the equilibrium rule “You take what you get”. However, one major exception was the large amount of 32:2 PE in LP which did not occur in the homogenate. Additionally, the two fully saturated species 34:0 (C16:0/C18:0) and 36:0 (C18:0/C18:0) appeared in PS from the LP fraction, which were obviously below the

detection limits in the total cell extract. Differences between the phospholipid species patterns of LP and homogenate from YPO grown cells were mainly in the PI and PS fractions.

Whereas PE and PC from LP exhibited a similar species pattern as the homogenate, the amount of 34:1 PI in LP was increased at the expense of 36:2 PI, and the level of 36:2 PS in LP was much higher than in homogenate. However, despite the strong influence of exogenous oleic acid on the formation of phospholipid species, the individual phospholipid classes retained at least some typical constituents.

Table 4: Degree of saturation of phospholipids from homogenate and LP isolated from cells grown on glucose or oleic acid

	% of saturated fatty acids			
	Homogenate		Lipid particle	
	Glucose	Oleate	Glucose	Oleate
PE	17.5	5.4	7.6	2.3
PC	17.2	5.6	10.6	3.0
PI	42.4	26.5	40.4	33.8
PS	35.9	20.7	34.9	10.8

Whereas species analysis of phospholipids yields a precise information of molecular components, the ratio of saturated to unsaturated species gives us an overview of properties which may be important for the physical status of membranes formed from different phospholipid species. It is remarkable that the different phospholipid classes exhibited marked differences in their degree of saturation (Table 4). As the most striking example, PI is the phospholipid containing most saturated fatty acids followed by PS irrespective of the carbon source used for the cultivation of cells. In LP from cells grown on glucose a slight selectivity seems to occur insofar as more unsaturated PE and PC species accumulate compared to the homogenate. Oleate as a carbon source led to a more than 3 fold reduction of saturated PE and PC species, but only to a moderate although pronounced effect on PI and PS. Also in oleate grown cells, LP attracted more unsaturated PE and PC species than bulk membranes, but this effect was also observed with PS. In contrast, the level of saturated PI species in LP remained high even when oleate was present as carbon source.

Influence of the carbon source on the pattern of lipid particle proteins

During the last decade, the yeast LP proteome was studied based on mass spectrometry (Athenstaedt *et al.*, 1999) and localization of GFP-tagged proteins (http://www.yeastgenome.org/12182009; http://yeastgfp.yeastgenome.org/12182009). Currently, 41 LP proteins were identified by these criteria (Table 5). Interestingly, most of these proteins are not exclusively localized to the LP but seem to have a dual localization with additional occurrence in microsomal and mitochondrial fractions.

Table 5: Proteome of yeast LP

[§]novel LP protein, found on glucose as well on oleate grown cells; [¥] numbers indicate fragments used for identification of proteins from cells grown on glucose (D) or oleate (O). C, cytosol; M, mitochondria; PM, plasma membrane; ER, endoplasmic reticulum; LP, lipid particle; End, endosomes; G, golgi; Mic, microsomes; V, vacuole; Px, peroxisome; N, nucleus; nEnv, nuclear envelope; ext, extrinsic to membrane; mem, integral to membrane; bud, cellular bud; rib, ribosomal subunit; CW, cell wall; R, ribosome; Retro, retrotransposon; mTub, microtubule

Gene name	Systematic name	SGD GFP YPL MIPs	This study	D[¥]	O[¥]	Localisation (SGD)	Description
ACH1	YBL015W		✓		2	C / M	CoA transferase activity
ADH1	YOL086C		✓		2	C / PM	Alcohol dehydrogenase
ALG9	YNL219C		✓	2		ER	Mannosyltransferase
ATF1	YOR377W	✓				LP / End	Alcohol acyltransferase
ATP2	YJR121W				9	M	Subunit of mitochondria ATP synthase
AYR1	YIL124W	✓ ✓	✓	15	12	C / ER / LP / M	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase
BSC2	YDR275W	✓ ✓ ✓				LP	Unknown
COY1	YKL179C	✓ ✓				G	Golgi membrane protein
CPR5[§]	YDR304C		✓	7	3	C / ER	Peptidyl-prolyl cis-trans isomerase
CSR1	YLR380W	✓				LP / C / M / Mic	Phosphatidylinositol transfer protein
CST26	YBR042C	✓ ✓ ✓ ✓				LP	Required for incorporation of stearic acid into phosphatidylinostiol
CWH43	YCR017C		✓	2		PM	Putative sensor/transporter protein
DFM1	YDR411C		✓	2		ER	ER localized derlin-like family member
DGA1	YOR245C	✓	✓		3	LP	Diacylglycerol acyltransferase
DPL1	YDR294C		✓	2		ER	Dihydrosphingosine phosphate lyase
DPM1	YPR183W		✓	2		ER / M	Dolichol phosphate mannose synthase

EHT1	YBR177C	✓		✓	✓	✓	19	18	LP / M	Acyl-coenzymeA:ethanol O-acyltransferase
ENO2	YHR174W					✓		2	V / PM / M	Enolase II
ERG1	YGR175C	✓		✓	✓	✓	5	15	ER / LP	Squalene epoxidase
ERG6	YML008C	✓	✓	✓	✓	✓	37	30	ER / LP / M	Delta(24)-sterol C-methyltransferase
ERG7	YHR072W	✓	✓	✓	✓	✓	7	2	ER /LP / PM	Lanosterol synthase
ERG27	YLR100W		✓	✓	✓	✓	9	4	ER / M	3-Keto sterol reductase
FAA1	YOR317W	✓				✓	20	15	LP / PM / M	Long chain fatty acyl-CoA synthetase
FAA3	YIL009W					✓	3		unknown	Long chain fatty acyl-CoA synthetase
FAA4	YMR246W	✓	✓	✓	✓	✓	13	6	LP / C	Long chain fatty acyl-CoA synthetase
FAT1	YBR041W	✓	✓	✓	✓	✓	13	9	PM / LP / Mic / PX	Fatty acid transporter
FMP52	YER004W					✓		4	ER / M	Unknown
GPT2	YKR067W					✓	12		C / ER	sn-1 Acyltransferase
GTT1[§]	YIR038C					✓	2	8	ER / M / PM	glutathione S-transferase
GVP36	YIL041W					✓		3	C / G	BAR domain-containing protein
HFD1	YMR110C	✓	✓		✓	✓	7	18	M / LP / End	Putative fatty aldehyde dehydrogenase
HSP12	YFL014W					✓		6	C / PM / N	Heat shock protein
KAR2	YJL034W					✓	9		ER	ATPase
LAP4	YKL103C					✓		3	V	Vacuolar aminopeptidase
LDB16	YCL005W	✓	✓		✓				LP / M	Unknown
MSC1	YML128C					✓	2		M / ER / PM	Unknown
NUS1	YDL193W	✓		✓		✓	9	4	ER / LP /nEnv	Putative prenyltransferase
OSH4[§]	YPL145C						3	5	C / G / ext	Oxysterol binding protein
OSW5	YMR148W		✓		✓	✓	2		mem	Unknown
PDI1	YCL043C					✓	6	7	ER	Disulfide isomerase
PDR16	YNL231C	✓	✓		✓	✓	5	9	LP / Mic / PM / C	Phosphatidylinositol transfer protein

PET10	YKR046C	✓ ✓ ✓ ✓	✓	12	24	LP	Unknown
PGC1	YPL206C	✓	✓	9		LP / M	Phosphatidylglycerol phospholipase C
PIL1	YGR086C	✓				C / M / PM	Primary component of eisosomes
PMA1	YGL008C		✓		3	PM / M / mem	Plasma membrane H+-ATPase
PMT1	YDL095W		✓	2		ER	Protein O-mannosyltransferase
PMT2	YAL023C		✓	3		ER	Protein O-mannosyltransferase
POR1	YNL055C		✓		2	M	Mitochondrial porin
POX1	YGL205W		✓		3	PX	Fatty-acyl coenzyme A oxidase
RHO1	YPR165W		✓	4		mem / PX / PM / M / bud	GTP-binding protein
RPL5	YPL131W		✓		3	rib	Protein component of the large (60S) ribosomal subunit
RPL10	YLR075W		✓		2	rib	Protein component of the large (60S) ribosomal subunit
RPS1B	YML063W		✓		2	rib	Ribosomal protein 10 (rp10) of the small (40S) subunit
RPS3	YNL178W		✓		5	rib	Protein component of the small (40S) ribosomal subunit
RPS19B	YNL302C		✓	2		rib	Protein component of the small (40S) ribosomal subunit
RPS31	YLR167W		✓		4	rib / C	Fusion protein that is cleaved to yield a ribosomal protein of the small (40S) subunit and ubiquitin
RRT8	YOL048C	✓ ✓ ✓	✓	4	2	LP	Unknown
RTN2	YDL204W		✓	3		ER / nEnv	Unknown
SEC61	YLR378C		✓	5		ER	Essential subunit of Sec61 complex
SEC63	YOR254C		✓	4		ER / M	Essential subunit of Sec63 complex

SHE10	YGL228W				✓	4		unknown	Putative glycosylphosphatidylinositol (GPI)-anchored protein of unknown function
SLC1	YDL052C	✓		✓	✓	✓	2	3	LP	1-Acyl-sn-glycerol-3-phosphate acyltransferase
SNA2	YDR525W-a	✓	✓		✓				Mem / C	Unknown
SNX41	YDR425W	✓			✓				End	Sorting nexin
SRT1	YMR101C	✓							LP	Cis-prenyltransferase
SSA1	YAL005C					✓		2	C / PM / N	ATPase
SSO1	YPL232W		✓		✓				PM	Plasma membrane t-snare
TDH1	YJL052W	✓				✓		4	C / LP / M / PM / CW	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 1
TDH2	YJR009C	✓							C / LP / M / PM / CW	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 2
TDH3	YGR192C	✓				✓		5	C / LP / M / PM / CW	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3
TEF1	YPR080W							5	M / R	Translational elongation factor EF-1 alpha
TGL1	YKL140W	✓			✓	✓	12	14	LP / mem	Steryl ester hydrolase
TGL3	YMR313C	✓	✓		✓	✓	4	5	LP	Triacylglycerol lipase
TGL4	YKR089C	✓	✓		✓	✓	3	4	LP	Triacylglycerol lipase
TGL5	YOR081C	✓	✓		✓	✓	5	3	LP	Triacylglycerol lipase
USE1	YGL098W		✓		✓				ER	SNARE protein
TSC10	YBR265W					✓	2		C / ER / M	3-ketosphinganine reductase
TUB2	YFL037W					✓		2	mTub	Beta-tubulin
UBX2[§]	YML013W					✓	5	3	ER / M	Protein involved in ER-associated protein degradation
VPS66[§]	YPR139C					✓	6	4	C	Cytoplasmic protein of unknown function involved in vacuolar protein sorting

WBP1	YEL002C		✓	3		ER / nEnv	Beta subunit of the oligosaccharyl transferase (OST) glycoprotein complex
YBR204C	YBR204C		✓		3	unknown	Serine hydrolase
YDR018C	YDR018C	✓				unknown	Similarity to acyltransferase
YEH1	YLL012W	✓ ✓ ✓				LP / mem	Steryl ester hydrolase
YIM1	YMR152W	✓ ✓	✓		4	LP / C / M	Unknown
YGR038C-B	YGR038C-B		✓	2		Retro	Retrotransposon TYA Gag and TYB Pol genes
YJU3	YKL094W	✓ ✓ ✓	✓	3	2	LP / C / M / PM	Serine hydrolase
YNL134C	YNL134C		✓		2	C / N	Unknown
YNL208W	YNL208W		✓		3	M / R	Unknown
YOR059C	YOR059C	✓				LP	Unknown
YOR246C	YOR246C	✓ ✓ ✓				LP	Similarity to oxidoreductase
YPT7[§]	YML001W		✓	4	2	V / M	GTPase
ZEO1	YOL109W		✓		9	M / PM / ext	Peripheral membrane protein

Previous experiments (our own unpublished data) had led us to the assumption that the existing list of LP proteins is not complete. We assumed that due to methodological limitations LP proteins of low abundance escaped identification so far. Therefore, we re-addressed this issue by employing a novel proteomic method of nano-LC-MS (see Methods section). Moreover, we tested whether change of growth conditions (YPD vs. YPO) had an impact on the LP protein pattern.

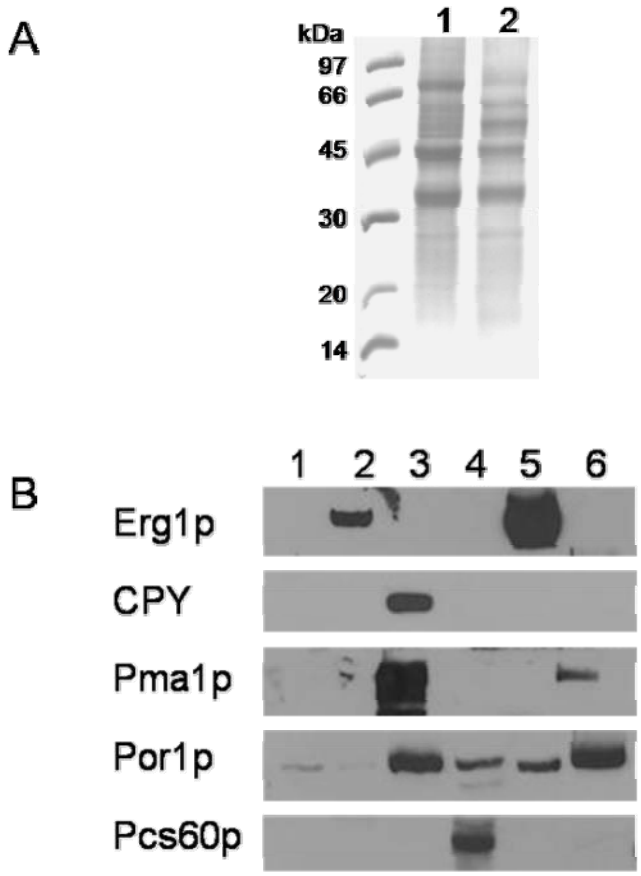


Figure 4: Protein analysis and quality control

(A) Protein patterns of lipid particle fraction from *Saccharomyces cerevisiae* wild type cells grown on glucose (1) or oleic acid (2). Low molecular weight standard was loaded in the first lane. Lanes were loaded with 15 µg total protein, each.

(B) Quality control of subcellular fractions from *Saccharomyces cerevisiae* wild type cells. Western blot analysis of cell fractions from BY4741 grown on glucose (1-3) and oleic acid (4-6). Homogenate (1, 4), lipid particle (2,5) and vacuole (3,6). Lanes were loaded with 15 µg total protein, each.

Protein samples were generated from LP originating from cells that were either grown on glucose or oleic acid as the carbon source. An initial analysis for these samples by SDS-Page (Fig. 4A) revealed already qualitative differences between the two LP variants. Both LP preparations were of high purity (Fig. 4B) which was important for the further proteome analysis.

A primary survey obtained data by NanoLC-MS is shown in Figure 5. With LP from cells grown on glucose 49 proteins were identified; 22 of these polypeptides had already been assigned to LP. Analysis of LP prepared from oleic acid grown cells resulted in the identification of 54 proteins; 24 of these proteins were already known as LP constituents. All proteins identified from glucose grown cells were also found with oleic acid as carbon source. It is tempting to speculate that these 20 proteins are the most abundant and standard proteins of LP.

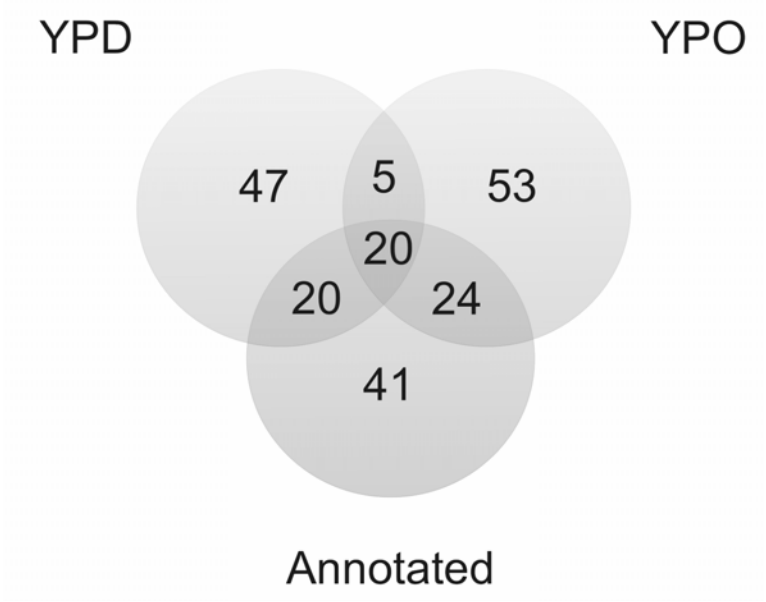


Figure 5: Venn diagram displaying results of MS-analysis of LP

The graph summarizes LP protein analysis from cells grown on glucose (YPD) and oleate (YPO).

In addition to the known set of LP proteins some other polypeptides were identified as novel although putative LP proteins. Surprisingly, only 6 proteins (see Table 5, proteins marked with §) that were so far not assigned to LP were identified on both variants (YPD and YPO) of LP, namely Cpr5p, Gtt1p, Osh4p, Ubx2p, Vps66p and Ypt7p. In data bases, these proteins had been assigned to other subcellular compartments, mainly the cytosol and the ER. Two aspects have to be taken into account for further and more precise localization of these proteins. First, we have to be aware of the dual localization of LP proteins as shown before which made biochemical and microscopic analysis difficult. Since LP are believed to originate from the ER (Rajakumari *et al.*, 2008) such localization scenarios may be expected. Secondly, of course, contamination of LP with other subcellular compartments has to be considered. This problem has to be ruled out by precise molecular investigations.

So far, proteins identified by this proteomic approach were not only assigned to LP, but also to the ER, mitochondria, cytosol and plasma membrane (Figure 6). Comparing localization patterns of the newly found proteins revealed that small variations can be seen depending on the carbon source used for cultivation.

Interestingly, the percentage of potential ER resided proteins was strongly reduced when LP from oleic acid grown cells were investigated, whereas the portion of proteins assigned to the cytosol was doubled. Notably, several ribosomal and nuclear proteins were found associated with LP when cells were grown on oleic acid. Another interesting aspect is function of proven and putative LP proteins. A large number of LP proteins are involved in lipid metabolism both from YPD and YPO grown cells (Figure 7). Other functions attributed to these proteins were protein glycosylation, cell wall organization or ER unfolded protein response. For a remarkably large number of proteins biological functions have not been annotated. Several clusters of proteins appear to be strongly affected by the change of carbon source. As examples, proteins involved in ER unfolded protein response were only found in LP from cells grown on YPD, and the big portion of proteins involved in protein glycosylation was drastically reduced in cells grown on YPO. In contrast, a large number of proteins involved in translation and energy providing processes were found in LP from cells cultivated on oleic acid

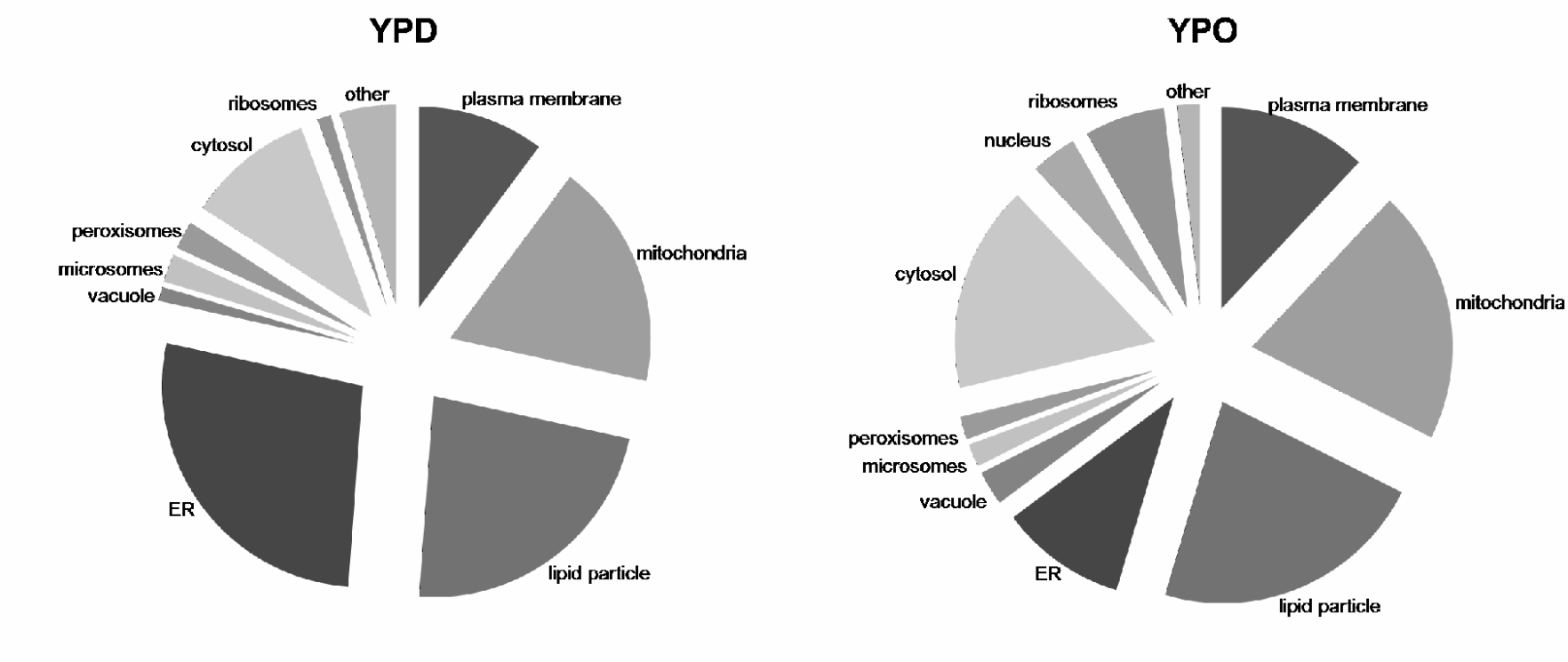


Figure 6: Assignment to subcellular fractions of LP proteins from wild type cells grown on glucose (YPD) or oleate (YPO)

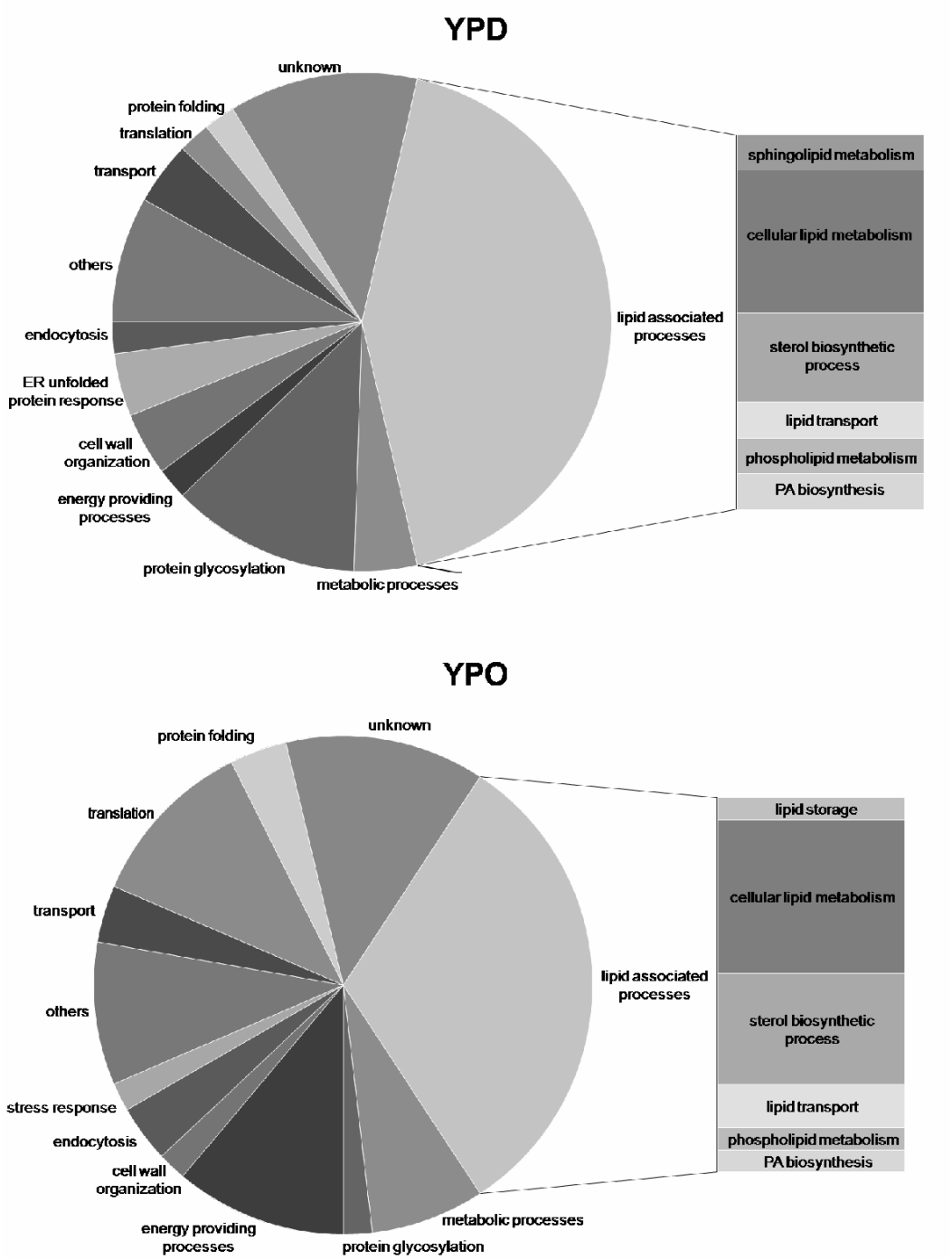


Figure 7: Assignment of newly found proteins of LP from wild type cells grown on glucose (YPD) or oleate (YPO) to biological processes

Identification of novel LP proteins

In our approach we found six novel proteins that have not been annotated for LP localization so far (Fig. 5 and Table 5). To verify data obtained by MS analyses, we used a setup of GFP constructs obtained from Invitrogen (Table 1) to further characterize and verify localization of the six new LP proteins. GFP was genomically tagged to the C-terminus of the proteins which were expressed under their endogenous promoters. This should minimize mislocalization due to overexpression. Yeast strains were grown in the presence of either glucose (YPD) or oleic acid (YPO) until early stationary phase and cell fractionation was performed as described in Material and Methods section. We found that five of the six constructs could be detected by Western blotting (Figure 8A and B). From these proteins, Cpr5p, Vps66p and Gtt1p were detected on both carbon sources clearly in the LP fraction (Figure 8A).

Interestingly, in the yeast strain expressing Cpr5p-GFP, an additional GFP signal was found in vacuoles when cells were grown on oleate. However, this signal was significantly smaller (approx. 30 kDa) than the Cpr5p-GFP construct indicating that Cpr5p-GFP is most likely transported to and depredated in vacuoles under oleic acid conditions but not when cells were grown on glucose. We suppose that special sorting mechanisms might be involved that control protein composition on LP under different conditions. It is, however, unknown, how these sorting mechanisms function and neither which cellular processes are involved. Protein Ypt7p-GFP was not detected under the conditions tested, however, this does not exclude localization on LP but can be due to low expression below the detection limit of the Western blotting. Two proteins of interest, Ubx2p-GFP and Osh4p, were also detected on the LP isolated from cells grown on oleic acid supplemented media (Figure 8B). To further characterize localization of the protein Osh4p, we performed multiple cell fractionations from cells grown on YPD as well as on YPO. Western blot analyses of the fractions confirmed localization of Osh4p-GFP mainly in the LP (Figure 8C). Interestingly, cells grown on glucose also contained some amount of Osh4p-GFP in the ER whereas cells grown on oleate showed no localization of Osh4p-GFP in the ER fraction but rather occurrence in the cytosol and to some extent in the vacuole. Since Osh4p has been claimed to be cytosolic protein (Fang *et al.*, 1996; Li *et al.*, 2002) these data indicate a diverse localization of Osh4p in the cell which has to be further analyzed. Further detailed studies on the other new LP proteins have to be carried out to confirm their localization on LP. In addition, functional studies should reveal their role on LP and the impact of multiple localizations within the cell.

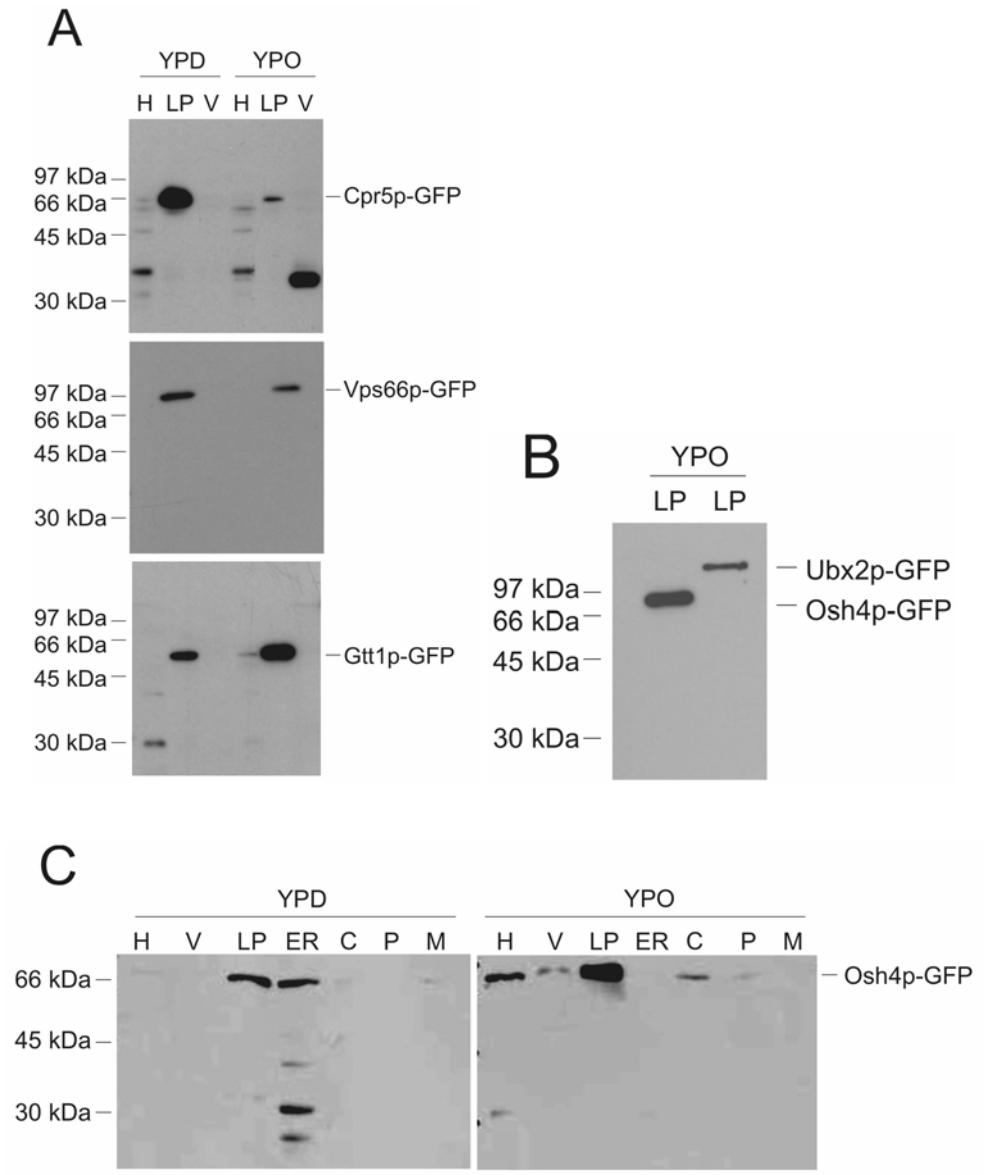


Figure 8: Localization studies of putative new Lipid particle proteins

Yeast strains containing genomically tagged GFP constructs were grown in liquid media in the presence of either glucose or oleate until early stationary phase. Cell fractionation was performed as described in Material and Methods. Presence of the respective protein was analyzed by SDS-PAGE and Western blotting using anti-GFP monoclonal antibody ().

(A) Genomically tagged GFP constructs of CPR5, VPS66 and GTT1 were expressed during growth on glucose or oleate as indicated. Cell fractionations were analysed by Western blotting for the occurrence of respective protein.

(B) LP from cells containing either UBX2-GFP or OSH4-GFP grown in the presence of oleate were analyzed by Western blotting for the presence of respective protein.

(C) Organelles from yeast strain harbouring OSH4-GFP were isolated from cells grown on glucose (YPD) or oleate (YPO). Western blotting analyses was performed to confirm localization of Osh4p-GFP construct in the different cell fractionations. H, homogenate; V, vacuole; LP, lipid particle; ER, 40,000xg microsomes; P, peroxisomes; M, mitochondria

In summary, the data provided in this study clearly show that the proteome of LP is not completely understood. With the method used in this approach we found novel LP proteins whose localization on LP was partly confirmed by independent methods. Together our data indicate that LP are a highly dynamic organelle, changing lipid as well as protein profile to environmental conditions. Underlying mechanisms are, however, unclear and have to be investigated in more detail in future studies.

Discussion

Lipid particles (lipid droplets, oil bodies) have gained much attention during the last decade due to their role as lipid buffer and involvement in human diseases and plant oil storage. Moreover, we are at the beginning to understand the metabolic functions that might be governed by LP. For all these reasons knowledge about structure and molecular equipment of LP is required. We are using the yeast *Saccharomyces cerevisiae* as a model for such studies because of the parallel features to other experimental systems and the convenience of genetic and nutritional manipulation of this microorganism.

Lipid particle lipidome

In the past, several attempts were made to obtain an overview of the lipid pattern of LP (Zinser *et al.*, 1993; Leber *et al.*, 1994; Schneiter *et al.*, 1999). These studies were mainly meant to explain lipid storage in this organelle. More recently, the technology of mass spectrometry enabled us to study lipid components of LP in molecular detail (Connerth *et al.*, 2009). These methods are based on systematic lipidome analyses of yeast lipids as published by Ejsing *et al.* (Ejsing *et al.*, 2009) and Xue Li Guan and Wenk (Guan and Wenk, 2006).

Recent studies from our laboratory (Rosenberger *et al.*, 2009; Connerth *et al.*, manuscript under revision) and by others (Petschnigg *et al.*, 2009; Garbarino and Sturley, 2009) showed that growth of yeast cells on oleate (YPO medium) greatly stimulated the proliferation of yeast LP. Under these conditions, the requirement for fatty acid storage increases and makes LP to a more prominent cell organelle. Hence, we decided to expand our studies of LP component analysis to variation of culture conditions, namely growth of cells on glucose or oleic acid. Two observations made with TAG from cells grown on glucose were interesting. First, there seems to be some selectivity regarding the fatty acid pattern of TAG (see Fig. 1) compared to total lipids insofar as TAG contain more C18:1 (oleic acid) than bulk lipids. The reason for this finding may be preference of the TAG synthesizing enzymes Dga1p and Lro1p (Oelkers *et al.*, 2002; Czabany *et al.*, 2007) for the respective substrates. Secondly, and result of the first observation was that a large portion of TAG contains unsaturated fatty acids. This portion of storage lipids appears to have a rather high degree of fluidity. The most obvious effect of growing cells on YPO was the huge increase in the amount of TAG, whereas the amounts of STE decreased dramatically close to detection limits.

We showed recently that neither a transcriptional regulation of the STE synthesizing enzymes Are1p and Are2p nor any posttranslational modification was the reason for the depletion of STE (Connerth *et al.*, manuscript under revision). We demonstrated that free oleic acid directly inhibits STE synthases. Interestingly, oleic acid had the opposite effect on the pattern of non-polar lipids in another yeast. Athenstaedt *et al.* (Athenstaedt *et al.*, 2006) observed in the yeast *Yarrowia lipolytica* grown on oleate a decrease of TAG but more STE compared to LP from glucose-grown cells. The other effect of oleate as a carbon source for *Saccharomyces cerevisiae* was that this fatty acid was obviously not only degraded by β-oxidation in peroxisomes under these conditions (Hiltunen *et al.*, 2003) but also directly incorporated as building block of complex lipids. This effect led to large amounts of oleic acid in TAG.

Although phospholipids are only a minor component of LP they may play an important role of this organelle. First, phospholipids of the LP surface monolayer determine the contact of the highly hydrophobic particle to the hydrophilic environment. Therefore, an appropriate shielding is required. Second, the small set of proteins embedded in the surface phospholipid monolayer of LP may need a specific membrane environment for functionality. Since we do not know details about the enzymology of LP proteins we can only speculate on this point. Finally, the surface membrane lipid composition is most likely result of the biogenesis process of LP. If we accept budding of LP from the ER as a realistic model for LP biosynthesis, at least large parts of the LP surface membrane have their origin in the ER. A combined selectivity for phospholipids and proteins (see below) may be anticipated.

The question arises whether or not there is something specific with LP phospholipids. As can be seen from Table 3, PI seems to be significantly enriched in LP, mainly at the expense of PS and PE. Given the fact that PI and PS belong to the group of negatively charged phospholipids, a replacement would most likely not lead to dramatic effects. However, the decrease of PE which is known as a bilayer disturbing phospholipid appears to make sense in the monolayer membrane, where such an effect may become superfluous. Whether or not the enrichment of 32:2 PE species in LP has a specific effect remains open. Enrichment of PI on the LP surface may increase the charge in the monolayer. It is known that inositol phospholipids including PI are also important for interfacial binding of proteins and regulating proteins at the cell interface. As PI is polyanionic, it can be very effective in creating unspecific electrostatic interactions with proteins (Shields and Arvan, 1999;

Gardocki *et al.*, 2005). The other remarkable feature of PI in total cell extracts and LP is its high degree of saturation (see Table 4).

In both fractions only minor amounts of double unsaturated PI species were detected. Specific requirements for PI may explain its stable amount and composition. Interestingly, PS exhibits a similar degree of saturation as PI. Biosynthesis of both phospholipids starts with the same precursor, CDP-DAG, catalyzed by two different enzymes although with similar substrate preference (Carman and Henry, 1999). During decarboxylation of PS by Psd1p and Psd2p some selectivity is introduced (Schuiki and Daum, 2009). It is known that PSDs exhibit a clear preference regarding the molecular species of their substrate (Burgermeister *et al.*, 2004) resulting in a higher degree of unsaturation of the product which is PE. A further step of selectivity is the stepwise methylation of PE which leads to the final product of the aminoglycerophospholipid pathway, PC. It has to be taken into account, however, the PE an PC are also formed through the CDP-Etn and CDP-Cho branches of the Kennedy Pathway with a different substrate usage and specificity (Kennedy and Weiss, 1956). Recently, a further pathway of PE formation involving the lyso-PE acyltransferase Slc4p was found (Jain *et al.*, 2007; Riekhof *et al.*, 2007) which may also contribute to the composition of the total cellular PE pool.

When yeast cells were grown on oleate, the total amount of phospholipids (see Table 2) was markedly increased. This increase was observed both with homogenate and the LP fraction. Interestingly, in the homogenate levels of PI and PC increased at the expense of PE. A similar effect was described for cells that showed a defect in unsaturation of fatty acids in an *rsp5* mutant(Kaliszewski *et al.*, 2008). Rsp5p strongly influenced the regulation of unsaturated fatty acid biosynthesis by governing the activation of two transcriptional activators Spt23p and Mga2p which in turn up-regulate the expression of the *OLE1* gene. In *rsp5-19* representing a low oleate condition the TAG level decreased whereas overproduction of the activator (high oleate conditions) led to an increase of TAG (Kaliszewski *et al.*, 2008). Neither a mutation in *RSP5* nor overproduction of the two activators mentioned above affected the level of STE.

Proteome of yeast lipid particles

The existing yeast LP proteome contained a small but specific set of 41 proteins (Athenstaedt *et al.*, 1999; Zweytick *et al.*, 2000). These proteins are embedded in the phospholipid monolayer of the lipid droplet. To our recent knowledge, yeast LP do not contain typical organelle proteins such as oleosins in plants (Capuano *et al.*, 2007) or perilipins in mammalian cells (Greenberg *et al.*, 1991; Greenberg *et al.*, 1993). The number of LP in different cells types is steadily increasing as methods employed get improved. As a recent example, Bartz *et al.* (Bartz *et al.*, 2007) expanded the proteome of mammalian lipid droplets by 70 further proteins.

The proteome approach with yeast LP presented here was aimed at the extension of the number of LP proteins but also to compare yeast LP proteins in cells growth under different conditions, namely on glucose or oleic acid. Oleic acid is the classical substrate to induce peroxisomes in *Saccharomyces cerevisiae*, (Smith *et al.*, 2002; Gurvitz and Rottensteiner, 2006; Smith *et al.*, 2006; Saleem *et al.*, 2008). However, oleic acid also leads to massive proliferation of LP due to the huge production of non-polar lipids, especially TAG, as described also in this study. The dramatic changes in the yeast LP lipidome of cells grown on oleate led us to speculate that also the LP proteome may be affected. Differences in LP proteins in lipid variants of this organelle have been shown before by Czabany *et al*. (Czabany *et al.*, 2008).

Interesting questions rising from this study were: (i) Do LP from YPD and YPO grown cells contain the same sets of proteins? (ii) Are there specific LP proteins present only in LP from YPD or YPO grown cells, respectively? If this was the case, can we learn something about the change in LP associated metabolism caused by the change of the substrate from (possible) functions of these specific LP proteins? (iii) Finally, does this strategy allow us to identify novel LP proteins.

Answers to the questions mentioned above can only be given in part. Uncertainties are due to the fact that despite the sophisticated method of proteome investigation employed in this study some polypeptides may have escaped detection. Moreover, slight contamination of LP with other organelles may still lead to false results. Although all samples used for protein mass spectrometry were rigorously tested for cross-contamination we are aware of the fact that 100 % purity can never be warranted. Despite these caveats, data summarized in Table 6 provide some valuable and novel information. In LP from YPD grown cells 49 polypeptides were identified, and in LP from YPO grown cells 54 proteins. As can be clearly seen from

Table 6 and Fig. 4, only 26 of these proteins occurred in both LP variants. Interestingly, most of these proteins are involved in lipid metabolism, such as Erg-proteins, lipases or fatty acid activating proteins. Six proteins detected in both types of LP have not been attributed to LP before, namely Cpr5p, Gtt1p, Osh4p, Ubx2p, Vps66p, Ypt7p. Two of these proteins, Osh4p and Vps66, were previously found to be localized to the LP of *Yarrowia lipolytica* (Athenstaedt *et al*., 2006). Preliminary results employing GFP-fluorescence microscopy showed that in *S. cerevisiae* Gtt1p, Osh4p, Vps66p and Ypt7p formed distinct punctuate structures when grown to stationary phase on glucose containing media (data not shown). Localization to LP was then also verified by sucellular fractionation and Western blotting (Figure 8). Interestingly, some dual localizations were found as well as carbon source dependent localization shifts.

The question as to the occurrence of specific groups of proteins in LP from YPD or YPO grown cells, respectively, remained. The major reason for this uncertainty is the insufficient annotation of many of these orphan proteins. Gene ontology annotations of the newly found putative LP proteins revealed that they covered a broad spectrum of biological processes under both growth conditions. One big group is associated with lipid metabolic processes. Surprisingly, a shift towards oleic acid brought about a decrease in proteins of this kind. On the other hand, a remarkable increase in proteins involved in processes of translation was found in LP from YPO grown cells. It has also to be noted that hardly any protein is known that is explicitly localized to LP. Erg6p for example, a very prominent LP protein, is known to localize to ER (McCammon *et al.*, 1984) and mitochondria (Sickmann *et al.*, 2003) besides the LP (Athenstaedt *et al.*, 1999). Another example is the long chain fatty acyl-CoA synthase Faa1p which localized to the LP (Athenstaedt *et al.*, 1999), mitochondrion (Sickmann *et al.*, 2003) and the plasma membrane (Delom *et al.*, 2006). This characteristic property of LP proteins complicates a direct assignment of putative new proteins to this cellular compartment, and data have therefore to be interpreted with caution.

Acknowledgements

This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (projects 18857 and W901-B05 to G.D.).

Reference List

Athenstaedt,K. and Daum,G. (2006). The life cycle of neutral lipids: synthesis, storage and degradation. Cell Mol. Life Sci. *63*, 1355-1369.

Athenstaedt,K., Jolivet,P., Boulard,C., Zivy,M., Negroni,L., Nicaud,J.M., and Chardot,T. (2006). Lipid particle composition of the yeast Yarrowia lipolytica depends on the carbon sources. Proteomics. *6*, 1450-1459.

Athenstaedt,K., Zweytick,D., Jandrositz,A., Kohlwein,S.D., and Daum,G. (1999). Identification and characterization of major lipid particle proteins of the yeast Saccharomyces cerevisiae. J. Bacteriol. *181*, 6441-6448.

Bartz,R., Li,W.H., Venables,B., Zehmer,J.K., Roth,M.R., Welti,R., Anderson,R.G., Liu,P., and Chapman,K.D. (2007). Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. J. Lipid Res. *48*, 837-847.

Bickel,P.E., Tansey,J.T., and Welte,M.A. (2009). PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. Biochim. Biophys. Acta *1791*, 419-440.

Broekhuyse,R.M. (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.

Burgermeister,M., Birner-Grunberger,R., Nebauer,R., and 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.

Capuano,F., Beaudoin,F., Napier,J.A., and Shewry,P.R. (2007). Properties and exploitation of oleosins. Biotechnol. Adv. *25*, 203-206.

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

Cho,S.Y. *et al.* (2007). Identification of mouse Prp19p as a lipid droplet-associated protein and its possible involvement in the biogenesis of lipid droplets. J. Biol. Chem. *282*, 2456-2465.

Clausen,M.K., Christiansen,K., Jensen,P.K., and Behnke,O. (1974). Isolation of lipid particles from baker's yeast. FEBS Lett. *43*, 176-179.

Connerth,M., Grillitsch,K., Kofeler,H., and Daum,G. (2009). Analysis of lipid particles from yeast. Methods Mol. Biol. *579*, 359-374.

Czabany,T., Athenstaedt,K., and Daum,G. (2007). Synthesis, storage and degradation of neutral lipids in yeast. Biochim. Biophys. Acta *1771*, 299-309.

Czabany,T., Wagner,A., Zweytick,D., Lohner,K., Leitner,E., Ingolic,E., and Daum,G. (2008). Structural and Biochemical Properties of Lipid Particles from the Yeast Saccharomyces cerevisiae. J. Biol. Chem. *283*, 17065-17074.

Delom,F., Szponarski,W., Sommerer,N., Boyer,J.C., Bruneau,J.M., Rossignol,M., and Gibrat,R. (2006). The plasma membrane proteome of Saccharomyces cerevisiae and its response to the antifungal calcofluor. Proteomics. *6*, 3029-3039.

Duplus,E., Glorian,M., and Forest,C. (2000). Fatty acid regulation of gene transcription. J. Biol. Chem. *275*, 30749-30752.

Ejsing,C.S., Sampaio,J.L., Surendranath,V., Duchoslav,E., Ekroos,K., Klemm,R.W., Simons,K., and Shevchenko,A. (2009). Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. Proc. Natl. Acad. Sci. U. S. A *106*, 2136-2141.

Erdmann,R., Veenhuis,M., Mertens,D., and Kunau,W.H. (1989). Isolation of peroxisome-deficient mutants of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *86*, 5419-23.

Fang,M., Kearns,B.G., Gedvilaite,A., Kagiwada,S., Kearns,M., Fung,M.K., and Bankaitis,V.A. (1996). Kes1p shares homology with human oxysterol binding protein and participates in a novel regulatory pathway for yeast Golgi-derived transport vesicle biogenesis. EMBO J. *15*, 6447-6459.

Farese,R.V., Jr. and Walther,T.C. (2009). Lipid droplets finally get a little R-E-S-P-E-C-T. Cell *139*, 855-860.

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

Garbarino,J. and Sturley,S.L. (2009). Saturated with fat: new perspectives on lipotoxicity. Curr. Opin. Clin. Nutr. Metab Care *12*, 110-116.

Gardocki,M.E., Jani,N., and Lopes,J.M. (2005). Phosphatidylinositol biosynthesis: biochemistry and regulation. Biochim Biophys Acta *1735*, 89-100.

Goodman,J.M. (2008). The gregarious lipid droplet. J. Biol. Chem. *283*, 28005-28009.

Greenberg,A.S., Egan,J.J., Wek,S.A., Garty,N.B., Blanchette-Mackie,E.J., and Londos,C. (1991). Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. J. Biol. Chem. *266*, 11341-11346.

Greenberg,A.S., Egan,J.J., Wek,S.A., Moos,M.C., Jr., Londos,C., and Kimmel,A.R. (1993). Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes. Proc. Natl. Acad. Sci. U. S. A *90*, 12035-12039.

Guan,X.L. and Wenk,M.R. (2006). Mass spectrometry-based profiling of phospholipids and sphingolipids in extracts from Saccharomyces cerevisiae. Yeast *23*, 465-477.

Guo,Y., Walther,T.C., Rao,M., Stuurman,N., Goshima,G., Terayama,K., Wong,J.S., Vale,R.D., Walter,P., and Farese,R.V. (2008). Functional genomic screen reveals genes involved in lipid-droplet formation and utilization. Nature *453*, 657-661.

Gurvitz,A. and Rottensteiner,H. (2006). The biochemistry of oleate induction: Transcriptional upregulation and peroxisome proliferation. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1763*, 1392-1402.

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

Hiltunen,J.K., Mursula,A.M., Rottensteiner,H., Wierenga,R.K., Kastaniotis,A.J., and Gurvitz,A. (2003). The biochemistry of peroxisomal beta-oxidation in the yeast Saccharomyces cerevisiae. FEMS Microbiol Rev *27*, 35-64.

Huang,C.Y., Chung,C.I., Lin,Y.C., Hsing,Y.I., and Huang,A.H. (2009). Oil bodies and oleosins in Physcomitrella possess characteristics representative of early trends in evolution. Plant Physiol *150*, 1192-1203.

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

Kaliszewski,P., Szkopinska,A., Ferreira,T., Swiezewska,E., Berges,T., and Zoladek,T. (2008). Rsp5p ubiquitin ligase and the transcriptional activators Spt23p and Mga2p are involved in co-regulation of biosynthesis of end products of the mevalonate pathway and triacylglycerol in yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta *1781*, 627-634.

Kennedy,E.P. and Weiss,S.B. (1956). The function of cytidine coenzymes in the biosynthesis of phospholipides. J. Biol. Chem. *222*, 193-214.

Kurat,C.F., Wolinski,H., Petschnigg,J., Kaluarachchi,S., Andrews,B., Natter,K., and Kohlwein,S.D. (2009). Cdk1/Cdc28-dependent activation of the major triacylglycerol lipase Tgl4 in yeast links lipolysis to cell-cycle progression. Mol. Cell *33*, 53-63.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-5.

Leber,R., Zinser,E., Zellnig,G., Paltauf,F., and Daum,G. (1994). Characterization of lipid particles of the yeast, Saccharomyces cerevisiae. Yeast *10*, 1421-1428.

Li,X., Rivas,M.P., Fang,M., Marchena,J., Mehrotra,B., Chaudhary,A., Feng,L., Prestwich,G.D., and Bankaitis,V.A. (2002). Analysis of oxysterol binding protein homologue Kes1p function in regulation of Sec14p-dependent protein transport from the yeast Golgi complex. J. Cell Biol. *157*, 63-77.

Lowry,O.H., Rosebrough,N.J., Farr A.L., and Randall R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

McCammon,M.T., Hartmann,M.A., Bottema,C.D., and Parks,L.W. (1984). Sterol methylation in Saccharomyces cerevisiae. J. Bacteriol. *157*, 475-483.

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

Ohsaki,Y., Cheng,J., Fujita,A., Tokumoto,T., and Fujimoto,T. (2006b). Cytoplasmic lipid droplets are sites of convergence of proteasomal and autophagic degradation of apolipoprotein B. Mol. Biol. Cell *17*, 2674-2683.

Ohsaki,Y., Cheng,J., Fujita,A., Tokumoto,T., and Fujimoto,T. (2006a). Cytoplasmic lipid droplets are sites of convergence of proteasomal and autophagic degradation of apolipoprotein B. Mol. Biol. Cell *17*, 2674-2683.

Ohsaki,Y., Cheng,J., Suzuki,M., Shinohara,Y., Fujita,A., and Fujimoto,T. (2009). Biogenesis of cytoplasmic lipid droplets: from the lipid ester globule in the membrane to the visible structure. Biochim. Biophys. Acta *1791*, 399-407.

Olofsson,S.O., Bostrom,P., Andersson,L., Rutberg,M., Levin,M., Perman,J., and Boren,J. (2008). Triglyceride containing lipid droplets and lipid droplet-associated proteins. Curr. Opin. Lipidol. *19*, 441-447.

Petschnigg,J., Wolinski,H., Kolb,D., Zellnig,G., Kurat,C.F., Natter,K., and Kohlwein,S.D. (2009). Good fat - essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast. J. Biol. Chem.

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

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

Robenek,H., Buers,I., Hofnagel,O., Robenek,M.J., Troyer,D., and Severs,N.J. (2009). Compartmentalization of proteins in lipid droplet biogenesis. Biochim. Biophys. Acta *1791*, 408-418.

Robenek,H., Hofnagel,O., Buers,I., Robenek,M.J., Troyer,D., and Severs,N.J. (2006). Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis. J. Cell Sci. *119*, 4215-4224.

Rosenberger,S., Connerth,M., Zellnig,G., and Daum,G. (2009). Phosphatidylethanolamine synthesized by three different pathways is supplied to peroxisomes of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta.

Saleem,R.A., Knoblach,B., Mast,F.D., Smith,J.J., Boyle,J., Dobson,C.M., Long-O'Donnell,R., Rachubinski,R.A., and Aitchison,J.D. (2008). Genome-wide analysis of signaling networks regulating fatty acid-induced gene expression and organelle biogenesis. J. Cell Biol. *181*, 281-292.

Schneiter,R. *et al.* (1999). Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of the lipid molecular species composition of yeast subcellular membranes reveals acyl chain-based sorting/remodeling of distinct molecular species en route to the plasma membrane. J. Cell Biol. *146*, 741-754.

Schuiki,I. and Daum,G. (2009). Phosphatidylserine decarboxylases, key enzymes of lipid metabolism. IUBMB. Life *61*, 151-162.

Serrano,R. (1988). H+-ATPase from plasma membranes of Saccharomyces cerevisiae and Avena sativa roots: purification and reconstitution. Methods Enzymol. *157*, 533-544.

Shields,D. and Arvan,P. (1999). Disease models provide insights into post-golgi protein trafficking, localization and processing. Curr. Opin. Cell Biol. *11*, 489-494.

Sickmann,A. *et al.* (2003). The proteome of Saccharomyces cerevisiae mitochondria. Proc. Natl. Acad. Sci. U. S. A *100*, 13207-13212.

Smith,J.J., Marelli,M., Christmas,R.H., Vizeacoumar,F.J., Dilworth,D.J., Ideker,T., Galitski,T., Dimitrov,K., Rachubinski,R.A., and Aitchison,J.D. (2002). Transcriptome profiling to identify genes involved in peroxisome assembly and function. J Cell Biol *158*, 259-71.

Smith,J.J., Sydorskyy,Y., Marelli,M., Hwang,D., Bolouri,H., Rachubinski,R.A., and Aitchison,J.D. (2006). Expression and functional profiling reveal distinct gene classes involved in fatty acid metabolism. Mol. Syst. Biol. *2*, 2006.

Tauchi-Sato,K., Ozeki,S., Houjou,T., Taguchi,R., and Fujimoto,T. (2002). The surface of lipid droplets is a phospholipid monolayer with a unique Fatty Acid composition. J. Biol. Chem. *277*, 44507-44512.

Thiele,C. and Spandl,J. (2008). Cell biology of lipid droplets. Curr. Opin. Cell Biol. *20*, 378-385.

Trotter,P.J. (2001). The genetics of fatty acid metabolism in Saccharomyces cerevisiae. Annu. Rev. Nutr. *21*, 97-119.

Walther,T.C. and Farese,R.V., Jr. (2009). The life of lipid droplets. Biochim. Biophys. Acta *1791*, 459-466.

Yamaguchi,T. and Osumi,T. (2009). Chanarin-Dorfman syndrome: deficiency in CGI-58, a lipid droplet-bound coactivator of lipase. Biochim. Biophys. Acta *1791*, 519-523.

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

Zinser,E., Paltauf,F., and Daum,G. (1993). Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. J. Bacteriol. *175*, 2853-2858.

Zweytick,D., Athenstaedt,K., and Daum,G. (2000). Intracellular lipid particles of eukaryotic cells. Biochim. Biophys. Acta *1469*, 101-120.

CHAPTER 6

FATE OF FAT: OLEIC ACID STORAGE AND BETA-OXIDATION REQUIRE NOVEL HYDROLASES IN YEAST

Connerth M.[1], Schatte J.[1], Kollroser M.[2], Rechberger G.[3], Hermetter A.[1] and Daum G.[1]

[1]Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, 8010 Graz, Austria; [2]Institute of Forensic Medicine, Medical University of Graz, Austria
[3]Institute of Molecular Biosciences, Karl Franzens University, Graz, Austria

Running title: Yeast hydrolases

Key words: lipases, hydrolases, peroxisomes, lipid particles, yeast

Abbreviations: CL, cardiolipin; ERG, ergosterol; ER, endoplasmic reticulum; FA, fatty acid(s); HE-HP, hexanoyl-hexyl phosphonate; NL, neutral lipid; NBD, nitobenz-2-oxa-1,3-diazole; LP, lipid droplet/particle(s); RT, room temperature; SE, steryl ester; TAG, triacylglycerol; TGP, triglyceride phosphonate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; Px, peroxisomes

Abstract

Peroxisomes are small ubiquitous organelles involved in degradation of toxic substances and fatty acid β-oxidation. The mechanism of fatty acid traffic to their site of degradation is not yet completely understood. This study is aimed at the investigation of fatty acid supply to peroxisomes and the impact of yeast lipases and hydrolases. In contrast to mammalian cells, yeast β-oxidation exclusively takes place in peroxisomes. Thus, functional peroxisomes are crucial for growth of the yeast on oleic acid as carbon source. Yeast cells grown in the presence of exogenous oleic acid, however, do not only proliferate peroxisomes but at the same time also accumulate storage lipid particles (LP) at large size and abundance. A subset of lipases (Tgl3p, Tgl4p and Tgl5p) and hydrolases (Tgl1p, Yeh1p) is located on the surface of the LP for mobilization of the neutral lipids upon requirement. Employing different yeast mutants defective in neutral lipid turnover allowed us to dissect possible traffic routes of oleic acid in the yeast cell. We found that peroxisome function was not entirely dependent on LP formation and that oleic acid was preferentially incorporated into triacylglycerols (TAG). Interestingly, in a mutant deficient of all known TAG lipases (*tgl3*Δ*tgl4*Δ*tgl5*Δ) turnover of TAG did not come to an halt when cells were grown under peroxisome inducible conditions. Our data led to the assumption that an additional pathway may safeguard supply of fatty acids for specific cellular processes in the presence of oleic acid. *In vitro* experiments using a subset of specific fluorescently labeled inhibitors for TAG lipases and hydrolases allowed us to identify several candidate genes resembling most likely novel lipases/hydrolases which may be involved in TAG mobilization. In summary, our data provide strong evidence that oleic acid storage and turnover in the LP is a highly regulated mechanism providing pools of fatty acids for subsequent metabolic or regulatory functions in the cell. In addition, a new link between oleic acid storage and peroxisomal β-oxidation is shown.

Introduction

Peroxisomes are small ubiquitous organelles. Their main function involves decomposition of toxic substances like H_2O_2 as well as degradation of fatty acids (FA) via oxidative pathways. In humans, only very long chain fatty acids (VLCFAs) are oxidized in peroxisomes (Lazarow and DeDuve, 1976) and then transported to mitochondria which complete the degradation (Hettema and Tabak, 2000). On the contrary, in the yeast *Saccharomyces cerevisiae* peroxisomes are the only organelle capable of fatty acid oxidation (Kunau *et al.*, 1988; Hiltunen *et al.*, 2003). Therefore, yeast provides an ideal model organism to study peroxisome proliferation and function in the cell. Prior to β-oxidation fatty acids have to be activated by one of the six fatty acid activators, Faa1p, Faa2p, Faa3p, Faa4p, Fat1p and Fat2p which show different cellular localization and diverse substrate specificity. For Fat2p actually no substrate specificity has been shown (for review see: (Black and DiRusso, 2007). Different mechanisms have been described for uptake of fatty acids into peroxisomes. It is assumed that short and medium chain fatty acids can diffuse through the peroxisomal membrane whereas long chain and very long chain fatty acids need special import mechanisms. In the case of long chain fatty acids, Pex11p might play a role in importing either activated or non-activated fatty acids. For VLCAs the homo- or heterodimer of *PXA1/PXA2* is thought to facilitate FA uptake into peroxisomes (Shani *et al.*, 1995; Hettema *et al.*, 1996; Verleur *et al.*, 1997). To date, the underlying mechanisms of lipid traffic are still not completely understood.

Two possible pathways might direct fatty acids aimed at energy production to peroxisomal β-oxidation: either the exogenous route via plasma membrane transport is involved and/or the endogenous route from the lipid particle (LP) might play a role in FA supply to peroxisomes (Figure 1). Traffic via the plasma membrane involves vectorial acylation of FA depending on concerted activities of Fat1p and either Faa1p or Faa4p (DiRusso *et al.*, 2000; Faergeman *et al.*, 2001; Zou *et al.*, 2003). After activation of the FA, acyl-CoA has to be transported to and taken up by the peroxisome through a yet unknown mechanism. A possible trafficking route of FA from the LP to peroxisomes has not yet been shown. Hence, lately we found that cells grown on oleic acid not only induce peroxisome proliferation due to enhanced β-oxidation but at the same time LP were found to be highly enriched in size and abundance (Rosenberger *et al.*, 2009). Recently, a possible direct link between LP and peroxisomes has been proposed (Binns *et al.*, 2006) indicating a putative pathway for lipid supply to peroxisomes.

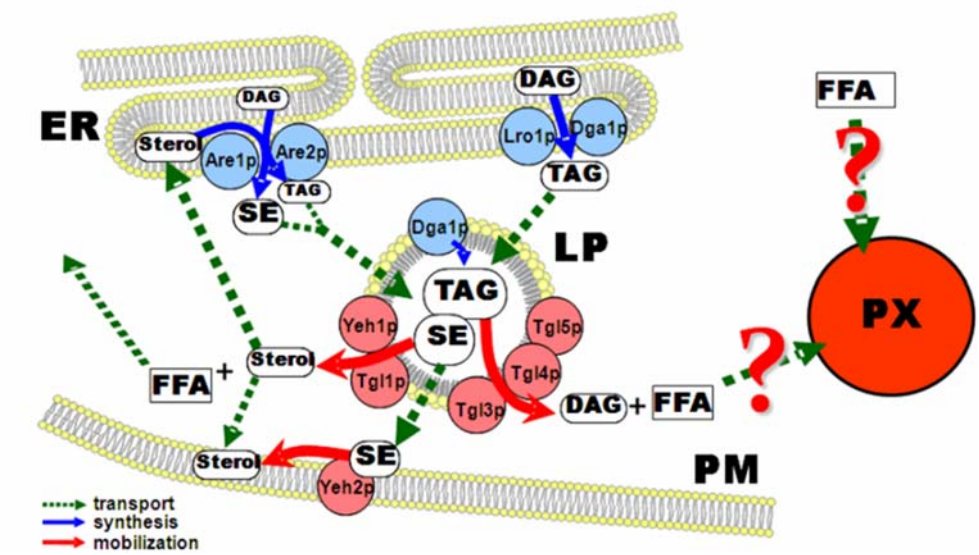


Figure 1: Overview of neutral lipid turnover in the yeast

Enzymes responsible for the formation of neutral lipids, Dga1p, Lro1p, Are1p and Are2p are localized to the ER. They form triacylglycerols (TAG) or steryl esters (SE), respectively, which are stored in the lipid particle (LP). Upon requirement, TAG can be mobilized by lipases Tgl3p, Tgl4p and Tgl5p located on the LP. SE are hydrolyzed by either Yeh1p or Tgl1p, located on the LP, or Yeh2p which is found on the plasma membrane. Mobilization of neutral lipids results in free fatty acids which then can be supplied to peroxisomes by a yet unknown mechanism for further decomposition. Alternatively, FFA might also directly enter peroxisomes without turnover of neutral lipids.

LP are the lipid storage organelle of the cell, incorporating excess of free fatty acids (FFA) into either triacylglycerols (TAG) or steryl esters (SE). Four enzymes are responsible for the synthesis of these neutral lipids and thus also for formation of LP and FA storage (Sorger *et al.*, 2004). Dga1p and Lro1p form TAG from free fatty acids (FFA) and diacylglycerol (DAG) or phospholipid (PL), respectively. SE are synthesized from FFA and sterols by Are1p and Are2p with Are2p being the most active protein under aerobic conditions (Valachovic *et al.*, 2001; Valachovic *et al.*, 2002). All of these enzymes are localized to the ER with only Dga1p also found on LP (Sorger and Daum, 2002). Mobilization of neutral lipids involves TAG lipases (Tgl3p, Tgl4p and Tgl5p) and hydrolases (Tgl1p and Yeh1p on LP and Yeh2p on the plasma membrane) and results in release of FFA (Athenstaedt and Daum, 2003; Athenstaedt and Daum, 2005; Jandrositz *et al.*, 2005; Koffel *et al.*, 2005; Kurat *et al.*, 2006; Wagner *et al.*, 2009).

The aim of the present study was to elucidate the fate of FFA in the cell and to investigate a possible link between storage, mobilization and β-oxidation of FFA (see Figure 1). Dissecting fatty acid traffic through the cell revealed that endogenous oleic acid present in the growth medium can easily enter the cell. Neutral lipid analysis showed that FFA are not incorporated into SE but rather into TAG. This finding is in line with previous studies from our laboratory (Connerth *et al.*, manuscript under revision). Assuming that TAG is the major source for FFA release in the cell, we created a knock out strain lacking all known TAG lipases, Tgl3p, Tgl4p and Tgl5p, as well as the two SE synthases, Are1p and Are2p, resulting in a pentuple mutant (PM, *tgl3*Δ*tgl4*Δ*tgl5*Δ*are1*Δ*are2*Δ). This mutant lacks the ability of SE formation and TAG mobilization. This strain was viable when grown on glucose or oleate, respectively. Surprisingly, TAG mobilization did not come to an halt in this strain upon cultivation on oleic acid indicating the presence of novel not yet characterized hydrolase(s). Proteome analyses using lipase and esterase inhibitors revealed a subset of candidate genes for yet unknown hydrolases on peroxisomes and lipid particles. These data suggest an important role of TAG from LP for FFA supply to peroxisomal β-oxidation and a novel link between LP and peroxisomes.

Material and Methods

Yeast strains and growth conditions

Strains used in this study are listed in Table 1. Yeast cells were grown in either full glucose media (YPD) containing 2 % peptone, 1 % yeast extract and 2 % glucose or oleic acid supplemented media (YPO) containing 0.5 % peptone, 0.3 % yeast extract, 0.5 % KH_2PO_4, 0.1 % glucose, 0.1 % oleic acid and 0.2 % Tween 80. If not stated otherwise cells were cultivated in liquid media and grown at 30 °C under vigorous shaking until early stationary phase.

Table 1: Strains used in this study

Name	Genotype	Origin
BY4741	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf, Frankfurt, Germany
TM TGL	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 tgl3Δ::KanMX4; tgl4Δ::KanMX4; tgl5::KanMX4*	Athenstaedt and Daum, 2005
PM	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 tgl3Δ::KanMX4; tgl4Δ::KanMX4; tgl5::KanMX4; are1Δ::; are2Δ::*	This study
TM YEH	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; tgl1Δ::KanMX4; yeh1Δ::KanMX4; yeh2Δ::KanMX4*	This study, kindly provided by A. Wagner

Construction of deletion mutants

A list of primers used for creating deletion cassettes can be found in Table 2. Genomic knock out of genes was performed following the protocol of Longtine *et al.*, (Longtine *et al.*, 1998). Correct integration of the knock out cassette was verified by growth auxotrophy as well as by colony PCR.

Table 2: Primers used in this study

Name	Sequence 5'-3'	Function
MC24	GTT CAG CAC GGC TTG CAG CAA GAG CGC CAA AAC AGA TTG CAA GAC AGC TGA AGC TTC GTA CGC	Deletion ARE1 FW
MC25	TAT ATC TAT CAA GGG CTT GCG AGG GAC ACA CGT GGT ATG GTG GCA GTG CAT AGG CCA CTA GTG GAT CTG	Deletion ARE1 REV
MC26	TAT ATC TAT CAA GGG CTT GCG AGG GAC ACA CGT GGT ATG GTG GCA GTG CAT AGG CCA CTA GTG GAT CTG	Deletion ARE2 FW

MC27	AAC AGA CAC ATT ACG TTA GCA AAA GCA ACA ATA ACA AAC ACA ACC CAG CTG AAG CTT CGT ACG C	Deletion ARE2 REV
Are1 wt FW TC	GAA AAA TGT GAG ATG GTG TAG AGT G	FW primer upstream ARE1 ORF
MC29	ATG GTT CTG CCC CAG ATT TAC C	Rev primer in LEU2 cassette
ARE2 wt FW	CTT TCA TCA ATA CAT CTA TAT ATT CG	FW primer upstream ARE2 ORF
ARE2 rev control	GTA ATT GTG GTA GCT GTG TGT TCA T	Rev primer in ARE2 ORF

Isolation of organelles

Subcellular fractionation was performed as described by previously (Rosenberger *et al.*, 2009; Connerth *et al.*, 2009). For peroxisome preparation, late exponential phase cultures grown in oleic acid medium were harvested by centrifugation at 5,000 rpm for 5 min in a Sorvall SLC3000 rotor. Cell pellet was washed in distilled water and resuspended in twice the volume of buffer A (0.1 M Tris/SO_4, pH 9.4). After addition of 0.66 mg dithiothreitol (DTT) per ml buffer, the cell suspension was incubated at 30 °C shaking for at least 10 min. Cells were washed and resuspended in pre-warmed buffer B (1.2 M sorbitol and 20 mM KH_2PO_4, pH 7.4). Preparation of spheroplasts was performed using 2 mg zymolyase 20T per g cell wet weight in seven volumes buffer B for approximately 1 h at 30 °C shaking.

Spheroplasts were then washed in buffer B and resuspended in twice the cell volume in breaking buffer containing 5 mM-MES-KOH, pH 6.0, 0.6 M sorbitol, 1 mM KCl and 0.5 mM EDTA. 1 mM PMSF was added as protease inhibitor and cell suspension was homogenized on ice using a Dounze homogenizer with a tight fitting pestle. Unbroken cells and nuclei were removed by centrifugation at 5,000 rpm for 5 min in a Sorvall SLC3000 rotor. In order to enhance the yield of peroxisomes, the resulting pellet was twice resuspended in breaking buffer, re-homogenized and centrifuged as described above. The combined supernatants were centrifuged at 15,000 rpm in an SS34 rotor for 15 min. The crude organelle pellet, consisting of mitochondria and peroxisomes, was gently resuspended in breaking buffer plus 1 mM PMSF, and centrifuged at low speed (5,000 rpm) to remove larger aggregates.

Then, the supernatant was centrifuged again at 15,000 rpm, the pellet was resuspended in breaking buffer as described above and loaded for further purification on a Nycodenz gradient (17-35%; w/v) in 5 mM-MES-KOH, pH 6.0, 1 mM KCl, 0.24 M sucrose. Centrifugation was carried out in a swing out rotor (Sorvall AH-629) at 26,000 rpm for 90 min. The white peroxisome layer was withdrawn using a syringe, diluted with 4 volumes of the breaking buffer and sedimented at 15,000 rpm in an SS34 rotor for 15 min at 4 °C. Mitochondria formed a separated band in the density gradient and were also collected and sedimented by centrifugation.

Lipid particles were isolated from cells grown on either YPD or YPO medium for 24 h (early stationary phase). Spheroplasts were prepared as described above and then resuspended in buffer LP-A (12 % Ficoll, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA{\cdot}2\ H_20$) followed by mechanical disruption in the presence 1 mM PMSF with 30 strokes using a 30 ml Dounce Homogenizer with a loose fitting pestle. The homogenate was centrifuged to remove cell debris in a Sorvall SS34 rotor at 7,000 rpm for 5 min and supernatant was collected. The pellet was homogenized again and after another centrifugation the supernatants were combined. These samples were then carefully overlaid with buffer LP-A in an Ultra-Clear Centrifuge Tube (Beckman). Ultracentrifugation at 28,000 rpm for 45 min using a swing out rotor Sorvall AH-629 yielded a white layer on top (crude LP) which was transferred with a moistened spatula to a 15 ml Dounce Homogenizer. After homogenizing with 8 strokes using a loose fitting pestle in the presence of 1 mM PMSF the sample was loaded onto a new ultracentrifuge tube and carefully overlaid with buffer LP-B (8 % Ficoll, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA{\cdot}2\ H_20$). Ultracentrifugation at 28,000 rpm for 30 min resulted in a top layer containing LP. Prior to the last ultracentrifugation step, buffer LP-D (0.25 M sorbitol, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA{\cdot}2\ H_20$) was filled into a fresh ultracentrifuge tube. Then, the homogenized sample was loaded to the bottom of the tube by injection under the buffer using a syringe. Ultracentrifugation at 28,000 rpm for 30 min led to a top layer consisting of highly purified LP. After homogenizing the isolated LP in a 5 ml Dounce Homogenizer samples were stored at -80 °C until required. The pellet from the last centrifugation step containing vacuoles was collected as well.

Lipid analysis

Lipid analysis were performed as described before (Connerth *et al.*, 2009). In brief, cell samples were harvested from a main culture grown in either YPD or YPO until early stationary phase. Lipid extracts were performed according to Folch *et al.* (Folch J. *et al.*, 1957). 3 ml $CHCl_3$:MeOH (2:1 v/v) and twice the volume of glass beads was added to the cells in a Pyrex glass tube. Crude homogenates were prepared and lipids were extracted to the polar organic phase by vortexing at room temperature (RT) for 1 h. Proteins and non-polar substances were removed by consecutive washing steps with 0.2 volumes 0.034 % $MgCl_2$, 1 ml 2 N KCl/MeOH (4:1; v/v) and 1 ml of an artificial upper phase ($CHCl_3$:MeOH:H_2O; 3:48:47; per vol.). These solutions were added to the extracts and incubated with shaking for 3 min. After centrifugation for 3 min at 3,000 rpm in a table-top centrifuge the aqueous phase was removed by aspiration. Finally, lipids were dried under a stream of nitrogen and stored at -20 °C.

Neutral lipid quantification was performed by thin layer chromatography (TLC). For the direct densitometric quantification of lipids on TLC plates authentic standards were used containing defined amounts of the respective lipids. Therefore, dried lipid extracts were dissolved in an appropriate volume of $CHCl_3$/MeOH (2:1; v/v) and spotted onto a TLC plate with a Hamilton syringe. Lipids were separated on a 10 cm TLC plate in an ascending manner using light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) as a solvent until the front has reached two thirds of the plate's height. Then, separation of lipids was continued in the same direction using light petroleum/diethyl ether (49:1; v/v) until the solvent front reaches the top of the plate. SE were quantified densitometrically using a TLC scanner (Shimadzu chromatoscanner CS-930) at 275 nm. TAG were irreversibly stained by charring prior to scanning. For this purpose, the TLC plate was incubated approximately 15 sec in a solution of 0.63 g $MnCl_2 \cdot 4H_2O$, 60 ml water, 60 ml methanol, 4 ml conc. sulfuric acid and stained in a heating chamber at 100°C for 30 min. Spot of neutral lipids visualized as described above are scanned at a wavelength of 400 nm, and spot areas are quantified relative to standards.

In vivo TAG mobilization assay

Yeast cells from wild type BY4741 and from the PM (*tgl3*Δ*tgl4*Δ*tgl5*Δ*are1*Δ*are2*Δ) from preculture were used to inoculate a main culture to an OD600 of 0.1 in 100 ml liquid YPO containing 10 µl [3H]oleic acid (50 µCi = 110,000,000 dpm).

Cells were grown for 20 h at 30 °C shaking. Then, cells were harvested and washed twice in 0.5 % sterile BSA (essentially fatty acid free; SIGMA) and resuspended in 100 ml non-labeled YPO. Cells were incubated at 30 °C and aliquots were taken at time points indicated. Density was measured from 1 ml sample, washed in 0.5 % BSA and taken up in 1 ml distilled water agains water as blank in a spectrophotometer at 600 nm wavelength. 10 ml aliquots were used for harvesting cells in a Greiner tube at 4,500 rpm for 5 min in a Hettich table top centrifuge. Cells were washed in 0.5 % BSA and cell pellet was frozen at -20 °C and then used for lipid extraction as described above. Dried lipid extract was separated by TLC as described above. Bands resembling TAG were scraped off and after addition of 7 ml scintillation cocktail were subjected to scintillation counting.

Protein analysis and inhibitor assays

A special subset of enzyme inhibitors was used to screen for the occurrence of TAG lipases (NBD- *sn1/3*-TGP, nitrobenz-2-oxa-1,3-diazole-*sn1/3*-triglyceride phosphonate) and hydrolases (NBD-HE-HP, nitrobenz-2-oxa-1,3-diazole hexanoyl-hexyl phosphonate) (Schmidinger *et al.*, 2005; Schmidinger *et al.*, 2006a; Schmidinger *et al.*, 2006b). Enzymes bind specifically to the reactive group of the inhibitor which cannot be cleaved. The covalently linked enzyme-inhibitor complex can be detected through the NBD-tag. For the inhibitor assay, purified LP or peroxisome samples were incubated with 0.1 % (final conc.) of the respective inhibitor and 0.6 % Triton X-100 in 50 mM TrisCl (pH 7.4) at 37 °C on a thermomix shaker at 600 rpm over night. Proteins were precipitated with 10 % (final conc.) TCA and washed in cold acetone to remove all unbound inhibitor. The remaining sample was dissolved in 10 µl 1 x SDS-sample buffer (63 mM TriCl, pH 6.8, 2 % SDS, 10 % glycerol, 0.0025 % bromphenol blue, 2 % β-mercaptoethanol) and separated by SDS-PAGE using the method of Laemmli (Laemmli, 1970). To identify protein-inhibitor complexes by the NBD-tag gels were scanned at a wavelength of 530 nm and at excitation of 488 nm in an Imager FX ProPlus (BIORAD). For total protein staining, gels were incubated with Sypro Ruby for 1 h at RT, washed in acetic acid/ ethanol for 2 h and scanned at 605 nm and at an excitation wavelength of 488nm.

LC-MS/MS analyses were performed as described by Birner-Gruenberger *et al.*, (Birner-Gruenberger *et al.*, 2005). Proteins quantification was carried out using the method of Lowry (Lowry *et al.*, 1951).

Results

Yeast strains defective in neutral lipid mobilization are not sensitive to oleic acid

Excess of endogenous free fatty acids can be stored as neutral lipids in the core of LP. At the same time and in the absence of glucose they also become an essential energy source and have to be metabolized by β-oxidation in the peroxisome (Hiltunen *et al.*, 2003). The present study aims at the elucidation of the route of exogenous free fatty acids (FFA) and a possible link of TAG storage and mobilization to FFA β-oxidation. To address this question we made use of a set of triple mutant strains, namely TM TGL (*tgl3*Δ*tgl4*Δ*tgl5*Δ) and TM YEH (*tgl1*Δ*yeh1*Δ*yeh2*Δ), which are incapable of either TAG or SE hydrolysis, respectively. Assuming that release of fatty acids (FA) from either of this storage lipids would be essential for β-oxidation, cells deficient in either of the pathways would not be able to grow on oleic acid. We found that growth of these mutants was not altered on oleic acid compared to wild type (Figure 2).

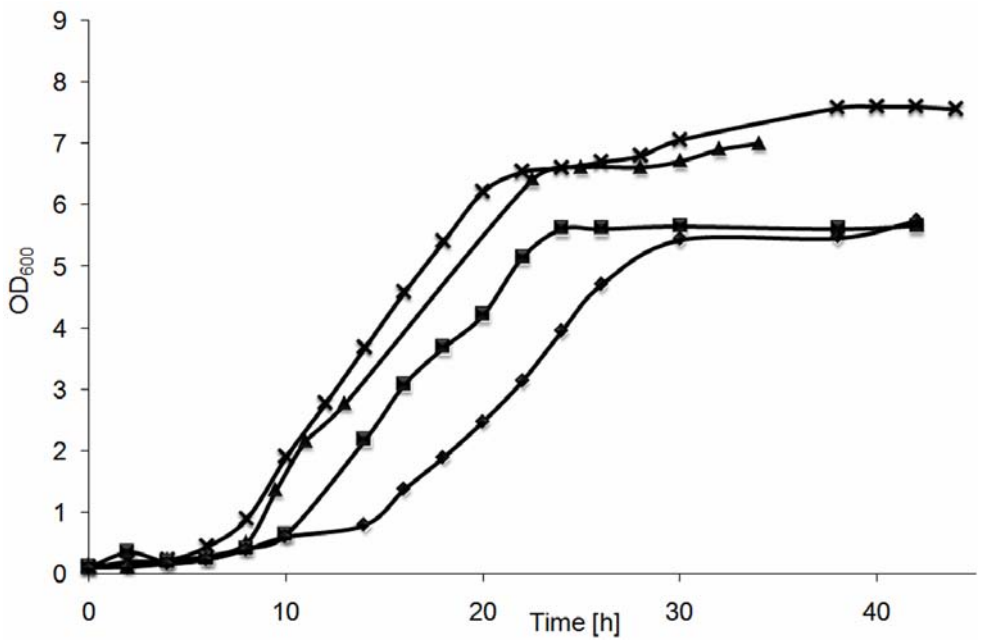


Figure 2: Growth analyses of mutant strains deficient in different routes of neutral lipid mobilization

Yeast cells from different strains were grown on YPD for 48 h and then used for inoculation of an YPO culture to an OD600 of 0.1. Aliquots were withdrawn at time points indicated, washed in 0.1 % BSA and cell density was determined in a spectrophotometer at 600 nm. Data shown are representative from at least three independent experiments.

Cells from either wild type BY4741 (♦), the TM TGL (■), TM YEH (▲) or the PM (x) were analyzed.

These results, however, did not exclude that both pathways of neutral lipid degradation might complement each other. To test this hypothesis we constructed a mutant strain lacking both acyl-CoA:sterol acyltransferases, Are1p and Are2p, as well as the major known TAG lipases, Tgl3p, Tgl4p and Tgl5p. This pentuple mutant (PM; *tgl3*Δ*tgl4*Δ*tgl5*Δ*are1*Δ*are2*Δ) only produces TAG but not SE. Since lipolysis of TAG is impaired in this strain, mobilization of fatty acids from neutral lipid depots should not be possible. Interestingly, growth of PM on oleic acid supplemented media revealed that this strain was not sensitive to the exogenous fatty acid (Figure 2) indicating that β-oxidation did not come to a halt in any of these strains.

To further characterize the mutant strains, lipid analyses were performed to investigate the incorporation of FFA into either TAG or SE, respectively. In line with previous studies (Czabany *et al.*, 2008; Grillitsch *et al.*, manuscript in preparation) we found that wild type cells exhibited a TAG to SE ratio of about 1 when grown on glucose (Figure 3). Yet, when cells were grown in the presence of oleic acid as carbon source, approximately 90 % of the cellular neutral lipids were present as TAG indicating that FFA were incorporated into this compound with strong preference. Similar results were obtained in TM TGL, TM YEH and PM. Growth on oleic acid led to massive accumulation of TAG accompanied with a dramatic reduction of SE in all four strains (see Figure 3). This finding was in line with recent studies from our laboratory showing that Are2p is competitively inhibited by oleic acid (Connerth *et al.*, manuscript under revision). This effect may account for the reduction of SE observed here.

Data presented so far indicate a need for TAG buffering the excess of FFA, but a link between FA release from TAG and FA degradation via β-oxidation remained elusive. Therefore, we tested the growth phenotype of strains lacking LP on fatty acids as carbon sources. Indeed, growth on oleic acid of a quadruple mutant (QM, *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ) lacking the essential genes for LP formation was initially found to be impaired (Lockshon *et al.*, 2007; Petschnigg *et al.*, 2009). This finding may be interpreted as link between functional peroxisomal β-oxidation and LP formation. Most importantly, however, prolonged exposure to oleic acid showed adaption of the cells to normal growth indicating that β-oxidation can function independently from TAG storage and mobilization (Connerth *et al*, manuscript under revision). We further tested formation of peroxisomes in the QM grown on oleic acid and palmitic acid. Growth of the mutant was delayed, but using a GFP-SKL construct expressed under glucose depletion we observed formation of peroxisomes by fluorescence microscopy (data not shown).

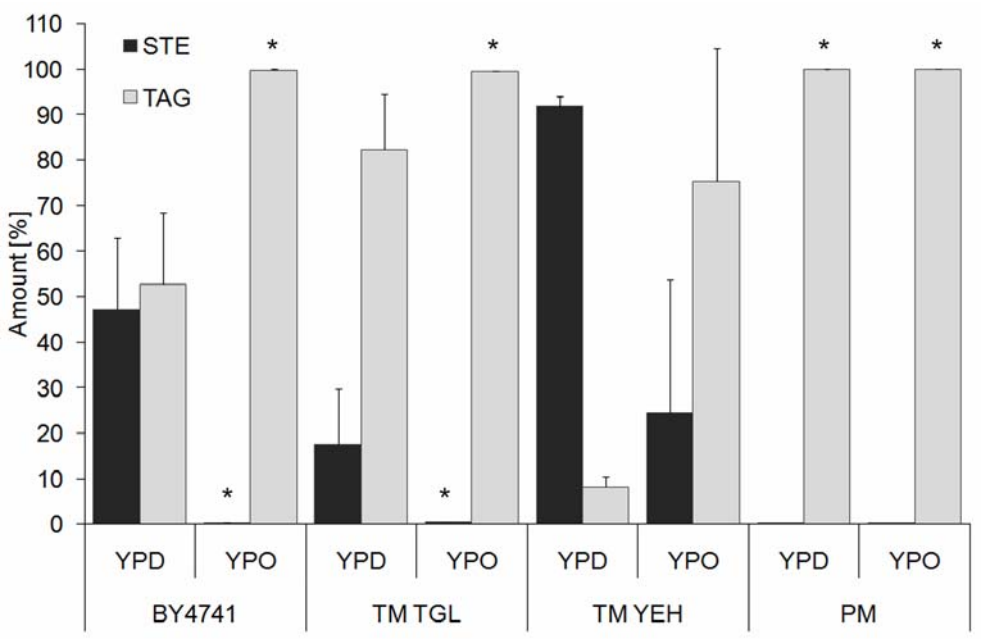


Figure 3: Neutral lipid analysis of different strains grown on YPD or YPO

Strains used for this study are wild type (BY4741), TM TGL (*tgl3*Δ*tgl4*Δ*tgl5*Δ), TM YEH (*tgl2*Δ*yeh1*Δ*yeh2*Δ) and PM (*tgl3*Δ*tgl4*Δ*tgl5*Δ*are1*Δ*are2*Δ). Cells from the same preculture were used to inoculate a YPD or a YPO liquid culture and grown until early stationary phase. Total cell and lipid extracts were performed as described in Material and Methods section. Lipids were separated by TLC and quantified after charring densitometrically. Results shown are average from at least three independent experiments with the standard deviation indicated. Asterisk indicates standard deviation <1%

A yeast strain lacking all known TAG lipases is able to mobilize TAG in vivo

Data described above suggested that peroxisomes can either receive FFA in a LP independent manner or TAG mobilization was not abolished under the conditions tested. In line with these questions w wondered whether the PM strain (*tgl3*Δ*tgl4*Δ*tgl5*Δ*are1*Δ*are2*Δ) would accumulate large amounts of TAG or whether a regulatory threshold limited the cellular amount of TAG. Therefore, we performed a pulse chase assay monitoring the fate of TAG in the PM strain *in vivo*. Cells were pulsed labeled with [^{3}H]oleic acid, and [^{3}H] incorporation into TAG was chased on unlabeled YPO (see Material and Methods). Since the PM was not able to produce detectable levels of SE but accumulated high levels of TAG (see Figure 3) we ensured that incorporation of [^{3}H]oleate occurred primarily into TAG and loss of label due to incorporation into other compounds was minimized. Both wild type and PM grew equally well under the given conditions (Figure 4A).

As expected, in wild type cells the label in TAG decreased over the time measured indicating that TAG was subjected to turnover (Figure 4B). Surprisingly, also in the PM lacking all known TAG lipases TAG mobilization was observed with a slight delay but at a similar rate as wild type. This finding was in contrast to previous analyses using cells grown on glucose where mobilization of TAG was not observed in the TM TGL strain (*tgl3*Δ*tgl4*Δ*tgl5*Δ) (Athenstaedt and Daum, 2005). Thus, we concluded that under oleic acid induced conditions one or more additional enzymes might become active accounting for TAG mobilization

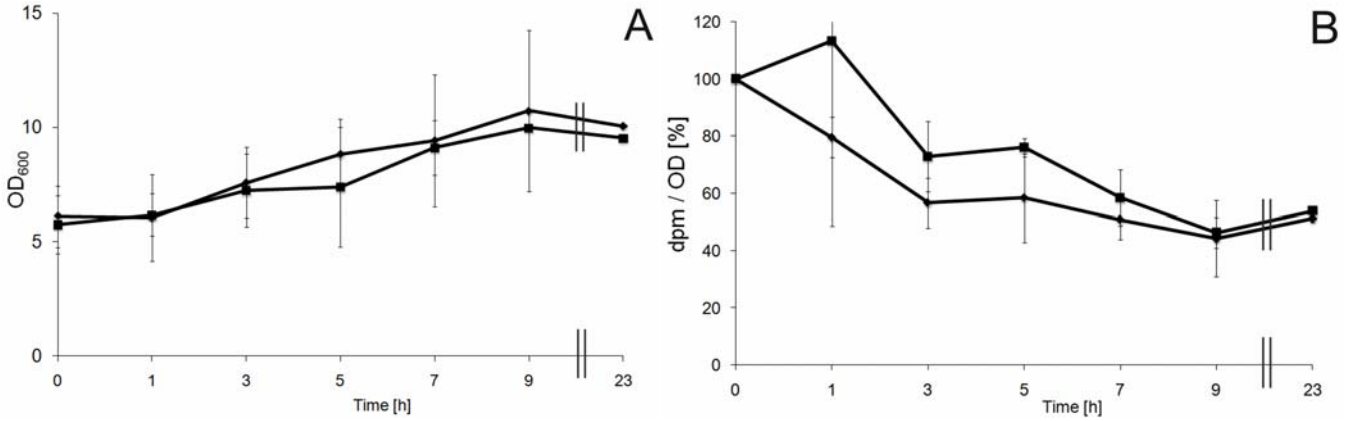


Figure 4: *In vivo* TAG mobilization

Cells from either wild type (♦) or the PM (*tgl3*Δ*tgl4*Δ*tgl5*Δ*are1*Δ*are2*Δ) (■) were grown in the presence of [^{3}H]oleate for 5 h and then shifted to fresh YPO media. At time points indicated, aliquots were withdrawn and analyzed for cellular density (A) or for the amount of [^{3}H]TAG (B). Data were obtained from two independent experiments with standard error as indicated.

Screening for novel lipases and hydrolases in LP and peroxisomes

Findings described above led us to search for novel TAG lipases/hydrolases in the yeast. To test whether additional enzyme(s) harboring TAG lipase/hydrolase activity in yeast grown on oleic acid were induced we employed a proteome assay making use of a subset of fluorescently labeled inhibitors of TAG lipases and hydrolases (Schmidinger *et al.*, 2005; Schmidinger *et al.*, 2006a). Isolated organelles from the yeast were used to screen for such novel enzymes. LP from cells grown on either glucose or oleic acid as well as peroxisomes from YPO grown cells were incubated with fluorescent phosphonic acid esters which covalently bind to the active sites of either TAG lipases (NBD-*sn1/3*-TGP) or hydrolases (NBD-HE-HP) (see Methods section).

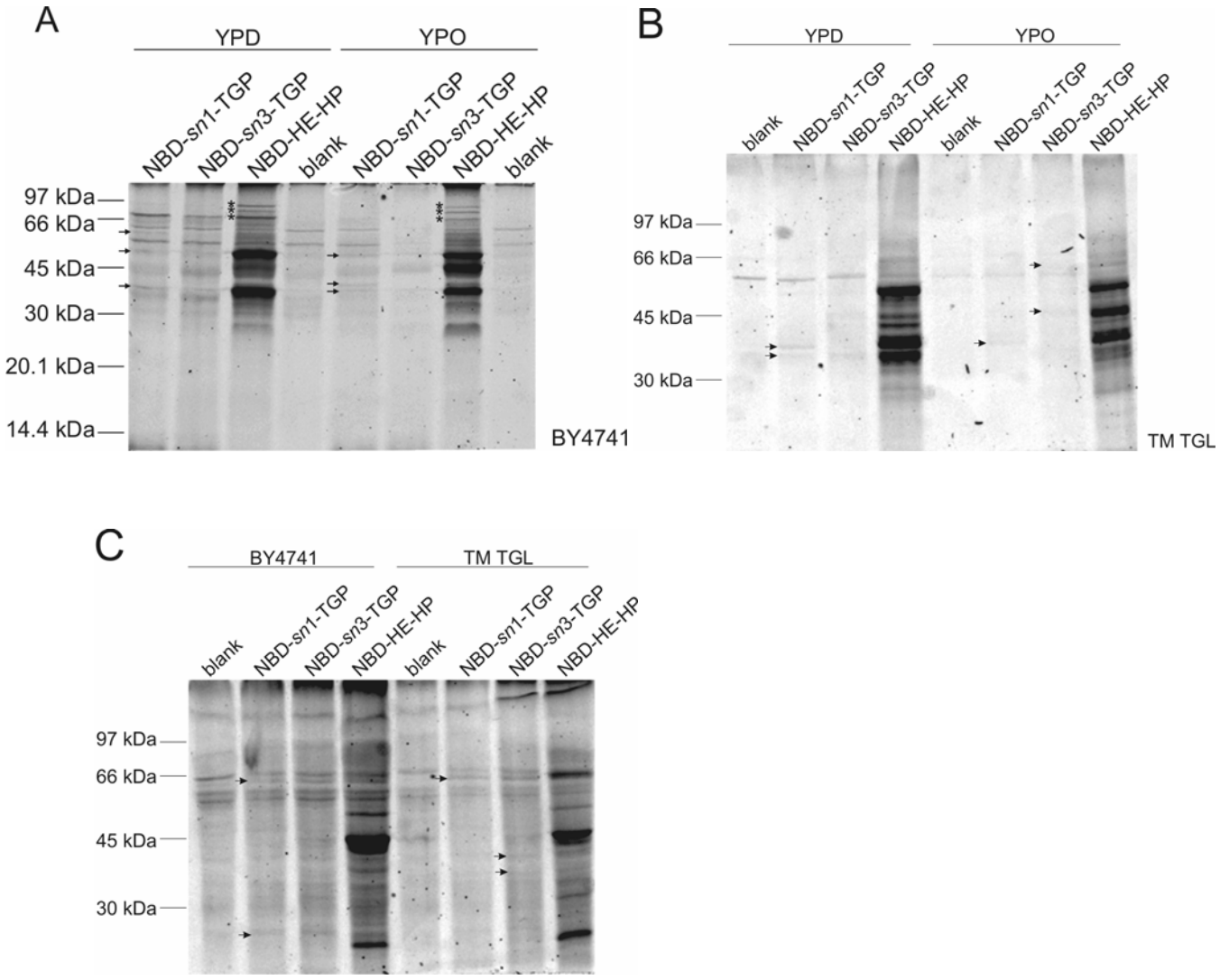


Figure 5*: In vitro* inhibitor assay screen for novel hydrolases in LP and Px

LP and peroxisomes from wild type and TM TGL cells were isolated from YPD or YPO grown cells as described in Material and Methods. Aliquots were incubated with inhibitors as incubated at 37°C over night. Proteins were then precipitated and separated by SDS-PAGE. Gels were scanned on a scanner at a wavelength for NBD.

(A) LP protein pattern from wild type cells after incubation with inhibitors for TAG lipases or hydrolases from YPD and YPO grown cells. Asterisks indicate the occurrence of the three known TAG lipases Tgl3p (73 kDa), Tgl4p (102 kDa) and Tgl5p (84 kDa). Arrows show the formation of an additional band indicating the presence of additional lipases or hydrolases. Blank shows the background of proteins incubated with no inhibitor.

(B) LP protein pattern from TM TGL grown cells on either YPD or YPO. Arrows indicate occurrence of new hydrolases /lipases.

(C) Peroxisomes from either wild type or TM TGL cells grown on YPO were incubated with a subset of inhibitors and analyzed for formation of protein bands indicating lipases or hydrolases (arrows). NBD-*sn*1(*sn*3)-TGP, nitobenz-2-oxa-1,3-diazole- *sn*1(*sn*3) – triglyglyceride phosphonate; NBD-HEHP, NBD- hexanoyl-hexyl phosphonate

After incubation proteins were separated by SDS-PAGE and reactive polypeptides were identified by the NBD-reporter tag. In LP from wild type BY4741 grown on YPD and YPO, protein bands of the size of the three lipases Tgl3p (~73 kDa), Tgl4p (~102 kDa) and Tgl5p (~85 kDa) were detected (Figure 5A, asterisks). Moreover, additional bands representing possibly new lipases were detected (Figure 5A, arrows). These proteins were mainly detectable with NBD-*sn*1-TGP indicating putative substrate specificity. Comparing the two growth conditions revealed that the protein pattern from YPO grown cells was different from YPD. This result became most obvious with the more hydrophilic inhibitor NBD-HE-HP. We conclude from this data that, indeed, a different subset of proteins might exist when cells were grown in the presence of oleic acid compared to glucose. To follow up this idea, we analyzed LP from TM TGL grown on YPD or YPO, respectively. Again, we were able to detect additional protein bands which did not correspond to the already known TAG lipases (Figure 5B, arrows). We also found an altered protein pattern with NBD-HE-HP when comparing YPD to YPO conditions which indicated that different hydrolases were found on LP upon oleic acid induction of cells.

Similar results were obtained when we analyzed peroxisomes from wt and TM TGL with the same subset of inhibitors (Figure 5C). Arrows indicate the occurrence of novel proteins exhibiting a hydrolase (NBD-HE-HP) or even lipase (NBD-*sn1/3*-TGP) activity. Proteins detected were of different size compared to those found on LP. Thus, we speculate that on LP as well as on peroxisomes distinct hydrolases may be expressed under oleic acid induced conditions. Altogether, it appears that our screening identified new and not yet characterized hydrolases or even lipases in LP and peroxisomes.

Recently, a new TAG lipase on peroxisomes has been identified (Thoms *et al.*, 2008). Deletion of the respective gene (*YOR084w*) in addition to the known TAG lipases showed no growth alteration in the presence of glucose or oleic acid as carbon source (data not shown). In addition, another subset of deletion strains deficient in putative yeast lipases (Δ*yor059c*, Δ*ykl094w*, Δ*ykl107w*) were analyzed for growth retardation on oleic acid supplemented medium. Since no growth phenotype of these strains was observed on glucose or oleic acid we concluded that either these genes were redundant or not essential (data not shown). Further examinations will be needed to analyze lipase function of these gene products.

Identification of putative novel hydrolases in yeast

To identify possible candidate genes for novel hydrolases found in the experiment described above, proteins reacting with NBD-HE-HP were separated by SDS-PAGE (Figure 6A) and subjected to MS analyses.

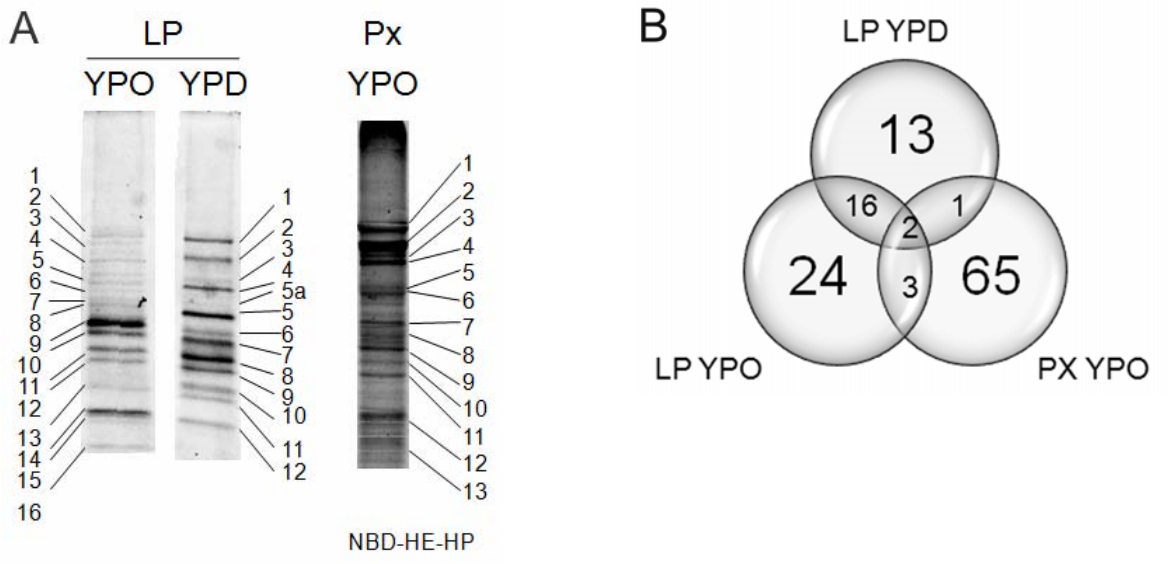


Figure 6: *In vitro* inhibitor assay for MS analyses

LP and peroxisomes were prepared and incubated with NBD-HE-HP which resembles a single chain carboxylic acid ester as described in Material and Methods. (A) Proteins were separated by SDS-PAGE and respective bands were cut from the gel, subjected to trypsin digestion and analyzed by MS analysis. (B) Venn diagram showing the proteins found in either LP from YPD, LP from YPO or Px from YPO indicating the overlapping hits.

A complete list of proteins identified in the respective spots is shown in the Supplementary Table 1. Gene products were identified according to the respective molecular weight of the protein band from the SDS-PAGE as well as by peptide length and the score value. Proteins which might account for hydrolase or lipase activity are listed in table 3. All known TAG lipases were identified with high abundance in LP samples. One exception was Tgl5p which was found only on LP from YPD grown cells.

Table 3: List of proteins found on yeast lipid particles and peroxisomes

ORFs found in this study on LP from cells grown on either YPD or YPO or peroxisomes from cells grown on YPO. Organelles were isolated and subjected to an inhibitor assay with NBD-HE-HP. Protein bands were scanned and subjected to MS analysis.

LP YPD	LP YPO	PX YPO
		AAT2
		ADE13
		ADH2
		AGX1
		AGX1
		ALD4
	ALG11	
	ATP1	
		ATP3
		ATP4
AYR1	*AYR1*	
		BBC1
		BPT1
	CAT2	*CAT2*
		CBR1
		CIT1
		CIT2
	CSR1	
		CTA1
DGA1	*DGA1*	
		DCI1
		DPM1
		ECI1
		EFT2
EHT1	*EHT1*	
ERG1	*ERG1*	
		ERG2
ERG6	*ERG6*	
	ERG7	
		ERG9
ERG27	*ERG27*	
		ERP1
FAA1	*FAA1*	
FAT1	*FAT1*	
		FAA2
		FMP52
		FOX2
		FSH3 (YOR280C)
	GDI1	
GTT1	*GTT1*	*GTT1*
	Gut2	
	HEF3	

HFD1	*HFD1*	
	HSP60	
		IDH1
		IDH2
		IDP3
		ILV5
	IML2	
KAR2		*KAR2*
	KES1	
	LPD1	
		LPX1
		LSP1
		LYS1
		MDH2
		MDH3
		MIR1
	MIP1	
		MLS1
		PCS60
		PDI1
PDR16	*PDR16*	
	PET9	*PET9*
PET10	*PET10*	
		PEX3
		PEX14
		PEX25
		PGK1
		PIL1
		PMA1
		PMA2
		POP1
		POT1
	POR1	
		POX1
PRB1		
PRC1		
QCR2		
RPL8a		
	RPS3	
	SDH1	
		SEC4
		SEC61
SEC63	*SEC63*	
		SMC1

		SMC4
		SPS19
TDH2		
		TDH3
	TEF2	*TEF2*
		TES1
		TFP1
TGL1		
TGL3	*TGL3*	
TGL4	*TGL4*	
TGL5		
	TSC10	
UBX2		
	URA2	
VAC8		
	VPS1	
		VPS13
VPS66		
		WBP1
	YBR056wp	

	YBR204c	
YDR196c	*YDR196c*	
		YGL185
	YIM1	
		YJR088c
		YJR111c
YJU3	*YJU3*	*YJU3*
	YKL050w	
YKL107w		
		YKL151c
		Yll023
YNL115c		
	YOR1	
YOR059c		
YPL206c	*YPL206c*	
		YPT32
	YSP1	
		ZTA1

Interestingly, only few gene products from this screen were detected in more than one organelle sample, and only two proteins were found in all three samples. One of those proteins was *GTT1* (*YIR038c*), known as an ER associated glutathione S-transferase (Choi *et al.*, 1998). This protein has been previously found to be localized primarily to the ER but also to mitochondria and plasma membrane enriched fractions. Localization to LP or peroxisomes has not yet been described. However, an *in silico* motif search did not reveal a putative active serine nor a lipase (GxSxG) or hydrolase motif (DxDxT/V; (Collet *et al.*, 1998). Also, *GTT1* was previously described to be repressed under peroxisome induced conditions (Smith *et al.*, 2006). It remains to be elucidated whether this enzyme is actually localized to peroxisomes and LP and what its function on these organelles may be.

The other protein found in this study in all three samples was *YJU3* (*YKL094w*) which was assumed to be a serine hydrolase with sequence similarities to mammalian monoglyceride lipase and was reported to be localized to LP already previously (Athenstaedt *et al.*, 1999; Baxter *et al.*, 2004; McPartland *et al.*, 2006). It has been found that *YJU3* is slightly upregulated upon addition of oleic acid to the growth medium (Smith *et al.*, 2006) which would support the theory of an additional hydrolase upon oleic acid induction. A specific role in TAG hydrolysis has to be investigated in more detail but has been already described in recent review (Kohlwein, 2010).

Only few proteins were found to be dually located in LP and peroxisomes (Figure 6B), with three proteins found on YPO (*CAT2, PET9, TEF2*) and only one when LP were isolated from YPD grown cells (*KAR2*). All of these proteins were already described previously, mainly localized to mitochondria or the ER and did not show any lipase or hydrolase motif. As expected, both LP samples showed highest overlap in protein candidates. 16 ORFs encoding for proteins were found on LP from cells grown on both carbon sources (Table 4 and Figure 6B). These proteins found on either YPD or YPO or both represent putative new candidate hydrolases or lipases under oleic acid induced conditions and are listed in Table 4. Among these candidates, five proteins were only found in cells grown on oleic acid whereas two were only found in cells grown on glucose. Six proteins contain a TAG lipase motif (GxSxG) and two show a putative hydrolase motif (DxDxT/V). Five ORFs were previously found in an independent screen for hydrolases (marked with $^{\#}$) (Wagner *et al.*, 2009). Eight of the proteins in the list are of unknown function and six were located to LP previously. The protein encoded by *YBR204c* was of special interest as this ORF contained both a lipase and a hydrolase motif, and has not yet been characterized for its localization and function. In addition, this ORF was found to be a putative hydrolase in a previous screen from our laboratory (Wagner *et al.*, 2009). This protein was identified in one of the most prominent bands from the NBD-HE-HP analysis when cells were grown on YPO (Figure 6A, LP YPO, band 9) indicating that this protein might be upregulated upon oleic acid induction. In an additional study, we tried to characterize localization and function of this protein. We found that it was localized to the LP and showed high hydrolase and minor lipase and phospholipase activity (Thoms *et al*., manuscript in preparation). Further cloning and characterization of the candidate proteins should reveal their potential hydrolase function *in vitro* and *in vivo*.

Table 4: Candidate proteins for novel hydrolases found on lipid particles

List of putative new lipases or hydrolases on LP from yeast cells grown on either YPD or YPO. TAG, lipase motif (GxSxG); HYD, hydrolase motif (DxDxT/V); x found in cells grown on the respective medium; LP, lipid particle; Mt, mitochondrion; Cyt, cytosol; ER, endoplasmic reticulum; N, nucleus; # found in a previous hydrolase screen by Wagner *et al.* (Wagner *et al.*, 2009)

ORF	Gene Name	YPD	YPO	Function	Localization	Size	Motif
YIL124w	*AYR1*	x	x	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase	ER, LP, Mt, Cyt	32.82	TAG
YBR177c #	*EHT1*	x	x	Acyl-coenzymeA:ethanol O-acyltransferase; possesses short-chain esterase activity	LP, Mt	51.25	TAG
YJL082w	*IML2*		x	Protein of unknown function	Cyt, Mt, N	82.53	
YKR046c #	*PET10*	x	x	Protein of unknown function; expression pattern suggests a role in respiratory growth	LP	31.24	
YBR056w			x	Putative cytoplasmic protein of unknown function	Cyt	57.82	HYD
YBR204c #		x	x	Serine hydrolase; YBR204C is not an essential gene	LP, Mt, Px	43.31	TAG HYD
YDR196c			x	Probable dephospho-CoA kinase (DPCK) that catalyzes the last step in coenzyme A biosynthesis	ER, Mt, N	27.3	
YMR152w #	*YIM1*		x	Protein of unknown function; null mutant displays sensitivity to DNA damaging agents	LP, Cyt, Mt	41.63	
YKL094w #	*YJU3*	x	x	Serine hydrolase with sequence similarity to monoglyceride lipase (MGL)	LP	35.56	TAG (2x)
YKL050c			x	Protein of unknown function;protein is a target of the SCFCdc4 ubiquitin ligase complex	unknown	103.1	TAG
YKL107w		x		Putative protein of unknown function; proposed to be a palmitoylated membrane protein	unknown	34.5	
YNL115c		x		Putative protein of unknown function	unknown	74.04	
YPL206c	*PGC1*	x	x	Phosphatidyl Glycerol phospholipase C	LP, Mt	37.07	
Ybr265w	*TSC10*		x	3-ketosphinganine reductase	Cyt, ER, Mt	35.98	TAG

Discussion

Fatty acid turnover

Proteomes of yeast LP and peroxisomes have been investigated quite intensively in the last years (Gabaldon *et al.*, 2006; Athenstaedt *et al.*, 2006). New proteins have been continuously found indicating that the list of proteins and their complex functions are not complete. In a recent study aimed at the completion of the LP proteome we found six additional putative LP proteins depending on the growth conditions used (Grillitsch *et al*., manuscript in preparation). Our laboratory also discovered a dual role of the known TAG lipases on LP. Tgl3p, Tgl4p and Tgl5p seem to possess high acyltransferase in addition to moderate lipase activity (Rajakumari and Daum, 2010a; Rajakumari and Daum, 2010b). These observations led us to the assumption that also other proteins might have dual function, maybe not only on LP but also on other organelles. One of the most prominent LP proteins, Erg1p, has dual localization within the yeast cell. Interestingly, only ER localization allows a fully functional protein (Leber *et al.*, 1998). Together, these data strongly indicate that LP are dynamic organelle, adapting to the environmental changes with alterations in lipid and protein composition. Thereby it is possible that LP serve as lipid as well as protein buffer regulating homeostasis of both under different cellular conditions. However, such a regulatory mechanism has not yet been proven.

In this study we found that TAG hydrolysis was not arrested under oleate stress conditions (see Figure 3B). The question remains, why TAG would be mobilized in cells that already suffer from FFA overflow. At the moment, we can only speculate that different pools of lipids which are used for different processes might exist in the cell. Thus, FFA mobilized from TAG may serve preferably in a specific turnover pathway for PL synthesis or β-oxidative degradation. However, a proof for this model of different pools of FFA for different purposes is still missing. Recently, we found that already growth on glucose led to accumulation of large amounts of FFA (Connerth *et al.*, manuscript under revision). From this finding we speculate that FFA do not play an essential role in regulating the neutral lipid turnover since TAG mobilization occurred normally under these conditions. Our data obtained in this study clearly demonstrate that peroxisomal β-oxidation does not exclusively rely on FFA from neutral lipid mobilization because peroxisomes were functional also in absence of LP. Our data also provide strong evidence for the presence of new, yet unknown hydrolases which are probably redundant

to each other. This finding once more indicates that neutral lipid mobilization is important and turnover of FFA is a highly regulated inter-connected process.

Proteome analysis and candidate hydrolases

Previous studies from our lab identified the three gene products of *TGL3*, *TGL4* and *TGL5* as TAG lipases being localized to the LP (Athenstaedt and Daum, 2003; Athenstaedt and Daum, 2005). In triple deletion strains lacking these three genes, TAG mobilization comes to a halt *in vivo*. It has to be noted that these studies were performed in cells grown on glucose with cerulenin added to prevent further FFA synthesis and therefore further TAG synthesis. Interestingly, our data showed a different effect when cells were grown on oleic acid. Not only was the TM TGL able to hydrolyze TAG *in vivo,* but this mobilization process seemed to be similar efficient as in wild type (see Figure 4B). Deletion of further candidate genes in the TM background could not abolish the TAG mobilization either. From these observations we conclude that most likely more than one protein is able to hydrolyze TAG in cells grown on oleic acid. To further analyze this hypothesis, we used specific fluorescent inhibitors of TAG lipases and hydrolases and found a subset of proteins that showed TAG lipase as well as hydrolase activity *in vitro* on isolated LP but also on peroxisomes (see Figure 5). This assay was very specific for catalytic serine residues but could as a matter of fact not exclude detection of false positives. We also have to be aware that non-catalytic enzymes might be detected by chance due to the overlap with reactive protein bands on the gel. However, all known TAG lipases were identified in this approach thus confirming the sensitivity and selectivity of the assay. Interestingly, *YEH1* encoding a major sterol hydrolase of LP was not found. Detection of prominent LP and peroxisomal proteins with this method was a proof for the purity of organellar preparations which is prerequisite for the localization of novel proteins. Hence, additional proteins so far not annotated as LP or peroxisome components were detected in this screen. These findings complement another study from our laboratory aimed at identification and completion of the LP proteome (Grillitsch *et al*., manuscript in preparation). In total 28 LP proteins were identified independently in both assays. Recently, we found a subset of six putative additional LP proteins of which we could also identify four proteins also in this assay (*KES1, UBX2, VPS66, GTT1*). These data indicate that the search for the entire proteome of organelles is not yet complete.

In addition, data provided in this study lead to the assumption that many proteins may have multiple localizations and also multiple functions in the cell. The underlying mechanisms regulating the localization of proteins in different compartments have yet to be elucidated but seem to be important, because function and localization of a protein are often connected for obvious reasons. From the list of proteins found in our screen we were able to identify a subset of proteins with putative hydrolase or lipase function (Table 4). Some of those proteins had already annotated functions and localization, but we speculate that additional functions of these polypeptides on LP are likely. Two proteins were found in the screen for novel hydrolases on both organelles, namely *GTT1* encoding a glutathione-S-transferase as well as *YJU3* encoding a putative homologue to the mammalian monoglyceride lipase. The latter protein might play an important role in TAG mobilization upon oleic acid growth. Detection of these proteins on both organelles also indicated that a possible link between LP and peroxisomes might exist enabling FFA release from storage to peroxisomal β-oxidation.

In summary, data provided in this study give rise to further and more detailed knowledge of the LP proteome and its role in FFA turnover. Although LP are not essential organelles for the cellular homeostasis of FFA we assume that they fulfill a regulatory function in separating and providing lipid pools for various lipid metabolic processes such as PL biosynthesis, neutral lipid turnover and β-oxidation. These processes seem to be strictly regulated and thus implying an important function of the LP. The subset of new candidate hydrolases will enable to investigate the FA hydrolyzing mechanism in yeast further and help to shed more light on LP function in the cell.

Acknowledgement

We thank Andrea Wagner for providing the yeast mutant strain. This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich Project W901-B05 to G. D. and A.H. and by GEN-AU GOLD3 to A.H.

Reference List

Athenstaedt,K. and Daum,G. (2003). YMR313c/TGL3 encodes a novel triacylglycerol lipase located in lipid particles of Saccharomyces cerevisiae. J. Biol. Chem. *278*, 23317-23323.

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

Athenstaedt,K., Jolivet,P., Boulard,C., Zivy,M., Negroni,L., Nicaud,J.M., and Chardot,T. (2006). Lipid particle composition of the yeast Yarrowia lipolytica depends on the carbon sources. Proteomics. *6*, 1450-1459.

Athenstaedt,K., Zweytick,D., Jandrositz,A., Kohlwein,S.D., and Daum,G. (1999). Identification and characterization of major lipid particle proteins of the yeast Saccharomyces cerevisiae. J. Bacteriol. *181*, 6441-6448.

Baxter,S.M., Rosenblum,J.S., Knutson,S., Nelson,M.R., Montimurro,J.S., Di Gennaro,J.A., Speir,J.A., Burbaum,J.J., and Fetrow,J.S. (2004). Synergistic computational and experimental proteomics approaches for more accurate detection of active serine hydrolases in yeast. Mol. Cell Proteomics. *3*, 209-225.

Binns,D., Januszewski,T., Chen,Y., Hill,J., Markin,V.S., Zhao,Y., Gilpin,C., Chapman,K.D., Anderson,R.G., and Goodman,J.M. (2006). An intimate collaboration between peroxisomes and lipid bodies. J Cell Biol *173*, 719-31.

Birner-Gruenberger,R. *et al.* (2005). The lipolytic proteome of mouse adipose tissue. Mol. Cell Proteomics. *4*, 1710-1717.

Black,P.N. and DiRusso,C.C. (2007). Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. Biochim. Biophys. Acta *1771*, 286-298.

Choi,J.H., Lou,W., and Vancura,A. (1998). A novel membrane-bound glutathione S-transferase functions in the stationary phase of the yeast Saccharomyces cerevisiae. J. Biol. Chem. *273*, 29915-29922.

Collet,J.F., Stroobant,V., Pirard,M., Delpierre,G., and van,S.E. (1998). A new class of phosphotransferases phosphorylated on an aspartate residue in an amino-terminal DXDX(T/V) motif. J. Biol. Chem. *273*, 14107-14112.

Connerth,M., Grillitsch,K., Kofeler,H., and Daum,G. (2009). Analysis of lipid particles from yeast. Methods Mol. Biol. *579*, 359-374.

Czabany,T., Wagner,A., Zweytick,D., Lohner,K., Leitner,E., Ingolic,E., and Daum,G. (2008). Structural and Biochemical Properties of Lipid Particles from the Yeast Saccharomyces cerevisiae. J. Biol. Chem. *283*, 17065-17074.

DiRusso,C.C., Connell,E.J., Faergeman,N.J., Knudsen,J., Hansen,J.K., and Black,P.N. (2000). Murine FATP alleviates growth and biochemical deficiencies of yeast fat1Delta strains. Eur. J. Biochem. *267*, 4422-4433.

Faergeman,N.J., Black,P.N., Zhao,X.D., Knudsen,J., and DiRusso,C.C. (2001). The Acyl-CoA synthetases encoded within FAA1 and FAA4 in Saccharomyces cerevisiae function as components of the fatty acid transport system linking import, activation, and intracellular Utilization. J. Biol. Chem. *276*, 37051-37059.

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

Gabaldon,T., Snel,B., van Zimmeren,F., Hemrika,W., Tabak,H., and Huynen,M.A. (2006). Origin and evolution of the peroxisomal proteome. Biol Direct *1*, 8.

Hettema,E.H. and Tabak,H.F. (2000). Transport of fatty acids and metabolites across the peroxisomal membrane. Biochim Biophys Acta *1486*, 18-27.

Hettema,E.H., van Roermund,C.W., Distel,B., van Den Berg,M., Vilela,C., Rodrigues-Pousada,C., Wanders,R.J., and Tabak,H.F. (1996). The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of Saccharomyces cerevisiae. Embo J *15*, 3813-22.

Hiltunen,J.K., Mursula,A.M., Rottensteiner,H., Wierenga,R.K., Kastaniotis,A.J., and Gurvitz,A. (2003). The biochemistry of peroxisomal beta-oxidation in the yeast Saccharomyces cerevisiae. FEMS Microbiol Rev *27*, 35-64.

Jandrositz,A., Petschnigg,J., Zimmermann,R., Natter,K., Scholze,H., Hermetter,A., Kohlwein,S.D., and Leber,R. (2005). The lipid droplet enzyme Tgl1p hydrolyzes both steryl esters and triglycerides in the yeast, Saccharomyces cerevisiae. Biochim. Biophys. Acta *1735*, 50-58.

Koffel,R., Tiwari,R., Falquet,L., and Schneiter,R. (2005). The Saccharomyces cerevisiae YLL012/YEH1, YLR020/YEH2, and TGL1 genes encode a novel family of membrane-anchored lipases that are required for steryl ester hydrolysis. Mol. Cell Biol. *25*, 1655-1668.

Kohlwein,S.D. (2010). Triacylglycerol homeostasis: insights from yeast. J. Biol. Chem. *in press*.

Kunau,W.H., Buhne,S., de la,G.M., Kionka,C., Mateblowski,M., Schultz-Borchard,U., and Thieringer,R. (1988). Comparative enzymology of beta-oxidation. Biochem. Soc. Trans. *16*, 418-420.

Kurat,C.F., Natter,K., Petschnigg,J., Wolinski,H., Scheuringer,K., Scholz,H., Zimmermann,R., Leber,R., Zechner,R., and Kohlwein,S.D. (2006). Obese yeast: triglyceride lipolysis is functionally conserved from mammals to yeast. J. Biol. Chem. *281*, 491-500.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-5.

Lazarow,P.B. and DeDuve,C. (1976). A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. Proc. Natl. Acad. Sci. U. S. A *73*, 2043-2046.

Leber,R., Landl,K., Zinser,E., Ahorn,H., Spok,A., Kohlwein,S.D., Turnowsky,F., and Daum,G. (1998). Dual localization of squalene epoxidase, Erg1p, in yeast reflects a relationship between the endoplasmic reticulum and lipid particles. Mol. Biol. Cell *9*, 375-386.

Lockshon,D., Surface,L.E., Kerr,E.O., Kaeberlein,M., and Kennedy,B.K. (2007). The sensitivity of yeast mutants to oleic acid implicates the peroxisome and other processes in membrane function. Genetics *175*, 77-91.

Longtine,M.S., McKenzie,A.3., Demarini,D.J., Shah,N.G., Wach,A., Brachat,A., Philippsen,P., and Pringle,J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-61.

Lowry,O.H., Rosebrough,N.J., Farr A.L., and Randall R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

McPartland,J.M., Matias,I., Di,M., V, and Glass,M. (2006). Evolutionary origins of the endocannabinoid system. Gene *370*, 64-74.

Petschnigg,J., Wolinski,H., Kolb,D., Zellnig,G., Kurat,C.F., Natter,K., and Kohlwein,S.D. (2009). Good fat - essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast. J. Biol. Chem.

Rajakumari,S. and Daum,G. (2010a). Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. Mol. Biol. Cell *21*, 501-510.

Rajakumari,S. and Daum,G. (2010b). Multiple functions as lipase, steryl ester hydrolase, phospholipase and acyltransferase of Tgl4p from the yeast Saccharomyces cerevisiae. J. Biol. Chem.

Rosenberger,S., Connerth,M., Zellnig,G., and Daum,G. (2009). Phosphatidylethanolamine synthesized by three different pathways is supplied to peroxisomes of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta.

Schmidinger,H., Birner-Gruenberger,R., Riesenhuber,G., Saf,R., Susani-Etzerodt,H., and Hermetter,A. (2005). Novel fluorescent phosphonic acid esters for discrimination of lipases and esterases. Chembiochem. *6*, 1776-1781.

Schmidinger,H., Hermetter,A., and Birner-Gruenberger,R. (2006a). Activity-based proteomics: enzymatic activity profiling in complex proteomes. Amino. Acids *30*, 333-350.

Schmidinger,H., Susani-Etzerodt,H., Birner-Gruenberger,R., and Hermetter,A. (2006b). Inhibitor and protein microarrays for activity-based recognition of lipolytic enzymes. Chembiochem. *7*, 527-534.

Shani,N., Watkins,P.A., and Valle,D. (1995). PXA1, a possible Saccharomyces cerevisiae ortholog of the human adrenoleukodystrophy gene. Proc Natl Acad Sci U S A *92*, 6012-6.

Smith,J.J., Sydorskyy,Y., Marelli,M., Hwang,D., Bolouri,H., Rachubinski,R.A., and Aitchison,J.D. (2006). Expression and functional profiling reveal distinct gene classes involved in fatty acid metabolism. Mol. Syst. Biol. *2*, 2006.

Sorger,D., Athenstaedt,K., Hrastnik,C., and Daum,G. (2004). A yeast strain lacking lipid particles bears a defect in ergosterol formation. J. Biol. Chem. *279*, 31190-31196.

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

Thoms,S., Debelyy,M.O., Nau,K., Meyer,H.E., and Erdmann,R. (2008). Lpx1p is a peroxisomal lipase required for normal peroxisome morphology. FEBS J. *275*, 504-514.

Valachovic,M., Hronska,L., and Hapala,I. (2001). Anaerobiosis induces complex changes in sterol esterification pattern in the yeast Saccharomyces cerevisiae. FEMS Microbiol. Lett. *197*, 41-45.

Valachovic,M., Klobucnikova,V., Griac,P., and Hapala,I. (2002). Heme-regulated expression of two yeast acyl-CoA:sterol acyltransferases is involved in the specific response of sterol esterification to anaerobiosis. FEMS Microbiol. Lett. *206*, 121-125.

Verleur,N., Hettema,E.H., van Roermund,C.W., Tabak,H.F., and Wanders,R.J. (1997). Transport of activated fatty acids by the peroxisomal ATP-binding-cassette transporter Pxa2 in a semi-intact yeast cell system. Eur J Biochem *249*, 657-61.

Wagner,A., Grillitsch,K., Leitner,E., and Daum,G. (2009). Mobilization of steryl esters from lipid particles of the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta *1791*, 118-124.

Zou,Z., Tong,F., Faergeman,N.J., Borsting,C., Black,P.N., and DiRusso,C.C. (2003). Vectorial acylation in Saccharomyces cerevisiae. Fat1p and fatty acyl-CoA synthetase are interacting components of a fatty acid import complex. J. Biol. Chem. *278*, 16414-16422.

Supplementary

Supplementary Table 1: Protein hits found in an MS screen

LP from yeast cells grown either in the presence of oleate or glucose as well as peroxisomes from cells grown on oleate were isolated as described in Material and Methods section. Then, organelle samples were incubated with the inhibitor NBD-HE-HP at 37 °C over night. Protein precipitation and SDS-PAGE were performed and the resulting gel was scanned for the NBD-tag revealing proteins that covalently bound to the inhibitor thus possessing hydrolase activity. Protein bands were cut out of the gel, subjected to trypsin digestion and analyzed by MS. Proteins found in the respective bands are liste in the Table below. Ei, eiosome; End, endosome; ER, endoplasmic reticulum; G, Golgi apparatus; LP, lipid particle; Mem, membrane; Mt, mitochondria; N, nucleus; PLM, plasma membrane; Px, peroxisome; R, ribosome; V, vacuole; Ves, vesicle

LP YPD						
Band No.	**Approx. band size**	**Gene Name**	**Function**	**Lokalization**	**kDa**	**Peptides**
1	77	*FAA1*	LCFA- CoA ligase activity	LP, Mt	77.9	13
		KAR2	ATPase	ER	74.4	8
		FAT1	FA transporter, VLCFA-CoA synthetase	LP, PX, PM	77.1	5
2	70	*SEC63*	ATPase activator	ER, Mt	75.3	3
		TGL3	TAG lipase	LP	73.6	1
3	60	*VAC8*	Required for the cytoplasm-to-vacuole targeting (Cvt) pathway	V	63.2	5
		PRC1	Carboxypeptidase C	V, C, ER	59.8	1
4	60	*HFD1*	Putative fatty aldehyde dehydrogenase	LP, Mt	60.0	2
		TGL4	TAG lipase	LP	102.7	2
5	55	*TGL5*	TAG lipase	LP, C	84.7	6
		ERG1	Squalene monooxygenase	ER, C, LP	55.1	6
		TGL4	TAG lipase	LP	102.7	3
		ERG6	Sterol-24-c methyltransferase	LP, Mt, C	43.4	1
5a	55	*ERG6*	Sterol-24-c methyltransferase	LP, Mt, C	43.4	1
6	50	*TGL1*	Sterol esterase and TAG lipase	LP, C	63.0	8
		ERG6	Sterol-24-c methyltransferase	LP, Mt, C	43.4	6
		ERG1	Squalene monooxygenase	ER, C, LP	55.1	1
7	45	*ERG6*	Sterol-24-c methyltransferase	LP, Mt, C	43.4	8
		PDR16	Phophatidylinositol bonding/transferring	LP, ER, C, PM	40.7	8

		QCR2	Ubiquitin-cytochrome C reductase	Mt	40.5	3
		DGA1	Diacylglycerol acyl-transferase	LP, ER, C	47.7	1
8	40	*AYR1*	Ketoreductase	LP, Mt, C	32.8	9
		TDH2	Glyceraldehyde-3-phosphate dehydrogenase	LP, C, N	35.8	3
		YJU3	Serine hydrolase	LP, ER, C, Mt	35.6	2
9						
10	30	*YKL107w*	Putative protein of unknown function	C	34.5	12
		ERG27	3-Keto sterol reductase	LP, Mt, ER	39.7	8
		VPS66	Protein of unknown function, acyltransferase	C	33.8	4
		YNL115c	Unknown function	V	74.0	3
		YOR059c	Putative serine esterase	unknown	51.1	2
11	25	*YKL107w*	Putative protein of unknown function	cytoplasm, vacuole	34.5	5
		UBX2	Involved in ER-associated protein degradation	ER, Mt	66.7	4
		EHT1	Acyltransferase, Esterase	LP, Mt, ER, C	51.3	3
		PRB1	Peptidase, Hydrolase	C, V	69.6	2
		RPL8a	Unknown function		28.2	2
		PET10	Unknown function	LP	31.2	2
12	20	*GTT1*	Gluthatione-s-transferase	Mt, C, ER	26.8	6
		YDR196c	Putative dephospho-CoA kinase	Mt, N, ER, C	27.3	4
		YPL206c	Contains glycerophosphodiester phosphodiesterase motifs	Mt, ER	37.1	3
13						
14						
15						
16						

LP YPO						
Band No.	**Approx. band size**	**Gene Name**	**Function**	**Localization**	**kDa**	**peptides**
1	80	*FAA1*	LCFA-CoA synthetase	LP, Mt	77.9	10
		FAT1	Fatty acid transporter and	LP, PLM,	77.1	10

			VLCFA-CoA synthetase	ER, Px		
		ERG7	Lanosterol synthase	ER, LP, PLM	83.5	9
		SEC63	channel protein for targeting and import into the ER	ER, Mt	75.3	5
		ERG1	Squalene epoxidase	ER, LP	55.1	3
		IML2	Protein of unknown function	C, Mt, N	82.5	2
2	75	*TGL3*	TAG lipase	LP	73.6	3
		FAA1	LCFA- CoA ligase	LP, Mt	77.9	3
		TGL4	TAG lipase	LP	102.7	3
		FAT1	Fatty acid transporter and VLCFA-CoA synthetase	LP, PLM, ER, Px	77.1	3
		ERG7	Lanosterol synthase	ER, LP, PLM	83.5	3
		SDH1	Flavoprotein subunit of succinate dehydrogenase	Mt	70.2	2
		ERG1	Squalene epoxidase	ER, LP	55.1	2
		IML2	Protein of unknown function	C, Mt, N	82.5	2
		VPS1	Dynamin-like GTPase required for vacuolar sorting	C, Px, Mt	78.7	2
3	70	*HSP60*	Tetradecameric mitochondrial chaperonin	Mt	60.8	4
		GUT2	Mitochondrial glycerol-3-phosphate dehydrogenase	Mt	72.4	3
		CAT2	Carnitine acetyl-CoA transferase	Mt, Px	77.2	2
		FAA1	LCFA-CoA synthetase	LP, Mt	77.9	2
		URA2	Bifunctional carbamoylphosphate synthetase (CPSase)-aspartate transcarbamylase (ATCase)	C, Mt	245.1	2
4	65	*HFD1*	Putative fatty aldehyde dehydrogenase	End, Mt, LP	59.9	7
		LPD1	Dihydrolipoamide dehydrogenase	Mt	54.0	3
		ERG1	Squalene epoxidase	ER, LP	55.1	2
		YBR056wp	Putative cytoplasmic protein of unknown function	C	57.8	2
5	60	*ERG1*	Squalene epoxidase	ER, LP	55.1	4
		ATP1	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase	Mt	58.6	3
		ALG11	Alpha-1,2-mannosyltransferase	ER	63.1	2
		MIP1	Catalytic subunit of the	Mt	143.	2

			mitochondrial DNA polymerase		5	
5a						
6	60	*TEF2*	Translational elongation factor EF-1 alpha	R	50.0	5
		GDI1	GDP dissociation inhibitor, regulates vesicle traffic in secretory pathways	Mem	51.2	4
		ERG1	Squalene epoxidase	ER, LP	55.1	3
		CSR1	Phosphatidylinositol transfer protein	C, LP, ER, Mt	47.5	3
7	55	*ERG1*	Squalene epoxidase	ER, LP	55.1	2
8	55	*ERG6*	Sterol-24-c methyltransferase	LP, Mt, C	43.4	2
9	35	*ERG6*	Sterol-24-c methyltransferase	LP, Mt, C	43.4	15
		PDR16	Phophatidylinositol bonding /transferring	LP, ER, C, PLM	40.7	6
		YBR204c	Uncharacterized serine hydrolase	LP, Px	43.3	4
10	45	*ERG6*	Sterol-24-c methyltransferase	LP, Mt, C	43.4	20
		ERG1	Squalene epoxidase	ER, LP	55.1	10
		AYR1	Ketoreductase	LP, Mt, C	32.8	9
		YJU3	Serine hydrolase	LP, ER, C, Mt	35.6	8
		HFD1	Putative fatty aldehyde dehydrogenase	End, Mt, LP	59.9	7
		KES1	Member of the oxysterol binding protein family	C, G	49.5	4
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	3
		Yim1	Protein of unknown function	LP, C, Mt	41.6	2
		DGA1	Diacylglycerol acyl-transferase	LP, ER, C	47.7	1
11	40	*ERG6*	Sterol-24-c methyltransferase	LP, Mt, C	43.4	8
		ERG1	Squalene epoxidase	ER, LP	55.1	4
		HFD1	Putative fatty aldehyde dehydrogenase	End, Mt, LP	59.9	4
		AYR1	Ketoreductase	LP, Mt, C	32.8	3
		TSC10	3-Ketosphinganine reductase	C, ER, Mt	36.0	2
		YSP1	Mitochondrial protein with a potential role in promoting mitochondrial fragmentationdeath	Mt	143.6	2
12	35	*POR1*	Mitochondrial porin (voltage-dependent anion channel)	Mt	30.4	5
		PET9	Major ADP/ATP carrier of the mitochondrial inner membrane	Mt	34.4	4

		TEF2	Translational elongation factor EF-1 alpha	R	50.0	3
		ERG1	Squalene epoxidase	ER, LP	55.1	2
		ERG27	3-Keto sterol reductase	ER, Mt	39.7	2
		YKL050w	Protein of unknown function	Unknown	103.1	2
		YOR1	Plasma membrane ATP-binding cassette (ABC) transporter	PLM, Mem	166.7	2
13	30	*PET10*	Protein of unknown function	LP	31.2	5
		ERG1	Squalene monooxygenase	ER, C, LP	55.1	3
14	25	*GTT1*	Gluthatione-s-transferase	Mt, C, ER	26.8	7
		PET10	Protein of unknown function	LP	31.2	5
		EHT1	Acyltransferase, Esterase	LP, Mt, ER, C	51.3	5
		ERG27	3-Keto sterol reductase	LP, Mt, ER	39.7	3
		YDR196c	Putative dephospho-CoA kinase	Mt, N, ER, C	27.3	3
		YPL206c	Contains glycerophosphodiester phosphodiesterase motifs	Mt, ER	37.1	2
15	20	*RPS3*	apurinic/apyrimidinic (AP) endonuclease activity	R	26.5	3
		PET10	Protein of unknown function	LP	31.2	2
		HEF3	Translational elongation factor EF-3	R	116	2
16	14	*PET10*	Protein of unknown function	LP	31.2	3

PX YPO						
Band No.	**Approx. band size**	**Gene Name**	**Function**	**Localization**	**kDa**	**peptides**
1						
2	75	*POX1*	Fatty-acyl coenzyme A oxidase	Px	84.0	10
		FAA2	LCFA-CoA synthetase	Px, Mt	83.4	8
		VPS13	Protein of unknown function	End, ext Mem, Mt	357.8	2
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase	Px	98.7	2
3	70	*POX1*	Fatty-acyl coenzyme A oxidase	Px	84.0	2
4	65	*POX1*	Fatty-acyl coenzyme A oxidase	Px	84.0	8

		FAA2	LCFA-CoA synthetase	Px, Mt	83.4	4
		CAT2	Carnitine acetyl-CoA transferase	Mt, Px	77.2	2
		PMA2	Plasma membrane H+-ATPase	PLM, Mt	102.2	2
5	60	*PCS60*	Peroxisomal AMP-binding protein	Px, C	60.5	6
		CTA1	Catalase A	Mt, Px	58.6	4
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	4
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase	Px	98.7	2
		ALD4	Mitochondrial aldehyde dehydrogenase	Mt	56.7	2
		EFT2	Elongation factor 2 (EF-2)	R	93.3	2
		SMC1	Subunit of the multiprotein cohesin complex	N	141.3	2
		BPT1	ABC type transmembrane transporter of MRP/CFTR family	V	176.9	1
5a						
6	55	*FOX2*	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	4
		ADE13	Adenylosuccinate lyase	unknown	54.5	3
		PCS60	Peroxisomal AMP-binding protein	Px, C	60.5	3
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	2
7	50	*POX1*	Fatty-acyl coenzyme A oxidase	Px	84.0	6
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	5
		CIT1	Citrate synthase	Mt	53.4	5
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	4
		TFP1	Vacuolar ATPase V1 domain subunit A containing the catalytic nucleotide binding sites	V	118.6	6
		CIT2	Citrate synthase	Px, Mt	51.4	4
		PMA1	Plasma membrane H+-ATPase	PLM, Mt, Mem	99.6	3
		SMC4	Subunit of the condensin complex	N	162.2	3
		PEX25	Peripheral peroxisomal	Px	44.9	2

			membrane peroxin			
		WBP1	Beta subunit of the oligosaccharyl transferase (OST) glycoprotein complex	ER, N	49.4	2
		POT1	3-Ketoacyl-CoA thiolase	Px	44.7	2
		POP1	Subunit of both RNase MRP, which cleaves pre-rRNA	R	100.4	2
8	45	*IDP3*	Peroxisomal NADP-dependent isocitrate dehydrogenase	C, Mt, Px	47.9	17
		CIT2	Citrate synthase	Px, Mt	51.4	12
		POT1	3-Ketoacyl-CoA thiolase	Px	44.7	8
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	7
		MDH2	Cytoplasmic malate dehydrogenase	C	40.7	6
		FOX2	Multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway	Px	98.7	6
		PGK1	3-Phosphoglycerate kinase	Mt, C	44.7	6
		PMA1	Plasma membrane H+-ATPase	PLM, Mt, Mem	99.6	5
		MLS1	Malate synthase	Px, C	62.8	5
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	4
		Pil1	Primary component of eisosomes	Ei, C, Mt	38.3	3
		CTA1	Catalase A	Mt, Px	58.6	3
		PEX3	Peroxisomal membrane protein (PMP)	ER, Px	50.7	2
		LPX1	Oleic acid-inducible, peroxisomal matrix localized lipase	Px	43.7	1
9	40	*IDP3*	Peroxisomal NADP-dependent isocitrate dehydrogenase	C, Px, Mt	47.9	15
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	12
		LPX1	Oleic acid-inducible, peroxisomal matrix localized lipase	Px	43.7	11
		MDH2	Cytoplasmic malate dehydrogenase	C	40.7	8
		PMA1	Plasma membrane H+-ATPase	PLM, Mt, Mem	99.6	8
		POT1	3-Ketoacyl-CoA thiolase	Px	44.7	8

		LYS1	Saccharopine dehydrogenase	C	41.5	9
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	7
		MLS1	Malate synthase	Px, C	62.8	6
		CIT2	Citrate synthase	Px	51.4	5
		FAA2	Long chain fatty acyl-CoA synthetase	Px, Mt	83.4	5
		AAT2	Cytosolic aspartate aminotransferase	C, Px	56.0	4
		ILV5x	Acetohydroxyacid reductoisomerase	Mt	44.4	4
		ADH2	Glucose-repressible alcohol dehydrogenase II	C	36.7	3
		AGX1	Alanine:glyoxylate aminotransferase (AGT)	Mt	41.9	3
		ERG9	Farnesyl-diphosphate farnesyl transferase (squalene synthase)	ER, Mt	51.7	2
10	35	*TES1*	Peroxisomal acyl-CoA thioesterase	Px, Mt	40.3	13
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	11
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	10
		ZTA1	Zeta-crystallin homolog	C, N	37.0	6
		LPX1	Oleic acid-inducible, peroxisomal matrix localized lipase	Px	43.7	5
		MDH3	Peroxisomal malate dehydrogenase	Px	37.2	5
		PMA1	Plasma membrane H+-ATPase	PLM, Mt, Mem	99.6	4
		LSP1	Primary component of eisosomes	C, Mt	38.1	4
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	3
		SEC61	channel protein for targeting and import into the ER	ER	52.9	3
		IDH2	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	Mt	39.7	3
		IDH1	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	Mt	39.3	3
		MLS1	Malate synthase	Px, C	62.8	3
		KAR2	ATPase involved in protein import into the ER	ER	74.5	3

		ILV5	Acetohydroxyacid reductoisomerase	Mt	44.4	3
		PEX3	Peroxisomal membrane protein (PMP)	ER, Px	50.7	2
		AAT2	Cytosolic aspartate aminotransferase	C, Px	56.0	2
		AGX1	Alanine:glyoxylate aminotransferase (AGT)	Mt	41.9	2
		YKL151c	Putative protein of unknown function	C	37.4	1
11	30	*MDH3*	Peroxisomal malate dehydrogenase	Px	37.2	14
		TDH3	Glyceraldehyde-3-phosphate dehydrogenase	C, LP, Mt	35.7	8
		PCS60	Peroxisomal AMP-binding protein	Px, C	60.5	6
		ZTA1	Zeta-crystallin homolog	C, N	37.0	6
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	3
		PIL1	Primary component of eisosomes	Ei, C, Mt	38.3	3
		LSP1	Primary component of eisosomes	C, Mt	38.1	2
		LPX1	Oleic acid-inducible, peroxisomal matrix localized lipase	Px	43.7	2
		YJU3	Serine hydrolase with sequence similarity to monoglyceride lipase (MGL)	LP, ER, C, Mt	35.6	2
12	25	*ECI1*	Peroxisomal delta3,delta2-enoyl-CoA isomerase	Px	31.7	12
		PET9	Major ADP/ATP carrier of the mitochondrial inner membrane	Mt	34.4	12
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities	Px	98.7	10
		SPS19	Peroxisomal 2,4-dienoyl-CoA reductase	Px	31.1	9
		LSP1	Oleic acid-inducible, peroxisomal matrix localized lipase	Px	43.7	9
		MIR1	Mitochondrial phosphate carrier, imports inorganic phosphate into mitochondria	Mt	32.8	9
		TEF2	Translational elongation factor EF-1 alpha; also encoded by TEF1	R	50.0	7

		DPM1	Dolichol phosphate mannose (Dol-P-Man) synthase	ER, Mt	30.4	5
		BBC1	Protein possibly involved in assembly of actin patches	Ct	128.3	5
		CBR1	Microsomal cytochrome b reductase	ER, Mt	31.5	5
		ATP3	Gamma subunit of the F1 sector of mitochondrial F1F0 ATP synthase	Mt	34.4	4
		MDH2	Cytoplasmic malate dehydrogenase	C	40.7	5
		FSH3 (YOR280 C)	serine hydrolase; similar to FSH1, FSH2 and FSH3		30.4	2
		YJR088c	Putative protein of unknown function	ER	33.9	2
		YJR111c	Putative protein of unknown function	Mt	32.2	1
		YGL185	Putative protein with sequence similarity to hydroxyacid dehydrogenases	C	43.0	1
13	20	*FOX2*	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	10
		PET9	Major ADP/ATP carrier of the mitochondrial inner membrane	Mt	34.4	9
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	8
		LSP1	Oleic acid-inducible, peroxisomal matrix localized lipase	Px	43.7	6
		SEC4	Secretory vesicle-associated Rab GTPase essential for exocytosis	Ves, Mt	23.5	6
		DCI1	Peroxisomal delta(3,5)-delta(2,4)-dienoyl-CoA isomerase	Px	30.1	6
		ATP4	Subunit b of the stator stalk of mitochondrial F1F0 ATP synthase	Mt	26.9	6
		ECI1	Peroxisomal delta3,delta2-enoyl-CoA isomerase	Px	31.7	6
		YPT32	GTPase of the Ypt/Rab family, very similar to Ypt31p	End, G, Mt	24.5	5
		GTT1	ER associated glutathione S-transferase capable of homodimerization	ER, Mt	26.8	4

		LPX1	Oleic acid-inducible, peroxisomal matrix localized lipase	Px	43.7	4
		SPS19	Peroxisomal 2,4-dienoyl-CoA reductase	Px	31.1	3
		PCS60	Peroxisomal AMP-binding protein	Px, C	60.5	3
		PDI1	Protein disulfide isomerase	ER	58.2	3
		FMP52	Protein of unknown function	ER, Mt	25.1	3
		MDH3	Peroxisomal malate dehydrogenase	Px	37.2	2
		ERP1	Protein that forms a heterotrimeric complex with Erp2p, Emp24p, and Erv25p	Ves, Mt	24.7	2
		ERG2	C-8 sterol isomerase	ER	24.9	1
		PEX14	Peroxisomal membrane peroxin	Px	38.4	1
		Yll023	Protein of unknown function	ER, R	32.2	1
14						
15						
16						

General Discussion

Lipids are one of the major components of cellular membranes and essential for their functionality. Thus, it is important that level and composition of lipids in distinct subcellular membranes are well balanced. Consequently, lipid homeostasis and turnover need to be tightly regulated as malfunction of these processes are often linked to severe defect such as the human diseases atherosclerosis, diabetes, cardiovascular diseases and obesity (Unger and Orci, 2002; Schaffer, 2003). Within the last decades, sites of biochemical pathways leading to biosynthesis or degradation of lipids were pinpointed. However, not all lipids are synthesized in the place where they are needed. Certain organelles have a limited capacity to synthesize lipids, whereas others completely lack this property. Therefore, lipid transport from sites of synthesis to proper destinations is another important process involved in cellular lipid homeostasis, and regulation of this translocation process may strongly affect the biogenesis of organelles. Despite intense efforts, mechanisms of lipid traffic in the cell are still ill defined although they are clearly essential for cellular function.

Work presented in this Thesis was aimed at elucidating some selected aspects of intracellular lipid traffic. In the following, novel findings of this study will be summarized and discussed in the light of our recent knowledge from the literature.

Fatty acid traffic

Fatty acids, besides being building blocks for complex lipids, also function as signaling molecules or even as energy source. The role of free fatty acids (FFA) in the cell is still controversial. This becomes most evident with the model system used in this study, the baker's yeast *Saccharomyces cerevisiae.* On one hand, yeast is able to grow on oleic acid as carbon source which leads to proliferation of peroxisomes and depends on peroxisomal functions. (Hiltunen *et al.*, 2003). On the other hand, it appears that excess of oleic acid has to be prevented by incorporation into neutral lipids to avoid a so-called lipotoxic effect (Kohlwein and Petschnigg, 2007; Garbarino and Sturley, 2009). It almost sounds like a paradox, that the cell needs supply of fatty acid to peroxisomes as carbon source and at the same time has to circumvent a putative toxic accumulation of these components.

To solve this problem, strict regulation of both processes and crosstalk between fatty acid transport to the site of β-oxidation and storage in the form of neutral lipids is required. The storage organelle, the lipid particle (LP), seems to play a central role in this link. This organelle forms a cellular buffer for free fatty acids in the form of harmless TAG and SE (Daum *et al.*, 2007). In this Thesis it was shown that LP similar to peroxisomes were strongly induced in size and abundance when yeast cells were grown on oleic acid (Chapter 1). In line with other studies we found that exogenously supplied FFA was primarily incorporated into TAG which served as a major depot of FA (Chapters 3-6) (Petschnigg *et al.*, 2009). Interestingly, we also found that this buffer was rather dynamic, and TAG was mobilized even in the presence of exogenous oleic acid (Chapter 6). Similar findings were made in hepatic cells (Reid *et al.*, 2008) assuming that FA released from TAG stores are essential for FA oxidation pathways under normal conditions. These data indicate that a pool of FA different from the exogenously supplied oleic acid might exist that specifically serves for cellular processes such as phospholipid synthesis or β-oxidation. In contrast to mammalian cells, however, our findings clearly showed that FA released from TAG are not essentially used for β-oxidation, because in the absence of TAG, caused by distinct mutations, growth on oleic acid did not come to an halt and functional peroxisomes were formed (Chapters 4 and 6). It remains to be examined whether other processes such as phospholipid biosynthesis or cellular signaling depend exclusively or predominantly on FA released from TAG hydrolysis. Only recently such a link between TAG hydrolysis and phospholipid synthesis was shown (Rajakumari and Daum, 2010a; Rajakumari and Daum, 2010b).

In addition, a novel yet unknown and unexpected regulatory mechanism of oleic acid in the yeast was found during this Thesis work. FFA are known to play a role in cellular and especially transcriptional regulation (Gurvitz and Rottensteiner, 2006). We found that oleic acid had a specific inhibitory effect on acyl-CoA:acyltransferase activity (Chapter 4). We observed that oleic acid competitively inhibited the steryl ester synthase Are2p *in vitro* and *in vivo* thus leading to a decreased level of steryl esters (SE) in the yeast. We speculate that this process enables the cell to deal with increased membrane fluidity due to an altered degree of saturation in phospholipids (Chapter 3 and 4).

We found that the degree of saturation dramatically changes upon growth of the yeast cell on oleic acid which may also affect membrane properties. We further assume from our studies that the cell requires more sterols embedded in the membrane for higher rigidity, which can be achieved by inhibition of SE formation. Altered membrane fluidity has been claimed in previous studies to be responsible for the lipotoxic effect (Lockshon *et al.*, 2007), but so far a proof has been missing. Here, we show for the first time a direct link between oleic acid overflow and membrane alteration as well as a novel regulatory effect by direct enzyme inhibition. Together our data provide a new facet of how lipid homeostasis is regulated in the yeast cell which may also be applicable to other cell types such as plants and mammals.

Phospholipid traffic

Phospholipid biosynthesis is a tightly regulated and well understood mechanism of interconnected pathways in different subcellular compartments (Carman and Henry, 1989; Daum *et al.*, 1998; Carman and Henry, 1999). Hence not all organelles are capable of phospholipid synthesis and thus depend on the supply of lipids from their sites of synthesis. This implies tightly regulated shuttle mechanisms which are hardly understood until today. Main sites of phospholipid synthesis are the ER and the mitochondria but also to some extend the Golgi apparatus and the LP (Zinser *et al.*, 1991; Trotter and Voelker, 1995; Leber *et al.*, 1995; Daum and Vance, 1997). Different theories account for phospholipid traffic in the cell. One of the best investigated is the secretory pathway via vesicle flux from the ER along the Golgi which eventually does not only transport proteins but also lipids to the plasma membrane (Kornberg and McConnell, 1971; Hrafnsdottir *et al.*, 1997; Boon *et al.*, 2002; Lee *et al.*, 2004). However, for some organelles, such as the peroxisome, participation in this vesicle flux is controversial and very unlikely (Titorenko *et al.*, 2000). A growth and division model is rather accepted for this organelle (Titorenko and Mullen, 2006; Motley and Hettema, 2007) which requires phospholipid supply for membrane expansion. One of the most discussed hypothesis for lipid transfer throughout the cell describes the involvement of phospholipid transfer proteins (Wirtz *et al.*, 1990; Wirtz, 1991; Wirtz, 2006) in lipid translocation and membrane assembly. Although such proteins perfectly transport lipids *in vitro*, no clear evidence for their activity *in vivo* has been presented.

It also remains uncertain whether the comparably low efficiency of such a transport mechanism could account for membrane expansions or whether this process would rather be important for remodeling or signaling processes. However, data provided in this Thesis did not reveal any hints supporting the theory of lipid transfer proteins trafficking phospholipids to peroxisomes. On the contrary, our data supported a third hypothesis of lipid traffic which requires direct membrane contact (Levine and Loewen, 2006). Investigations dealing with PE traffic to peroxisomes revealed that peroxisomes are clearly in contact with phospholipid synthesizing membranes such as ER, mitochondria and LP (Chapter 1). These data also support the theory that new peroxisomes may bud from the ER (Titorenko and Rachubinski, 1998; Hettema *et al.*, 2000). We speculate that membrane contact sites, probably similar to mitochondrial contacts with the ER, so called MAM (Ardail *et al.*, 1993; Achleitner *et al.*, 1995; Achleitner *et al.*, 1999), found in previous studies may also exist between peroxisomes and other organelles and may account for phospholipid traffic.

So far our attempts to directly visualize these contact sites failed. The method used for isolating MAM fractions was not applicable to peroxisomes as the pH shift required was already used during standard peroxisome isolation procedure. Further studies may lead to modification and improvement of the protocol and might result in the isolation of peroxisome associated membranes (PXAMs). In another approach we tried to make use of a fluorescence microscopy technique which was applied to plant chloroplasts visualizing direct membrane contact with the ER (Andersson *et al.*, 2007). Investigating yeast peroxisomes with this method clearly showed that optical tools available at date are not sensitive enough for the size of yeast organelles even under induced conditions. In addition, plant peroxisomes were isolated but were also not found to be applicable for the optical tweezers tool. Improvement of the laser beam size and resolution limitations would be needed for further more precise studies in this direction. Since involvement of direct membrane contact has also been found in lipid trafficking in plants (Levine, 2004) it is tempting to speculate that it also plays a role in yeast cells.

During our studies of phospholipid supply to peroxisomal membranes we found that one of the essential phospholipids, PE, can be supplied to the peroxisomes from all three known biosynthetic pathways present in three different organelles (see Introduction). This finding led to the speculation that not only peroxisomal contact with the ER plays a role for lipid delivery but perhaps also with mitochondria and the Golgi (Chapter 1). A clear and direct experimental

proof for a delivery mechanism via direct membrane contact is still missing. It was also not clear from this study whether PE was directly supplied from mitochondria or prior imported to the ER and then delivered to peroxisomes.

In a further attempt to elucidate the route of PE to peroxisomes we made use of a reporter enzyme assay. Opi3p, the major methyltransferase producing PC from PE, was introduced into peroxisomes carrying a reporter GFP-SKL-tag for peroxisomal localization (Chapter 2). This strategy enabled us to test the flux and efficiency of the substrate PE to peroxisomes from its sites of synthesis. It was found in *semi in vitro* studies using permeabilized cells that the hybrid reporter enzyme was fully functional in peroxisomes and exhibited similar PE methylation efficiency as wild type. This finding implies that PE was not limiting in peroxisomal membrane and thus has to be constantly transported to the organelle. This assay forms the basis for further investigations of PE traffic to peroxisomes in more detail in the future. Applying mutant strains deficient in different PE pathways to the assay will enable to elucidate important or even essential traffic routes. In a further step, also the export of peroxisomal PC as well as the impact of co-factors and cytosolic compounds can be tested in the same experimental set up. Thus, the reporter enzyme assay may become a powerful tool for the investigation of PE and PC traffic between peroxisomes and other organelles.

Lipid particles and lipid homeostasis in the cell

Neutral lipids, as described above, serve as energy reservoir for the cell. They are formed when overflow of nutrients occurs and mobilized upon requirement (Wagner and Daum, 2005; Daum *et al.*, 2007; Czabany *et al.*, 2007). Using yeast as a model system we were able to elucidate formation and turnover of neutral lipids in a systematic manner. Yeast cells grown on oleic acid as carbon source need functional peroxisomes for energy production (Erdmann *et al.*, 1989; Hiltunen *et al.*, 2003; Poirier *et al.*, 2006). As described above, oleic acid may also become toxic for the cell and thus is incorporated into neutral lipids of LP as prerequisite for cell survival (Listenberger *et al.*, 2003; Schaffer, 2003; Kohlwein and Petschnigg, 2007; Garbarino and Sturley, 2009). This organelle has long been regarded to be only a depot for excess of fatty acids. Recent investigations, however, supported the idea of LP as dynamic organelle (Leber *et al.*, 1994; Zweytick *et al.*, 2000; Sorger *et al.*, 2004).

As our studies showed that oleic acid induces proliferation of LP (Chapters 1 and 3) we made use of this effect to study alterations in LP formation including lipid and protein composition under different conditions (Chapters 3 and 5). Both, the proteome and lipidome of LP, were affect by oleate inductive conditions. Lipids of LP were not only changed in their amount but also in their species composition. Although it seemed at first glance that excess of oleic acid had led to a massive incorporation into all lipid species we found an exception which was phosphatidylinositol (PI). In addition, formation of SE was dramatically decreased by the presence of oleate in the medium, whereas TAG was found to serve as major pool for FFA. Interestingly, additional compounds such as ethyl ester (EE) were detected which may also be buffers for the surplus of oleic acid.

Analysis of proteins from LP revealed that amount and pattern were changed when cells were grown on oleic acid. It is tempting to speculate that specific protein functions may become required on the LP under these growth and metabolic conditions (Chapters 3, 5 and 6). As mentioned above we assume that regulated mobilization of neutral lipids is one of the major functions of the LP and its proteins. Indeed, analyzing TAG mobilization from oleate grown cells *in vivo* revealed a striking difference to previous studies performed with cells grown on glucose. Under oleate induction, TAG mobilization did not come to an halt even in the absence of the major known yeast lipases, Tgl3p, Tgl4p and Tgl5p (Chapter 6). In an attempt to investigate this effect we applied a fluorescence assay using inhibitors directed against lipases and hydrolases. With this assay we were able to identify additional lipase and hydrolase candidate proteins and showed an up-regulated hydrolase activity upon oleic acid induction. These findings imply that even though not essential, highly regulated TAG mobilization seems to play an important role even when oleic acid is present in excess. In cells bearing defects in LP formation (Sandager *et al.*, 2002) cell viability was impaired on oleic acid. The underlying regulatory mechanisms have to be further elucidated. Data presented in this Thesis will provide the basis for investigating the regulatory network of lipid homeostasis.

Conclusions

A major question of cell biology is the understanding of the organelle interplay in metabolic processes. This interplay is prerequisite for cellular function and has therefore to be tightly regulated as dysfunction can lead to severe defects. Although many cellular processes have been intensively investigated in the last years underlying regulatory mechanisms still remain ambiguous. The data presented in this Thesis aims at the investigation of lipid trafficking within the yeast cell. Thereby experiments were focused on cellular alterations in response to growth on oleic acid. Organelles affected mostly by this change of conditions were peroxisomes and LP which were therefore analyzed in more detail at the lipid as well as the protein level. New information addressing the phospholipid traffic in the yeast and especially supply of PE to peroxisomal membranes will help to elucidate the biogenesis of this organelle. Furthermore, data obtained in this study comprising the complete lipid and protein composition of LP will contribute to our knowledge of the role of this organelle. Finally, all these findings will lead one step forward to the understanding of lipid dynamic processes which may play an important role in future approaches of applied cell biology.

Reference List

Achleitner,G., Gaigg,B., Krasser,A., Kainersdorfer,E., Kohlwein,S.D., Perktold,A., Zellnig,G., and Daum,G. (1999). Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. Eur J Biochem *264*, 545-53.

Achleitner,G., Zweytick,D., Trotter,P.J., Voelker,D.R., and Daum,G. (1995). Synthesis and intracellular transport of aminoglycerophospholipids in permeabilized cells of the yeast, Saccharomyces cerevisiae. J Biol Chem *270*, 29836-42.

Andersson,M.X., Goksor,M., and Sandelius,A.S. (2007). Optical manipulation reveals strong attracting forces at membrane contact sites between endoplasmic reticulum and chloroplasts
1. J. Biol. Chem. *282*, 1170-1174.

Ardail,D., Gasnier,F., Lerme,F., Simonot,C., Louisot,P., and Gateau-Roesch,O. (1993). Involvement of mitochondrial contact sites in the subcellular compartmentalization of phospholipid biosynthetic enzymes. J. Biol. Chem. *268*, 25985-25992.

Boon,J.M., Shukla,R., Smith,B.D., Licini,G., and Scrimin,P. (2002). Selective phosphatidylethanolamine translocation across vesicle membranes using synthetic translocases. Chem Commun (Camb) 260-1.

Carman,G.M. and Henry,S.A. (1989). Phospholipid biosynthesis in yeast. Annu. Rev. Biochem. *58*, 635-669.

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

Czabany,T., Athenstaedt,K., and Daum,G. (2007). Synthesis, storage and degradation of neutral lipids in yeast. Biochim. Biophys. Acta *1771*, 299-309.

Daum,G., Lees,N.D., Bard,M., and Dickson,R. (1998). Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae. Yeast *14*, 1471-510.

Daum,G. and Vance,J.E. (1997). Import of lipids into mitochondria. Prog Lipid Res *36*, 103-30.

Daum,G., Wagner,A., Czabany,T., Grillitsch,K., and Athenstaedt,K. (2007). Lipid storage and mobilization pathways in yeast. Novartis. Found. Symp. *286*, 142-151.

Erdmann,R., Veenhuis,M., Mertens,D., and Kunau,W.H. (1989). Isolation of peroxisome-deficient mutants of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *86*, 5419-23.

Garbarino,J. and Sturley,S.L. (2009). Saturated with fat: new perspectives on lipotoxicity. Curr. Opin. Clin. Nutr. Metab Care *12*, 110-116.

Gurvitz,A. and Rottensteiner,H. (2006). The biochemistry of oleate induction: Transcriptional upregulation and peroxisome proliferation. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1763*, 1392-1402.

Hettema,E.H., Girzalsky,W., van den,B.M., Erdmann,R., and Distel,B. (2000). Saccharomyces cerevisiae pex3p and pex19p are required for proper localization and stability of peroxisomal membrane proteins. EMBO J. *19*, 223-233.

Hiltunen,J.K., Mursula,A.M., Rottensteiner,H., Wierenga,R.K., Kastaniotis,A.J., and Gurvitz,A. (2003). The biochemistry of peroxisomal beta-oxidation in the yeast Saccharomyces cerevisiae. FEMS Microbiol Rev *27*, 35-64.

Hrafnsdottir,S., Nichols,J.W., and Menon,A.K. (1997). Transbilayer movement of fluorescent phospholipids in Bacillus megaterium membrane vesicles. Biochemistry *36*, 4969-78.

Kohlwein,S.D. and Petschnigg,J. (2007). SLipid-induced cell dysfunction and cell death: lessons from yeast. Curr. Hypertens. Rep. *9*, 455-461.

Kornberg,R.D. and McConnell,H.M. (1971). Lateral diffusion of phospholipids in a vesicle membrane. Proc Natl Acad Sci U S A *68*, 2564-8.

Leber,A., Hrastnik,C., and Daum,G. (1995). Phospholipid-synthesizing enzymes in Golgi membranes of the yeast, Saccharomyces cerevisiae. FEBS Lett. *377*, 271-274.

Leber,R., Zinser,E., Zellnig,G., Paltauf,F., and Daum,G. (1994). Characterization of lipid particles of the yeast, Saccharomyces cerevisiae. Yeast *10*, 1421-1428.

Lee,M.C., Miller,E.A., Goldberg,J., Orci,L., and Schekman,R. (2004). Bi-directional protein transport between the ER and Golgi. Annu Rev Cell Dev Biol *20*, 87-123.

Levine,T. (2004). Short-range intracellular trafficking of small molecules across endoplasmic reticulum junctions. Trends Cell Biol *14*, 483-90.

Levine,T. and Loewen,C. (2006). Inter-organelle membrane contact sites: through a glass, darkly. Curr. Opin. Cell Biol. *18*, 371-378.

Listenberger,L.L., Han,X., Lewis,S.E., Cases,S., Farese,R.V., Jr., Ory,D.S., and Schaffer,J.E. (2003). Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc. Natl. Acad. Sci. U. S. A *100*, 3077-3082.

Lockshon,D., Surface,L.E., Kerr,E.O., Kaeberlein,M., and Kennedy,B.K. (2007). The sensitivity of yeast mutants to oleic acid implicates the peroxisome and other processes in membrane function. Genetics *175*, 77-91.

Motley,A.M. and Hettema,E.H. (2007). Yeast peroxisomes multiply by growth and division. J. Cell Biol. *178*, 399-410.

Petschnigg,J., Wolinski,H., Kolb,D., Zellnig,G., Kurat,C.F., Natter,K., and Kohlwein,S.D. (2009). Good fat - essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast. J. Biol. Chem.

Poirier,Y., Antonenkov,V.D., Glumoff,T., and Hiltunen,J.K. (2006). Peroxisomal beta-oxidation--a metabolic pathway with multiple functions. Biochim. Biophys. Acta *1763*, 1413-1426.

Rajakumari,S. and Daum,G. (2010a). Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. Mol. Biol. Cell *21*, 501-510.

Rajakumari,S. and Daum,G. (2010b). Multiple functions as lipase, steryl ester hydrolase, phospholipase and acyltransferase of Tgl4p from the yeast Saccharomyces cerevisiae. J. Biol. Chem.

Reid,B.N., Ables,G.P., Otlivanchik,O.A., Schoiswohl,G., Zechner,R., Blaner,W.S., Goldberg,I.J., Schwabe,R.F., Chua,S.C., Jr., and Huang,L.S. (2008). Hepatic overexpression of hormone-sensitive lipase and adipose triglyceride lipase promotes fatty acid oxidation, stimulates direct release of free fatty acids, and ameliorates steatosis. J. Biol. Chem. *283*, 13087-13099.

Sandager,L., Gustavsson,M.H., Stahl,U., Dahlqvist,A., Wiberg,E., Banas,A., Lenman,M., Ronne,H., and Stymne,S. (2002). Storage Lipid Synthesis Is Non-essential in Yeast. J. Biol. Chem. *277*, 6478-6482.

Schaffer,J.E. (2003). Lipotoxicity: when tissues overeat. Curr. Opin. Lipidol. *14*, 281-287.

Sorger,D., Athenstaedt,K., Hrastnik,C., and Daum,G. (2004). A yeast strain lacking lipid particles bears a defect in ergosterol formation. J. Biol. Chem. *279*, 31190-31196.

Titorenko,V.I., Chan,H., and Rachubinski,R.A. (2000). Fusion of small peroxisomal vesicles in vitro reconstructs an early step in the in vivo multistep peroxisome assembly pathway of Yarrowia lipolytica. J Cell Biol *148*, 29-44.

Titorenko,V.I. and Mullen,R.T. (2006). Peroxisome biogenesis: the peroxisomal endomembrane system and the role of the ER. J Cell Biol *174*, 11-7.

Titorenko,V.I. and Rachubinski,R.A. (1998). The endoplasmic reticulum plays an essential role in peroxisome biogenesis. Trends Biochem Sci *23*, 231-3.

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

Unger,R.H. and Orci,L. (2002). Lipoapoptosis: its mechanism and its diseases. Biochim. Biophys. Acta *1585*, 202-212.

Wagner,A. and Daum,G. (2005). Formation and mobilization of neutral lipids in the yeast Saccharomyces cerevisiae. Biochem. Soc. Trans. *33*, 1174-1177.

Wirtz,K.W. (1991). Phospholipid transfer proteins. Annu. Rev. Biochem. *60*, 73-99.

Wirtz,K.W. (2006). Phospholipid transfer proteins in perspective. FEBS Lett *580*, 5436-41.

Wirtz,K.W., Snoek,G.T., Gadella,T.W., van Paridon,P.A., and Somerharju,P.J. (1990). Properties and possible function of phosphatidylinositol-transfer proteins. Biotechnol. Appl. Biochem. *12*, 485-488.

Zinser,E., Sperka-Gottlieb,C.D., Fasch,E.V., Kohlwein,S.D., Paltauf,F., and Daum,G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. J Bacteriol *173*, 2026-34.

Zweytick,D., Athenstaedt,K., and Daum,G. (2000). Intracellular lipid particles of eukaryotic cells. Biochim. Biophys. Acta *1469*, 101-120.

Waltendorfer Haupstr. 35 • 8010 Graz, Austria • phone: 0043 681 10617847 • e-Mail: Melanie.Connerth@tugraz.at

Melanie Connerth

Curriculum vitae

Personal Information

Date of birth	04th October 1981
Nationality	German
Gender	female

Education

PhD in Biochemistry **2006 - present**
Institute of Biochemistry
Graz University of Technology, Austria
Interdisciplinary PhD program 'Molecular Enzymology'
Title: Lipid Traffic in the Yeast

Research Stay **Oct. 2008 – May 2009**
Institute of Plant and Environmental Sciences
University of Gothenburg, Sweden

Master of Science in Biochemistry **2004 – 2006**
Institute of Physiological Chemistry,
Department of System-biochemistry
Ruhr-University Bochum, Germany
Title: Identification of Phospholipid- Flippase Activity
in the Yeast *Saccharomyces cerevisiae*

Erasmus Studies **Oct. 2004 – Mar. 2005**
University of Sussex, Brigthon, UK

Bachelor of Science in Biochemistry **2001 – 2004**
Institute of Physiological Chemistry
Ruhr-University Bochum, Germany
Title: Functional studies on peroxisomal ABC-transporters
in the yeast *Saccharomyces cerevisiae*

General Higher Education Entrance Qualification (Abitur) **June 2001**
Gymnasium Sedanstrasse
Wuppertal, Germany

Publications

Rosenberger S., **Connerth M.**, Zellnig G. and Daum G. (2009)
Phosphatidylethanolamine synthesized by three different pathways is supplied to the peroxisomes of the yeast Saccharomyces cerevisiae, BBA, 1791(5); 379-387

Connerth M., Grillitsch K., Köfeler H. and Daum G. (2009)
Methods in Molecular Biology, Vol. 579, Armstrong D. (Ed.), Lipidomics 1: Chapter 18: Analysis of Lipid Particles from Yeast, Methods in Molecular Biology, Vol. 579: 359-374. Armstrong D. (Ed.) Springer, ISBN: 978-1-60761-321-3

Connerth M., Czabany T., Wagner A., Steyrer E., Zellnig G., Daum, G. (2010)
Oleate inhibits steryl ester synthesis and causes liposensitivity in the yeast;
JBC, manuscript under revision

Oral Presentations

Connerth M., Czabany T., Wagner A., Zellnig G., Leitner E., Steyrer E. and G. Daum
The Oily Truth: Oleate Inhibits Steryl Ester Synthesis and Causes Liposensitivity in the Yeast.
FEBS Workshop Microbial Lipids: From Genomics to Lipidomics, Vienna, Austria, 13-15 May 2010

Connerth M., Czabany T., Wagner A., Zellnig G., Leitner E., Steyrer E. and G. Daum
Fatty Acid Lipotoxicity in the Yeast: To Store or not to Store, This is the Question.
7th Euro Fed Lipid Congress, Graz, Austria, 18-21 October 2009

Connerth M., Czabany T., Zellnig G., Leitner E. and Daum G.
Those declared dead live longer: New insights into yeast lipotoxicity
9th Yeast Lipid Conference, Berlin, Germany, 21-23 May 2009

Connerth M., Schatte J., Zellnig G., Hermetter A., Kollroser M. and Daum G.
Lipid Traffic to Yeast Peroxisomes
Joint Annual Meeting of ÖGBM, ÖGGGT, ÖGBT and ANGT, Graz, Austria, 21-24 September 2008

Poster Presentations

Connerth M., Schatte J., Zellnig G., Kollroser M., Hermetter A. and Daum G.
Lipid Traffic to Yeast Peroxisomes
Frontier Lipidology: Lipidomics in Health and Disease, Gothenburg, Sweden, 10-13 May 2009

Connerth M., Schatte J., Zellnig G., Kollroser M., Hermetter A. and Daum G.
Lipid Traffic to Yeast Peroxisomes
Gordon Research Conference on Plant Lipids: Structure, Metabolism and Function, Galveston, USA, 1-6 February 2009

Connerth M., Schatte J., Zellnig G., Hermetter A., Kollroser M. and Daum G.
Lipid Traffic to Peroxisomes from the Yeast Saccharomyces cerevisiae
49th International Conference on the Bioscience of Lipids, Maastricht, Netherlands, 26– 30 August 2008

Connerth M., Rosenberger S., Zellnig G. and Daum G.
Supply of phospholipids to peroxisomes of Saccharomyces cerevisiae
2nd International Symposium on Lipid and Membrane Biology: Focus on Lipotoxicity, Graz Austria, 13-15 March 2008

Connerth M., Rosenberger S., Zellnig G. and Daum G.
Supply of phospholipids to peroxisomes of Saccharomyces cerevisiae
FEBS EMBO advanced lecture course: Cellular and Molecular biology of membranes, Cargèse, Corsica, France, 18-29 June 2007

Rosenberger S., **Connerth M.**, Zellnig G., Daum G.
Supply of phosphatidylethanolamine to the peroxisomes of Saccharomyces cerevisiae
8th Yeast Lipid Conference, Turino, Italy, May 10-12 2007

Additional Experiences

Tutor **2007 - 2009**
Lab course teaching ‘Methods in Immunology’

Tutor **2007 - 2009**
Supervision of undergraduate students in the lab

Participant **June 2007**
Summer school for research and transferable skills training
(Personal and Group Management, Scientific integrity,
Career Development, Communication, Project Development)

Awards

FEBS travel grant **June 2007**

Sokrates/Erasmus mobility scholarship **Oct. 2004 – Mar. 2005**

Technical Experiences

Lipid Biochemistry
- Lipid isolation, purification and analyses including 1D- and 2D-TLC, densometric scanning and GLC
- *In vivo* and *in vitro* labeling studies with radiolabeled or fluorescence samples
- *In vitro* membrane reconstitution including preparation and analyses of liposomes and proteoliposomes

Protein Biochemistry
- Protein expression and isolation in yeast and *E. coli*
- SDS-PAGE and Western blotting
- *In vivo* and *in vitro* enzyme assays in yeast and plants
- IgG isolation with protein A sepharose column
- Protein quantification and solid phase assay

Molecular Biology
- Isolation of plasmid and genomic DNA from yeast and bacteria
- DNA cloning including restriction and ligation
- PCR techniques
- Yeast genetics including generation of genomic knock-outs, construction of tagging and expression constructs
- Yeast and *E. coli* transformation

Cell Biology
- Subcellular fractionation and organelle isolation using differential centrifugation as well as density gradient centrifugation from yeast and plant cells
- Fluorescence and electron microscopy
- Fluorescence and UV/Vis spectroscopy

Bioinformatics
- *In silico* cloning analyses (Vector NTI, pDRAW32)
- Protein and nucleic acid analyses using BLAST, multiple sequence alignments, motif and domain search

Computational
- Microsoft Office (Word, Excel, Powerpoint)
- Macromedia Freehand, Corel Photo-Paint, Adope Photoshop
- ChemBioOffice

Language Skills

German	native
English	fluent
Swedish	basic
French	basic
Latin	High school level

References

Prof. Günther Daum
Institute of Biochemistry
Graz University of Technology
Petersgasse 12/2, 8010 Graz, Austria
Phone: ++43 316 873 6462
Fax: ++43 316 873 6952
e-Mail: guenther.daum@tugraz.at

Prof. Anna Stina Sandelius
Institute of Plant and Environmental Science
University of Gothenburg
Box 461
40530 Gothenburg, Sweden
Phone: ++46 031-786 2611
Mobile: ++46 705324151
Fax: ++46 031-786 25 60
e-Mail: annastina.sandelius@dpes.gu.se

Prof. Ralf Erdmann
Institute of Physiological Chemistry
Dept. Systems Biochemistry
Faculty of Medicine
Ruhr-University of Bochum
44801 Bochum, Germany
Phone: ++49 234 322 4943
Fax: ++49 234 321 4266
e-Mail: ralf.erdmann@rub.de

Contact details

Melanie Connerth
Freiligrathstrasse 62
42289 Wuppertal
Germany
Melanie.Connerth@rub.de