DISSERTATION

Molecular Enzymology of lipolytic enzymes in the yeast *Saccharomyces cerevisiae*

vorgelegt von

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zur Erlangung des akademischen Grades
Doktor der Naturwissenschaften

eingereicht an der
Technischen Universität Graz

durchgeführt am Institut für Biochemie

unter der Betreuung von
Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. Günther Daum

Graz, Mai 2014

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Danksagung

Die spannende Phase meiner Dissertationszeit ist mit dem Vorliegen der folgenden Arbeit nun beendet. Damit ist es an der Zeit, mich bei den vielen Menschen zu bedanken, die mich währenddessen unterstützt und begleitet haben.

Allen voran gilt mein besonderer Dank meinem Dissertationsbetreuer Herrn Ao.Univ.-Prof. DI Dr. Günther Daum. Durch sein Angebot an mich, diese Stelle in seiner Arbeitsgruppe anzunehmen, und seinen Einsatz für ein schnelles Anlaufen meines Projekts hat er meinen wissenschaftlichen Weg bereitet. Ich danke ihm für alle fachlichen Anregungen und seine uneingeschränkte Unterstützung bis hin zu meiner Post-Doc Stelle.

Auch den Mitgliedern meines Thesis commitees, bestehend aus meinem Zweitbegutachter, Herrn Univ.-Doz. DI Dr. Karl Lohner, dem Vorsitzenden meines Prüfungssenats, Herrn Ao.Univ.-Prof. DI Dr. Albin Hermetter, und Assoc.Prof. DI Dr. Harald Pichler, möchte ich für ihren wissenschaftlichen Rat und die äußerst angenehme Arbeitsatmosphäre herzlich danken.

Univ.Doz. DI Dr. Karin Athenstaedt möchte ich für viele hilfreiche Tipps und Tricks besonders in der Anfangsphase meiner Dissertation herzlich danken!

Ebenso geht mein Dank an all meine lieben Laborkollegen, die durch ein freundschaftliches und motivierendes Arbeitsklima ein optimales Umfeld für das Gelingen dieser Arbeit geschaffen haben. Ein besonderer Dank gilt in diesem Zusammenhang dem „Neutrallipidteam“ Claudia und Barbara, außerdem meiner Lieblingspausenpartnerin Lisa, die mich in den vergangenen Jahren mit bereichernden Tipps und Diskussionsbeiträgen wiederholt angespornt und mich durch ihre Freundschaft auch während Durststrecken motiviert haben. An dieser Stelle möchte ich auch meiner Masterstudentin Martina Korber für ihren unermüdlichen Einsatz und Fleiß, ihre Diskussionsbereitschaft und Wissbegierde danken.

Mein ganzes Leben hat mich meine Familie fürsorglich begleitet. Meinen Eltern gebührt der größte Dank von allen, da sie mich mit ihrer uneingeschränkten Unterstützung und Liebe über alle Hürden getragen haben.

Eine herausragende Stellung in jeglicher Hinsicht nimmt mein Partner Werner ein. Ohne seine liebevolle Unterstützung auf allen Ebenen wäre mein Leben, insbesondere während meiner Dissertationszeit, nicht so glücklich, wie es ist!

Mein Dank gilt auch vielen namentlich hier nicht Genannten, meinen Freunden und Unterstützern, von denen ich Impulse, Kraft und Teilhabe an deren Wissen gewonnen habe.

ZUSAMMENFASSUNG

Die wichtigsten Speicherlipide der Hefe *Saccharomyces cerevisiae* sind die Neutrallipide Triglyceride und Sterolester. Beide werden im Endoplasmatischen Reticulum synthetisiert und anschließend in organell-ähnlichen Strukturen, Lipidpartikel genannt, gespeichert. Die Synthese der Sterolester wird von den beiden Acyltransferasen Are1p und Are2p katalysiert, Triglyceride werden hauptsächlich von Lro1p und Dga1p gebildet. Um während des Wachstums oder während Hungerphasen genügend Energie und Membrankomponenten bilden zu können, werden Neutrallipide von Triglyceridlipasen bzw. Sterolesterhydrolasen mobilisiert. Die bis zu diesem Zeitpunkt identifizierten Hauptlipasen in Hefe sind Tgl3p, Tgl4p und Tgl5p. Ziel dieser Dissertation war einerseits die Identifizierung von weitern lipolytischen Enzymen, die im Neutrallipidmetabolismus für die Mobilisierung von Triglyceriden verantwortlich sind, andererseits die Erforschung von regulatorischen Prinzipien im Sterolestermetabolismus. Um weitere Triglyceridlipasen zu identifizieren, wurde eine funktionelle Proteomanalyse durchgeführt, und die daraus resultierenden vielversprechendsten Kandidaten wurden durch *in vitro* und *in vivo* Enzymassays, Genexpressionsanalysen sowie Lipidanalysen untersucht. Dies führte zur Identifizierung von Ayr1p als weitere Triglyceridlipase in Hefe. Basierend auf diesen Ergebnissen, wurde eine mögliche Verbindung zwischen Lipidspeicherung, -mobilisierung und der peroxisomalen Nutzung von Fettsäuren diskutiert. Um den regulatorischen Prinzipien des Sterolestermetabolismus auf den Grund zu gehen, wurde eine Hefe-Mutante (*Δtgl1Δyeh1Δyeh2*) ohne Sterolesterhydrolasen hinsichtlich Bildung, Hydrolyse und Zusammensetzung der Lipide untersucht. Diese Studien zeigten, dass es eine Feedback-Regulation auf die beiden Sterolester-bildenden Enzyme Are1p und Are2p gibt, die aber weder auf Genexpressions- noch auf Proteinebene stattfindet, sondern direkt die enzymatische Aktivität der beiden Acyltransferasen beeinträchtigt. Zusammenfassend tragen die Ergebnisse dieser Dissertation grundlegend zum Verständnis des komplexen Neutrallipidmetabolismus in Hefe bei.

SUMMARY

The main storage lipids of the yeast *Saccharomyces cerevisiae* are the non-polar lipids triacylglycerols and steryl esters. Both are synthesized in the endoplasmic reticulum and are then stored in organelle-like structures called lipid droplets. The synthesis of steryl esters is catalyzed by the two acyltransferases Are1p and Are2p, triacylglycerols are formed mainly by Lro1p and Dga1p. Upon requirement, i.e. during growth or starvation, non-polar lipids can be mobilized by triacylglycerol lipases and steryl ester hydrolases, respectively. The main triacylglycerol lipases identified in yeast are Tgl3p, Tgl4p and Tgl5p. The aim of this Thesis was, on the one hand, the identification of further lipolytic enzymes that are responsible for the mobilization of triacylglycerols, and, on the other hand, the study of regulatory principles in steryl ester metabolism. To identify further triacylglycerol lipases, a functional proteome analysis was performed and the resulting most promising candidates were studied by *in vitro* and *in vivo* enzyme assays, gene expression and lipid analysis. This strategy led to the identification of Ayr1p as novel triacylglycerol lipase in yeast. Based on these results, a possible link between lipid storage, mobilization and the utilization of fatty acids by peroxisomes was discussed. To investigate regulatory principles of steryl ester metabolism, a *Δtgl1Δyeh1Δyeh2* yeast mutant deficient of all three steryl ester hydrolases was analyzed regarding formation, hydrolysis and composition of lipids. These studies showed that there is a feedback regulation on the two steryl ester-forming enzymes Are1p and Are2p. Moreover, neither gene expression nor protein levels of the two acyltransferases were affected, but the activity of the two enzymes was depleted. In summary, results obtained in this Thesis contribute significantly to the understanding of the complex network of non-polar lipid metabolism in yeast.

TABLE OF CONTENTS

1

THESIS AIMS AND OUTLINE

Over the last few decades the traditional view on lipids as membrane building blocks and energy storage molecules has changed to an increased recognition of lipids as dynamic and vital molecules involved in a variety of cellular processes. Especially the fact that many lipid-associated disorders such as cardiovascular diseases, type-II-diabetes, obesity and insulin resistance have become increasing health risks for humans has repositioned fundamental lipid research. *Saccharomyces cerevisiae* is a well-established, powerful model system to study lipid metabolism, not only because principles of lipid metabolism are well conserved between all eukaryotes but also because this microorganism can be easily handled and manipulated. The use of *S. cerevisiae* as model system provides many advantages: (i) The complete genome is sequenced and subsequently well-curated databases are available. Additionally, a huge set of deletion mutants and plasmids is obtainable. (ii) Yeast is a robust organism, generally regarded as safe, which can easily be cultivated and shows very short generation times. (iii) Compared with other organisms, genetic manipulations are easy to handle. (iv) Most important for this study, the lipidome of yeast is, compared with the mammalian lipidome, quite simple comprising just a few hundred lipid species whereas mammalian cells have thousands. This feature allows designing a simplified landscape of lipid metabolism, which can be seen as a general compendium for higher eukaryotes. Although outstanding achievements have been made in recent years to identify the main cellular routes of lipid synthesis and degradation as well as the enzymes involved therein, still many gaps remained. Especially the knowledge of regulatory mechanisms, how cells can sense and manage their lipid composition of different organelles, and the cross-talk between different metabolic routes is limited.

This Thesis was aimed at shedding more light on the complex network of lipid metabolism in the yeast *S. cerevisiae*. The focus was on the synthesis, storage, degradation and regulation

of non-polar lipids. Specific attention was paid to the identification of novel enzymes involved in the mobilization of lipid storage pools, under which conditions cells have to resort on these minor lipolytic activities, and to provide more insight into regulatory aspects of non-polar lipid metabolism.

This Thesis is organized in the following chapters:

Chapter 2 provides an overview of metabolic pathways and lipid classes in yeast. This chapter comprises examples of recently discovered key players in yeast lipid metabolism, descriptions of regulatory networks, multifunctional enzymes and lipids that serve as modulators of their own synthesis. One section is dedicated to the increasing numbers of biotechnological processes based on lipid metabolism. Besides the prominent model organism *Saccharomyces cerevisiae*, also other especially oleaginous yeasts are discussed.

Chapter 3 describes a versatile protocol for the isolation of lipid droplets at high purity and addresses specific demands for handling the three different yeasts *Saccharomyces cerevisiae, Yarrowia lipolytica* and *Pichia pastoris*. Moreover, the analysis of proteome and lipidome of lipid droplets based on standard methods such as thin layer chromatography, gas liquid chromatography, mass spectrometry as well as GLC/MS is discussed. Similarities and disparities between the three different yeasts are pointed out.

Chapter 4 reports on the identification of a novel lipase responsible for the mobilization of triacylglycerols in *S. cerevisiae*. Based on a functional proteomic approach, sequence analysis, lipid analysis, *in vitro* and *in vivo* enzyme activity assays, Ayr1p, described as a 1-acyl-dihydroxyacetone-phosphate reductase, was identified as additional yeast triacylglycerol lipase.

Chapter 5 is dedicated to regulatory aspects of steryl ester formation. It describes a possible feedback regulation of steryl ester hydrolysis and mobilization on steryl ester formation.

Chapter 6 gives a summary of the results of this Thesis with concluding remarks.

2

LIPID METABOLISM AND LIPIDOMICS

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Published in: *Molecular Mechanisms of Yeast Carbon Metabolism*; Springer Berlin Heidelberg; ed. Jure Piškur and Concetta Compagno, 2014 (VIII), 169-216

KEY WORDS

Lipids, yeast, fatty acids, phospholipids, sterols, triacylglycerols, steryl esters, non-polar lipids, sphingolipids, lipid droplets, peroxisomes, mitochondria

ABBREVIATIONS

ABC, ATP-binding cassette; ATP, adenosine triphosphate; CDP, cytidinediphosphate; CL, cardiolipin; CoA, coenzyme A; CTP, cytidine triphosphate ; DAG, diacylglycerol; DGPP, diacylglycerol diphosphate; DMAPP, dimethylallyl diphosphate; ER, endoplasmic reticulum; ERMES, ER-mitochondria encounter structure; FA, fatty acids; FIT, fat storage-inducing transmembrane proteins; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPI, glycosylphosphatidylinositol; GPP, geranyl diphosphate; IMM, inner mitochondrial membrane; IPC, inositol phosphorylceramide; IPP, isopentenyl diphosphate; LD, lipid droplets; MAM, mitochondria associated membrane fraction; $M(IP)_2C$, mannosyl (inositol phosphoryl)$_2$ ceramide; MINOS, mitochondrial inner membrane organizing system; MIPC, mannosylinositol phosphorylceramide; Mt, mitochondria; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Nu, nucleus; OMM, outer mitochondrial membrane; PA, phosphatidic acid; PC, phosphatidylcholine; PDR, pleiotropic drug response; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PL, phospholipids; PS, phosphatidylserine; PUFA, polyunsaturated FA; Px, peroxisomes; SE, steryl ester(s); SPT, serine palmitoyltransferase complex; TG, triacylglycerol(s); TORC2, target of rapamycin complex 2; UAS_{INO}, inositol-responsive upstream activating sequence element

ABSTRACT

Lipids play several essential roles in the biology and metabolism of eukaryotic cells. In addition to their structural role as constituents of cell membranes, they have been increasingly recognized as dynamic and vital molecules, involved in a variety of cellular processes. Examples are cell signaling, membrane trafficking and influencing the stability of protein complexes in membranes. This chapter provides an overview of lipid classes and metabolic pathways in yeast. Lipid metabolism involves various organelles such as the endoplasmic reticulum (ER), mitochondria, peroxisomes and lipid droplets (LD), which will be highlighted. Specific attention is devoted to examples of recently discovered key players in yeast lipid metabolism, which illustrate our improved understanding of cells as an interconnected biological system. This chapter comprises descriptions of regulatory networks, multifunctional enzymes and lipids that serve as modulators of their own synthesis. The last part of the chapter is dedicated to the increasing numbers of biotechnological processes based on lipid metabolism. Besides the prominent model organism *Saccharomyces cerevisiae*, other predominantly oleaginous yeasts are also included.

INTRODUCTION

The link between central carbon metabolism and lipid synthesis is easily found: acetyl-CoA is the common precursor for all lipid biosynthetic pathways. Membrane biogenesis is, along with amino acid synthesis, one of the major consumers of acetyl-CoA and NADPH – the biosynthesis of one molecule of palmitic acid requires 8 acetyl-CoA and 14 NADPH molecules (Natter and Kohlwein, 2012). However, while the understanding of connections between different metabolic routes has already reached an advanced state at the level of metabolites, the identification of regulatory mechanisms is only at its infancy.

Lipids are essential constituents of every living cell. They were long seen as primarily structural components of cellular membranes. However, lipid research over the past decades has shown that they fulfill many more vital functions that are increasingly recognized. Prominent examples are their role as regulators of energy metabolism, cell integrity and membrane-based processes such as endocytosis and vesicular trafficking (Daum et al., 1998; Souza and Pichler, 2007). The accepted general definition of lipids is that they are relatively small, hydrophobic or amphiphilic molecules that are classified into eight distinct groups based on their chemical and biochemical properties: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterols and sterol derivatives, prenol lipids, glycolipids and polyketides. Altogether, more than 10,000 different lipid structures have been identified (Fahy et al., 2009). In this chapter we focus on the first five most commonly found lipid classes in yeast.

The field of lipid research has attracted more and more interest over the past decades as many lipid-associated disorders such as obesity, type-II-diabetes, insulin resistance and cardiovascular diseases have become increasing health risks in the Western world and recently also in developing countries. As the principles of lipid metabolism are well conserved between all eukaryotes and because of the many advantages of working with yeast, *Saccharomyces cerevisiae* has become a powerful model organism for lipid research. One established approach to dissect the complex network of enzymes and molecular mechanisms responsible for lipid homeostasis is the use of readily available single and multiple deletions mutants. One of the major resources that have enabled systematic studies in this direction is the repertoire of yeast deletion mutants of all non-essential genes,

which have helped, in combination with different cultivation conditions, to understand the basics of lipid synthesis, storage and degradation pathways (Winzeler et al., 1999).

Many different cellular compartments are involved in lipid metabolism (Natter et al., 2005). Lipid synthesis takes place mainly in the endoplasmic reticulum (ER) and the Golgi compartment, but also lipid droplets (LD), mitochondria and peroxisomes play influential roles and will therefore be highlighted in this introduction. LD and peroxisomes, especially with respect to their role in lipid metabolism, were recently reviewed (Kohlwein et al., 2012).

Lipid droplets (LD) are generally seen as a storage compartment for the non-polar lipids triacylglycerols (TG) and steryl esters (SE). They are small spherical organelles of approximately 400 nm in diameter consisting of a highly hydrophobic core of mainly TG, surrounded by shells of SE which are covered by a phospholipid monolayer with only a few embedded proteins (Athenstaedt et al., 1999a; Czabany et al., 2008). Proteome analysis revealed that these proteins are predominantly enzymes involved in lipid metabolism, for example TG lipases and SE hydrolases (Grillitsch et al., 2011). LD will be further described in the section on non-polar lipids.

Mitochondria are of special interest for lipid research. They provide an independent fatty acid synthesizing system (Tehlivets et al., 2007) and synthesize some phospholipids (Kuchler et al., 1986; Henry et al., 2012), but the majority of lipids are imported. Examples of autonomously formed mitochondrial lipids are phosphatidic acid (PA), cardiolipin (CL) or phosphatidylethanolamine (PE), whereas phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) as well as sterols and sphingolipids have to be imported. Various mechanisms have been proposed for the import of lipids, such as direct membrane contact between the ER and mitochondria via the mitochondria associated membrane (MAM) fraction, vesicular transport, and the involvement of specific lipid binding and transfer proteins (Daum and Vance, 1997). Mitochondria are also special regarding their lipid distribution, having an increased amount of CL and PI. The phospholipid cardiolipin comprises up to 15%, which is very high compared to other organelles. Therefore cardiolipin is often referred to as the typical mitochondrial phospholipid (Zinser and Daum, 1995). Mitochondria are compartmentalized into four different subcompartments: the outer mitochondrial membrane, the intermembrane space, and the inner mitochondrial

membrane with its cristae and the matrix. A mitochondrial complex connecting the inner boundary membrane to the cristae membrane was recently identified and termed MINOS (mitochondrial inner membrane organizing system) or MitOS (mitochondrial organizing structure), and shown to be responsible for maintaining mitochondrial morphology (Hoppins et al., 2011; van der Laan et al., 2012; Zerbes et al., 2012). Most interestingly, even among the four subcompartments, lipids are not distributed randomly (Daum, 1985). Therefore, intramitochondrial lipid transfer as well as the interorganelle transport of lipids is of outstanding interest. Some recent findings will be reported in the section on novel key players.

Peroxisomes deserve special attention in the description of lipid metabolism. They are spherical organelles with a diameter of about 0.1 µm, consisting of a fine granular matrix with a crystalline core, all surrounded by a single membrane. The protein content of these membranes is typically relatively low, whereas the matrix contains the highest protein concentration in eukaryotic cells with hydrogen peroxide-producing oxidase and catalase as prominent representatives (Kohlwein et al., 2012). Peroxisomes are ubiquitous and involved in various metabolic pathways, especially detoxification processes and degradation of fatty acids (FA). The latter process, termed β-oxidation, makes FA available as an energy source. In contrast to mammalian cells, where β-oxidation occurs in mitochondria and peroxisomes, yeast β-oxidation takes place exclusively in peroxisomes (Poirier et al., 2006). Prior to degradation, FA have to be activated by one of six specific activators Faa1p, Faa2p, Faa3p, Faa4p, Fat1p or Fat2p. The uptake of FA into peroxisomes can proceed by different mechanisms. Short and medium chain length FA are thought to be taken up by diffusion whereas long chain and very long chain FA require ABC (ATP binding cassette) transport proteins. In yeast peroxisomes, the two ABC transporters Pxa1p and Pxa2p are thought to be responsible for the uptake of FA. These transporters hydrolyze FA-CoA esters prior to their entry into peroxisomes, releasing CoA into the cytoplasm, whereas FA are then re-esterified by a peroxisomal synthetase (van Roermund et al., 2012). Lipid composition of peroxisomes comprises nearly 50% PC, 23% PE, 16% PI together with a remarkably high content of CL (7%) (Zinser et al., 1991).

Recently, techniques for lipid content analysis have advanced substantially. The prerequisite for lipid analysis is usually lipid extraction into organic solvents, followed by

chromatographic separation of lipid species that can then be detected by advanced spectrometric technologies. Currently, lipid research is shifting from basic molecular characterization to a global understanding of dynamic lipid regulation in the cell context. Lipids have been proposed to act as a molecular collective rather than as single molecules, best demonstrated by Guan *et al*. (2009), who showed that sphingolipids and sterols can interact functionally. In particular, lipidomic approaches and mathematical modelling are promising methods for interpreting lipid metabolism on a global scale (Alvarez-Vasquez et al., 2011; Santos and Riezman, 2012). Lipidomics, which involve mapping all lipids of an organism or a cell, is facilitated by sophisticated mass spectrometry techniques combined with state of the art data analysis software (Dennis, 2009; Ejsing et al., 2009). The absolute quantification of lipids depends on internal standards which are not always available. Quantitative analysis would be particularly important in finding out how cells adapt their lipid profile to changes in the environment. Specifically, points of regulation could be identified by mathematical modelling, although this approach is still just in its infancy. From the experimental point of view, however, mass spectrometry-based shotgun lipidomics have been applied to quantitatively and comprehensively asses the yeast lipidome (Ejsing et al., 2009). This approach was recently used to determine changes in the yeast lipidome under different growth conditions including growth on different carbon sources. Interestingly, different flexibility (defined as dispersion of a given lipidomic feature across the dataset) was determined for different classes of lipids (Klose et al., 2012). The authors observed marked differences in the lipidome between growth on glucose and non-glucose based media.

Another hot topic of lipid research is the investigation of membrane organization by visualizing specific lipids in the cell. Visualization techniques confirmed the view that lipids and proteins are not moving freely within a membrane but that their diffusion is restricted in certain domains called rafts, which are enriched in sphingolipids and sterols (Lingwood and Simons, 2010; Eggeling et al., 2009). However, the techniques are challenging, comprising high temporal and spatial super resolution microscopy, and are limited by the availability of appropriate probes.

More recently, other yeast genera besides *Saccharomyces* have been attracting interest in lipid research. In particular, oleaginous yeasts, such as *Candida curvata* and *Yarrowia lipolytica*, have been shown to be industrially relevant for the sustainable production of

lipids with compositions similar to those of vegetable oils and fats (Beopoulos et al., 2011). *Pichia pastoris*, an industrially highly relevant yeast especially for the expression of heterologous proteins, is another important model organism for lipid-related research, especially in organelle biology studies. Unless indicated otherwise, we will refer to *S. cerevisiae* in this chapter, but particularly in the section on biotechnological aspects other yeasts will also be mentioned.

The aim of this chapter is to provide a fundamental overview on yeast lipid metabolism, but also to point out novel findings and applications of the highly dynamic field of yeast lipid research. For detailed information beyond the scope of this chapter readers will be referred to other recent reviews (Henry et al., 2012; Jacquier and Schneiter, 2012; Kohlwein et al., 2012; Natter and Kohlwein, 2012; Rajakumari et al., 2008; Santos and Riezman, 2012).

LIPID CLASSES

Lipids are divided into classes based on their structure and function. The major classes discussed in this article are FA, glycerophospholipids, sphingolipids, sterols and the non-polar storage lipids triacylglycerols and steryl esters. These five classes will be described with special emphasis on a basic understanding of their metabolism and function of their members. Regulatory mechanisms, especially newly identified ones, will be discussed in the section on novel key players.

Fatty acids

Fatty acids (FA) are carboxylic acids with long hydrocarbon tails and differ from each other in chain length and degree of saturation. In *S. cerevisiae*, the overall composition of FA is rather simple, the members being mainly of C18:1 (oleate), C16:1 (palmitoleate) and C16:0 (palmitate) followed by C18:0 (stearate) and minor amounts of C14:0 (myristate) and C26:0 (cerotate) (Daum et al., 1998). The composition differs in the different yeast genera. In particular, in the oleaginous yeasts such as *Y. lipolytica,* the FA composition is highly diverse, comprising longer chain lengths and especially more double bonds, which makes such organisms useful for the production of nutritionally valuable polyunsaturated fatty acids (PUFA) (Beopoulos et al., 2011) as will be described below.

FA fulfill many different roles in cells. Most importantly, they serve as basic molecules for the biosynthesis of complex membrane and storage lipids (Tehlivets et al., 2007). Other functions include their role as signaling molecules, transcriptional regulators and posttranslational modifiers of proteins (Nadolski and Linder, 2007). One prominent example of the latter role is the palmitoylation of Ras proteins, but myristate is also often added as lipid moiety (Linder and Deschenes, 2004). FA metabolism in yeast is illustrated in Figure 2.1.

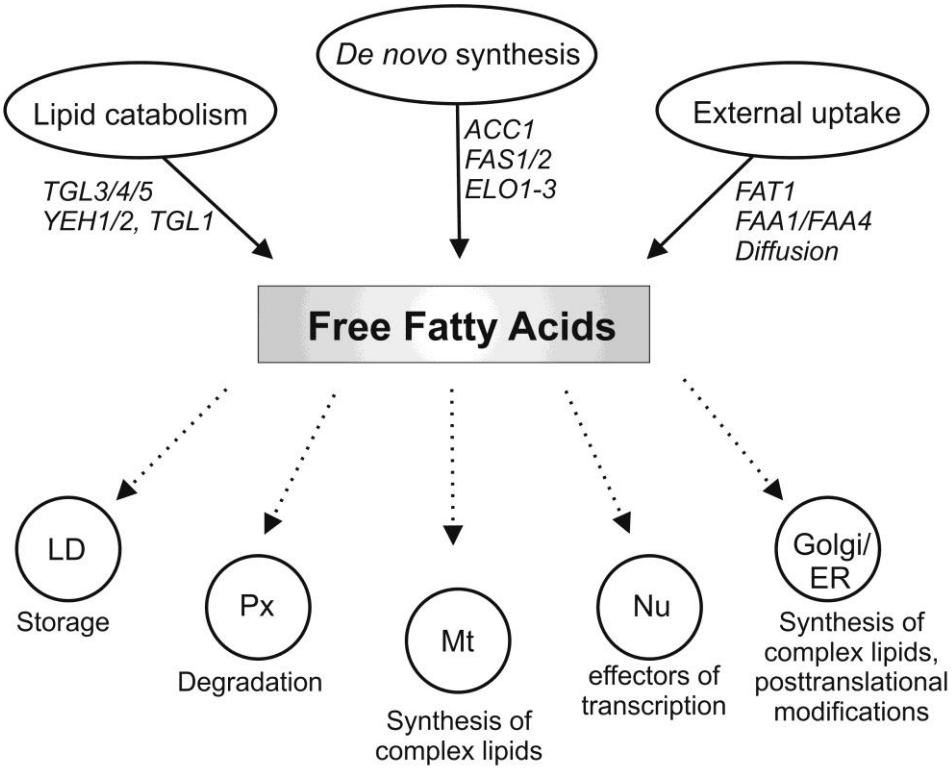

Figure 2.1: Overview of fatty acid metabolism in yeast. Fatty acids derive mainly from three routes: Catabolism of storage lipids, *de novo* synthesis and external uptake. They can be incorporated into storage lipids, degraded by β-oxidation to provide energy, or converted into complex lipids like phospholipids or sphingolipids. Fatty acids can also act as effectors of transcription. LD lipid droplets, Px peroxisomes, Mt mitochondria, Nu nucleus, ER endoplasmic reticulum. For details see text.

There are three main sources for FA: (i) *de novo* synthesis, (ii) uptake by specific transporters, and (iii) catabolism of complex lipids (Tehlivets et al., 2007). A small proportion of FA derives from the catabolism of proteins (Tehlivets et al., 2007). Two independent pathways exist for the biosynthesis of FA, the major cytosolic pathway and the mitochondrial pathway. The former pathway involves mainly three key enzymes, encoded by genes *ACC1*, *FAS1* and *FAS2* (for review see Henry et al., 2012). Biosynthesis of FA starts with the carboxylation by Acc1p of acetyl-CoA to give malonyl-CoA. Acc1p possesses three

different activities: it can act as a biotin carboxylase, as a biotin carboxyl-carrier protein and as a transcarboxylase. It is located on the cytoplasmic surface of the ER, contains one covalently bound biotin molecule and is essential for growth. Malonyl-CoA is metabolized by a series of reactions catalyzed by FA synthases (*FAS* genes) and elongases. *FAS1* and *FAS2* encode two different subunits of the FA synthase complex. The active FAS complex consists of six α-units and six β-units (Chirala et al., 1987). *FAS1* encodes the β-subunit, which comprises four different activities: acetyltransferase, enoyl reductase, dehydratase and malonyl-palmitoyl transferase activities. *FAS2* encodes the α-subunit that displays acyl carrier protein, 3-ketoreductase, 3-ketosynthase and phosphopantetheinyl transferase activities (reviewed by Tehlivets et al., 2007). In yeast, double bonds are introduced by a single acyl-CoA Δ9-desaturase encoded by *OLE1* (Stukey et al., 1990). Elongation is carried out predominantly by Elo1p, although elongation of very long FA, especially for sphingolipid synthesis, is catalyzed mainly by Elo2p and Elo3p. *De novo* synthesis of FA takes place mainly in the cytosol, whereas elongation and desaturation reactions are carried out in the ER (Tehlivets et al., 2007).

Imported FA, which can be taken up by diffusion or by transporters, can fully compensate for endogenously synthesized FA. Prerequisite for the uptake of FA is the activation of free FA with coenzyme A, which is carried out by the acyl-CoA synthetases Faa1p, Faa2p, Faa3p, Faa4p and Fat1p. These enzymes are also believed to be involved in the uptake of FA into the cell (reviewed by Black and DiRusso, 2007; Henry et al., 2012). While in *S. cerevisiae* machinery for utilization of extracellular complex lipids as energy or carbon source has not been identified, oleaginous yeast species produce extracellular lipases for this purpose. The best studied model for the utilization of hydrophobic substrates such as alkanes, TG and FA is *Y. lipolytica* (reviewed by Fickers et al., 2005). *Y. lipolytica* produces surfactants when grown on lipids as the only carbon source and changes the biophysical and morphological properties of the cell surface to enable adhesion of water-insoluble growth substrates. The cells produce both membrane-bound and extracellular lipases, the major one being Lip2p which catalyzes hydrolysis of TG to free FA and glycerol. Free FA are then taken up by a mechanism that is not completely understood, activated by specific acyl-CoA synthetases and further metabolized similarly as described below for *S. cerevisiae*.

In general, free FA are metabolized very quickly. Elevated levels of free FA are harmful to cells because they can perturb membrane properties due to changes in fluidity. Thus, FA are either incorporated into complex lipids, i.e. PL or the storage lipids TG and SE, or they are oxidized to provide energy. Regardless of the source of free FA, the prerequisite for further conversion is activation by thioesterification with coenzyme A, which requires the action of acyl-CoA synthetases (*Faa1-4*, *Fat1*) (Black and DiRusso, 2007). In mitochondria, the biosynthesis of FA is carried out by a totally different set of enzymes: Hfa1p, the mitochondrial acetyl-CoA carboxylase catalyzes the production of malonyl-CoA, which is then further processed by a different Fas complex (Hiltunen et al., 2010).

As mentioned in the introduction, catabolism of FA in yeast takes place exclusively in peroxisomes. Under standard growth conditions, the abundance of peroxisomes is quite low but can be increased by FA supplementation to the medium (van Roermund et al., 1995). The classical β-oxidation starts with the oxidation of acyl-CoA to *trans*-2-enoyl-CoA by Fox1p (frequently called Pox1p). This reaction releases hydrogen peroxide, which is detoxified by catalase. The second step is the conversion, by Fox2p of *trans*-2-enoyl-CoA to 3-ketoacyl-CoA. This compound is the substrate of Fox3p, a 3-ketoacyl-CoA thiolase, that yields acetyl-CoA and a C2-shortened FA (Einerhand et al., 1991; Hiltunen et al., 1992). The route of FA directed to the site of peroxisomal β-oxidation, either via plasma membrane transport from an exogenous source or from LD as an endogenous storage compartment, is still not completely understood.

Phospholipids

Phospholipids (PL) are regarded as bulk membrane constituents, since they can form lipid bilayers. They consist of a diacylglycerol backbone and a phosphate group at the *sn*-3 position that is linked to a polar head group. PL can be classified based on their different head groups. The major PL in yeast are phosphatidylcholine (PC), which comprises about 45% of the total phospholipid content, phosphatidylethanolamine (PE), which makes up to 20%, phosphatidylinositol (PI) with 15%, phosphatidylserine (PS) accounting for 5% and cardiolipin (CL) being present at 2% (Janssen et al., 2000; Zinser et al., 1991; Schneiter et al., 1999). However, the subcellular distribution of different phospholipids varies quantitatively and by origin. Especially PS and CL are present just at low amounts in most organelle membranes but are major components of the plasma membrane and the inner mitochondrial membrane, respectively (Zinser and Daum, 1995). In general, the lipid composition of membranes is not stochastic but very characteristic of each organelle.

In addition to the role of PL as major structural components of cellular membranes, they are involved in a variety of other processes. They provide precursors for the synthesis of membranes, act as reservoirs of second messengers, conduct the lipidation of proteins for membrane association and function as molecular chaperones (reviewed by Carman and Han, 2011; Dowhan and Bogdanov, 2009; van Meer et al., 2008). PL can also be differentiated according to their shape, which is dictated by their head to tail area ratio. PC, PS and phosphatidylglycerol are cylindrically shaped since they display a head group similar to fatty acid chain area. Cylindrical PL are known to favor bilayer structures, while PE and CL, which belong to the group of non-bilayer forming PL, are conically shaped, the result of a smaller head to tail area (Cullis et al., 1986).

A key molecule in PL synthesis is phosphatidic acid (PA) (Figure 2.2), which is also an important signaling molecule and regulator of lipid metabolism. PA derives from either glycerol-3-phosphate or dihydroxyacetone phosphate following fatty acyl-CoA dependent acyl transfer. These reactions are catalyzed by the *SCT1* (*GAT2*) and *GPT2* (*GAT1*) encoded glycerol-3-phosphate acyltransferases and the *SLC1* and *ALE1* encoded lysophospholipid acyltransferases (Athenstaedt and Daum, 1997; Athenstaedt et al., 1999b; Chen et al., 2007b; Jain et al., 2007; Riekhof et al., 2007). Dihydroxyacetone phosphate is reduced by Ayr1p, which is present in lipid droplets, the ER, and the mitochondrial outer membrane. PA

is a branch point between the CDP-DAG (cytidinediphosphate- diacylglycerol) pathway and the formation of DAG (Athenstaedt and Daum, 1999). In the first case, PA is metabolized to CDP-DAG under the catalytic action of the *CDS1* encoded CDP-DAG synthase (Shen et al., 1996). In the second case, the *PAH1* encoded PA phosphatase forms DAG (Han et al., 2006). CDP-DAG and DAG are both used in the synthesis of PE and PC, but by different pathways. The first biosynthetic route is the CDP-DAG pathway, whereas, in the Kennedy pathway, DAG is used as a substrate for the conversion (for reviews see Carman and Han, 2011; Henry et al., 2012). Both pathways are used in wild-type cells, but the CDP-DAG pathway is the major route for the synthesis of PE and PC when cells are grown in the absence of ethanolamine and choline. It starts with the conversion of CDP-DAG to PS by the ER localized *CHO1* encoded PS synthase. PS is further decarboxylated to PE by two PS decarboxylases, Psd1p and Psd2p. Psd1p is localized to the inner mitochondrial membrane and accounts for the major enzymatic activity, whereas Psd2p is associated with Golgi and vacuolar membranes (Trotter and Voelker, 1995; Clancey et al., 1993; Voelker, 2003). PE is methylated by Cho2p and Opi3p yielding PC. PE and PC can also be obtained from exogenously supplied lysoPE and lysoPC, which can be acylated by the *ALE1* encoded lysophospholipid acyltransferase (Riekhof and Voelker, 2006; Riekhof et al., 2007). CDP-DAG can also be converted to PI by reaction with inositol catalyzed by Pis1p (Fischl and Carman, 1983). The biosynthesis of CL takes place only in mitochondria, initiated by the transfer of the phosphatidyl moiety of CDP-DAG to glycerol-3-phosphate by Pgs1p and continued by dephosphorylation of phosphatidylglycerophosphate by Gep4p (Chang et al., 1998a; Osman et al., 2010). The *CRD1* encoded CL synthase finally produces CL (Chang et al., 1998b; Tuller et al., 1998, Jiang et al., 1997).

In the Kennedy pathway, exogenous ethanolamine and choline are transported into the cell by the choline/ethanolamine transporter Hnm1p. They are phosphorylated with ATP by the kinases Eki1p and Cki1p. They are then activated with CTP to form CDP-ethanolamine and CDP-choline, under the action of ethanolaminephosphate cytidylyltransferase Ect1p and cholinephosphate cytidylyltransferase Pct1p (Kennedy and Weiss, 1956; Kim et al., 1999; Henry et al., 2012). PE and PC are finally formed by the *sn*-1,2-diacylglycerol ethanolaminephospho-transferase Ept1p and the cholinephosphotransferase Cpt1p

catalyzing the reactions of CDP-ethanolamine and CDP-choline with DAG (Hjelmstad and Bell, 1992). DAG is provided by dephosphorylation of PA by Pah1p (Figure 2.2).

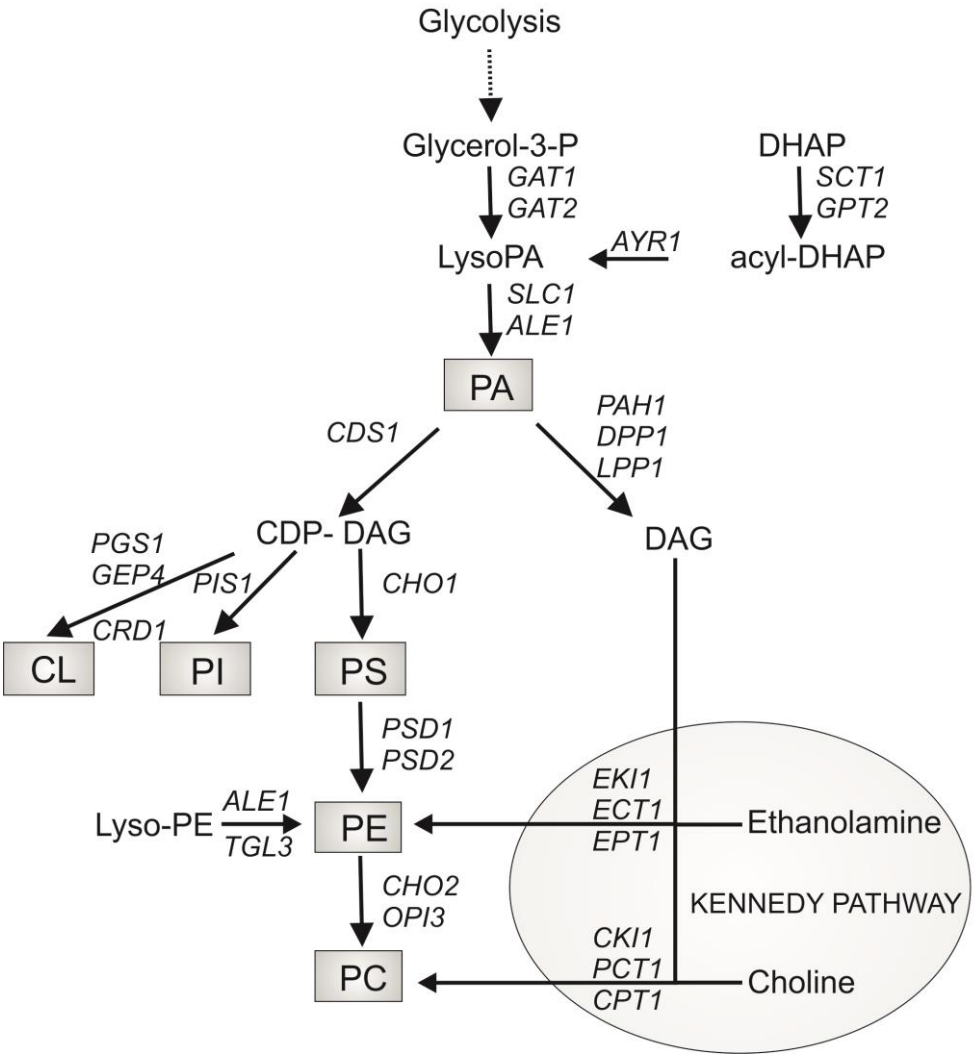

Figure 2.2: Simplified pathway of phospholipid synthesis in the yeast *S. cerevisiae*. For details see text. DHAP dihydroxyacetone phosphate, PA phosphatidic acid, CDP-DAG cytidinediphosphate diacylglycerol, DAG diacylglycerol, CL cardiolipin, PI phosphatidylinositol, PS phosphatidylserine, PE phosphatidyl-ethanolamine, PC phosphatidylcholine.

The organization of phospholipids within membranes is believed to occur via two principal mechanisms: lateral diffusion within the plane of a membrane and bidirectional, ATP-dependent movement facilitated by flippases. Intercompartmental phospholipid transport mainly occurs via vesicles and monomeric exchange (reviewed by Vehring and Pomorski, 2005). These transport mechanisms do not lead to a homogenous distribution of phospholipids. Moreover there is increasing evidence that distinct lipid domains which are

called rafts exist within certain cellular membranes (London and Brown, 2000; Simons and Sampaio, 2011).

The majority of PL undergoes rapid turnover and acyl-chain remodeling, which is catalyzed by specific acyltransferases, phospholipases and lipid phosphatases (reviewed in Henry et al., 2012).

Sterols

Sterols are important compounds in eukaryotic cells, serving as both structural and signaling molecules. Due to their rigid structure, they strongly affect membrane fluidity and permeability (Nes et al., 1993). It has been shown that yeast cells are not viable without sterols (Daum et al., 1998). They are often referred to as steroid alcohols that contain cyclopentanoperhydrophenanthrene as parent structure. The main sterol in yeast, and also the final product of sterol biosynthesis in other fungi, is ergosterol. Structural differences from the mammalian counterpart cholesterol are the double bonds between C-7,8 in the ring and C-22 in the side chain and the presence of a methyl group at C-24. The hydroxyl group at the C-3 position is the only hydrophilic component of the molecule which facilitates integration into membranes. The ergosterol biosynthetic pathway is one of the most complex biochemical pathways, comprising nearly thirty different biochemical reactions catalyzed by the so-called Erg-proteins (for recent reviews see Kristan and Rižner, 2012; Kuranda et al., 2010; Pichler, 2005). The most important steps are summarized in the following paragraph.

The ergosterol biosynthetic pathway is divided into the pre-squalene and post-squalene pathways, displayed in a very simplified scheme in Figure 2.3. Most Erg-proteins are located to the ER membrane, with the exception of Erg1p, Erg6p and Erg7p, which are localized mainly to LD (Athenstaedt et al., 1999a; Leber et al., 1994, 1998). The first steps of sterol synthesis are similar in fungi, plants and animals starting with the condensation of two acetyl-CoA molecules, catalyzed by Erg10p. This reaction yields acetoacetyl-CoA, which reacts with another acetyl-CoA molecule to form (3*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In the yeast, this important intermediate is subsequently reduced to mevalonate by HMG-CoA reductases 1 and 2 (*HMG1/2*). This reaction is not only the rate-limiting step of sterol biosynthesis but also one of the major control points, since HMG-CoA

reductase shows feedback inhibition by ergosterol (Bard and Downing, 1981). Polakowski *et al.* (1998) showed that overexpression of a truncated version of Hmg1p leads to an increase in early sterol precursors. A cascade of phosphorylations and decarboxylations, also known as the mevalonate pathway, leads to isopentenyl pyrophosphate (IPP), which is the precursor not only for squalene but also for other isoprenoids (Toth and Huwyler, 1996). Isomerization of IPP to dimethylallyl pyrophosphate (DMAPP) and a subsequent head-to-tail condensation reaction of IPP and DMAPP yield geranyl pyrophosphate (GPP). These reactions are catalyzed by Idi1p and Erg20p (Anderson et al., 1989a; Anderson et al., 1989b; Chambon et al., 1991). Erg20p also facilitates the formation of farnesyl pyrophosphate (FPP) by adding two IPP units to DMAPP. Finally, coupling of two molecules FPP by Erg9p leads to squalene.

The first step of the post-squalene pathway is epoxidation of squalene by Erg1p. This reaction is followed by a number of complex cyclization events, catalyzed by Erg7p, that form lanosterol, which is the first intermediate with the typical sterol structure. A cascade of demethylations, desaturations and subsequent reduction events (*ERG24-ERG28*) leads to zymosterol. The reaction steps yielding zymosterol are conserved in all eukaryotic cells. It was shown that deletion of genes downstream this biosynthetic sequence leads to sterol auxotrophy, whereas cells depleted of *ERG* genes acting later in the pathway are still viable. The further methylation of zymosterol at the C-24 position by Erg6p yields fecosterol, an intermediate which is unique to yeast and other fungi. Then, Erg2p catalyzes the shift of a double bond to the C-7 position, followed by the introduction of a further double bond at the C-5 position by Erg3p. The last steps of the pathway introducing and removing double bonds (*ERG5*, *ERG4*) yield the end product, ergosterol.

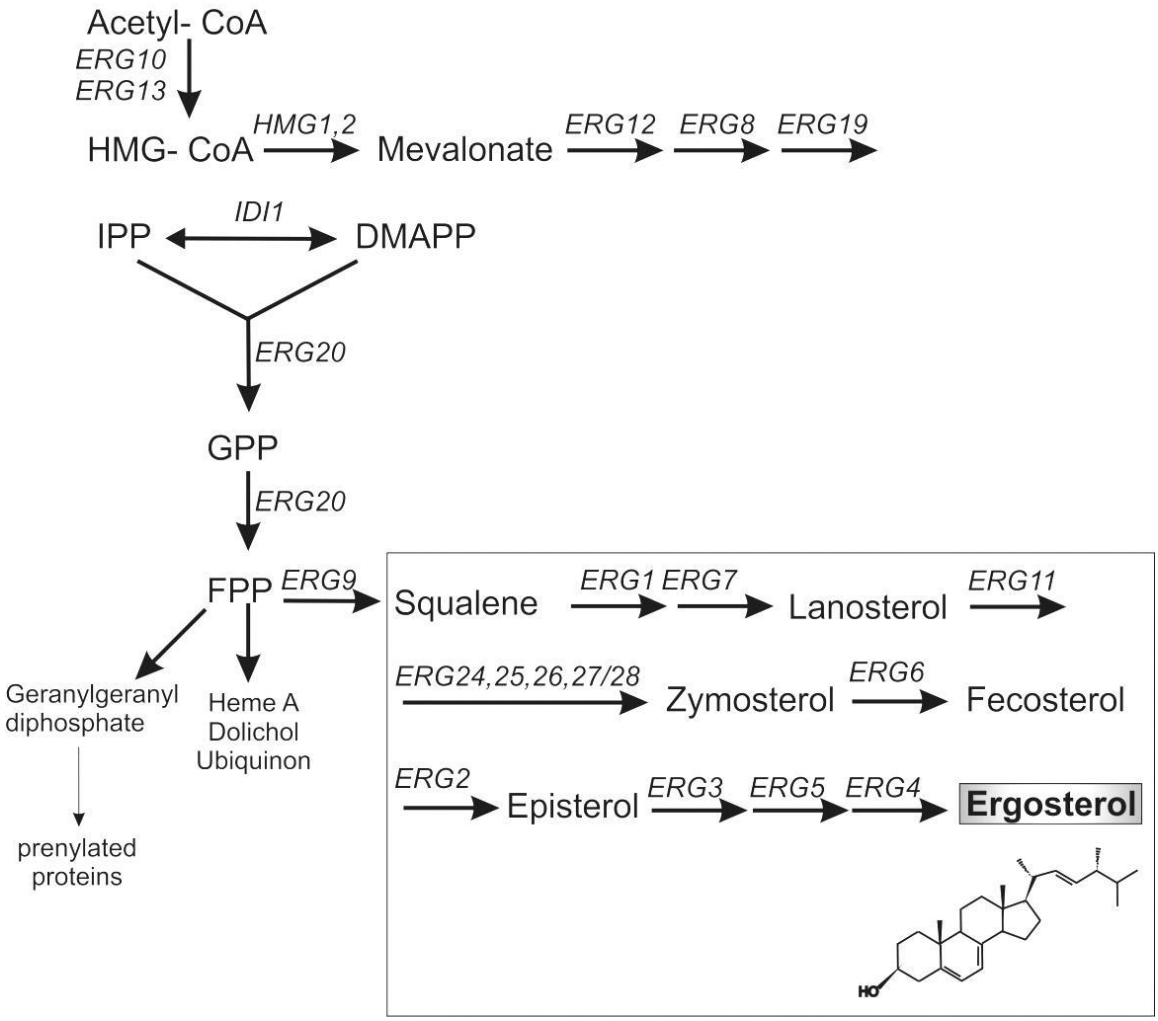

Figure 2.3: Simplified ergosterol biosynthesis divided into the pre-squalene and post-squalene pathways, the latter being highlighted in the box. Important metabolic intermediates as well as the chemical structure of ergosterol are shown. For details see text. CoA Coenzyme A, HMG (3*S*)-3-hydroxy-3-methylglutaryl-CoA, IPP isopentenyl diphosphate, DMAPP dimethylallyl diphosphate, GPP geranyl diphosphate, FPP farnesyl diphosphate.

Yeast cells usually synthesize sterols in excess. Since yeast is unable to degrade sterols, mechanisms of detoxification are required to avoid harmful influence on membranes. There are three main mechanisms to maintain sterol homeostasis: (i) esterification of free sterols with fatty acids by Are1p and Are2p and storage in LD (Yang et al., 1996; Yu et al., 1996; Zweytick et al., 2000); (ii) down regulation of sterol biosynthesis; and (iii) sterol acetylation by Aft2p, which enables yeast cells to efficiently secrete excess sterols in the form of sterol acetates into the medium. This last process is reversible and catalyzed by Say1p (Tiwari et al., 2007; Choudhary and Schneiter, 2009). Acetylation of sterols has also been discussed as a possible quality control mechanism. It was suggested that sterols which do not pass a quality control cycle are acetylated and secreted. Recently, the PRY proteins (pathogen-

related yeast proteins) that are involved in the secretion of acetylated sterols have been identified (Choudhary and Schneiter, 2012).

Since some steps of the sterol biosynthetic pathway require oxygen, yeast becomes strictly sterol auxotroph under anaerobic conditions (Lees et al., 1995). Uptake of external sterols from the exterior is mediated by two ATP-binding cassette transporters, encoded by *AUS1* and *PDR11*, both targets of the transcriptional activator Upc2p (Crowley et al., 1998; Wilcox et al., 2002).

The intracellular sterol concentration is lowest at its place of biosynthesis, the ER, and increases along the protein secretory pathway until it reaches its maximum at the plasma membrane (Zinser et al., 1993). The distribution of sterols between different cellular membranes has to be tightly regulated to maintain distinct membrane properties such as fluidity and thickness. Intracellular sterol transport involves both vesicular and non-vesicular routes but is mainly ATP-dependent. Non-vesicular sterol transport in addition requires the action of carrier proteins (for review see Jacquier and Schneiter, 2012). Over the last couple of years, specific yeast sterol carrier proteins have been identified, the so-called oxysterol binding proteins homologues Osh1-7 (reviewed by Schulz and Prinz, 2007). Deletion of all seven Osh-proteins was found to be lethal and accompanied by a 3.5-fold increase in the cellular level of ergosterol (Schulz and Prinz, 2007). Sterols can be either transported to the cell surface or sent to the *trans*-Golgi network where they associate with sphingolipids to form lipid rafts (Mesmin and Maxfield, 2009). The exact role of Osh-proteins in sterol transport still has to be elucidated. Georgiev *et al.* (2011) reported that Osh-proteins act as sterol sensors and regulate the organization of sterols at the plasma membrane rather than being involved in the transport of sterols between the ER and the plasma membrane. Intracellular sterol trafficking between membranes might also be governed by Arv1p, as reported by Tinkelenberg *et al*. (2000). Mutations of *ARV1* have been shown to render cells which are anaerobically non-viable, depend on sterol esterification and show altered intracellular sterol distribution. The balance of sterol synthesis, uptake, storage and mobilization as well as internal transport is very complex and a hot topic of lipid research.

Non-polar lipids: Triacylglycerols and steryl esters

Triacylglycerols (TG) and steryl esters (SE) are storage lipids preserving free FA and sterols in a biologically inert form. All eukaryotic cells store excess fatty acids in specific organelle-like compartments, often referred to as lipid droplets (LD), lipid particles, or oil bodies, used as energy depots. Yeast cells accumulate only little TG as long as they proliferate but can reach high TG levels in the stationary phase. When required, e.g. during growth or starvation, TG and SE can be mobilized to provide building blocks for membrane biosynthesis. Under these conditions the released fatty acids are channelled into phospholipid biosynthesis (Zanghellini et al., 2008). In yeast, lipid droplets (LD) are about 400 nm in diameter and consist of a highly hydrophobic core of TG, surrounded by shells of SE and a phospholipid monolayer containing a distinct set of proteins (Czabany et al., 2008; Athenstaedt et al., 2006; Grillitsch et al., 2011; Kohlwein et al., 2012). TG are synthesized by the acyltransferases Dga1p and Lro1p, and SE by the steryl ester synthases Are1p and Are2p. All TG and SE synthesizing enzymes are located at the ER. Additionally, Dga1p is also found in LD. The direct precursor for TG is diacylglycerol (DAG), that can derive from different routes: (i) dephosphorylation of *de novo* synthesized phosphatidic acid, (ii) degradation of PL by phospholipases, and (iii) deacylation of TG (see Figure 2.4) (Henry et al., 2012). For synthesis of TG, DAG is acylated in the *sn*-3 position by Dga1p, Lro1p and with low efficiency by Are1p and Are2p. In *S. cerevisiae*, the acyl-CoA:diacylglycerol acyltransferase Dga1p is the most efficient TG synthesizing enzyme. Lro1p is an acyl-CoA independent enzyme which uses the *sn*-2 acyl group from glycerophospholipids as co-substrate for the acylation of DAG (Czabany et al., 2007; Rajakumari et al., 2008; Horvath et al., 2011). SE of *S. cerevisiae* are synthesized by the two acyl-CoA:cholesterol acyltransferase (ACAT) related enzymes, Are1p and Are2p (Yang et al., 1996; Yu et al., 1996). Both proteins are located to the ER and harbor multiple transmembrane domains. Are1p and Are2p are 49% identical in sequence, but have different substrate specificities. Under standard cultivation conditions, Are2p accounts for approximately 70% of the total SE synthase activity and esterifies preferentially ergosterol. Are1p esterifies mainly sterol intermediates with a slight preference for lanosterol and becomes particularly important under hypoxic conditions (Zweytick et al., 2000). The esterification takes place at the hydroxyl group at the C3-atom with C16:1 as preferred fatty acid substrate followed by C18:1. Both TG and SE accumulate mainly during the stationary growth phase.

Storage of non-polar lipids would be useless without the possibility to mobilize them as required in order to provide sterols, DAG and FA for membrane synthesis and energy production. TG are mobilized by triacylglycerol lipases. Currently, four LD resident TG lipases are known, namely Tgl3p, Tgl4p, Tgl5p and Ayr1p (Athenstaedt and Daum 2003,2005; Ploier et al., 2013). SE are hydrolyzed by the three SE hydrolases Yeh1p, Yeh2p and Tgl1p (Köffel et al., 2005; Müllner et al., 2005), the highest activity being attributed to Yeh2p. The cycle of esterification of free sterols and the hydrolysis of SE are of utmost importance for a balanced level of free ergosterol (Wagner et al., 2009). Yeh1p and Tgl1p are localized to LD, whereas Yeh2p was surprisingly detected in the plasma membrane. The existence of further hydrolytic enzymes is currently under investigation (our own unpublished results). Especially peroxisomal enzymes might be involved in the mobilization of non-polar lipids (Thoms et al., 2011; Debelyy et al., 2011). TG and SE have long been viewed just as storage molecules, but this view has changed in recent years. TG in particular appear to be important for various cellular processes and their levels have been found to influence lipotoxicity, iron and phospholipid metabolism and cell cycle progression (Kohlwein, 2010).

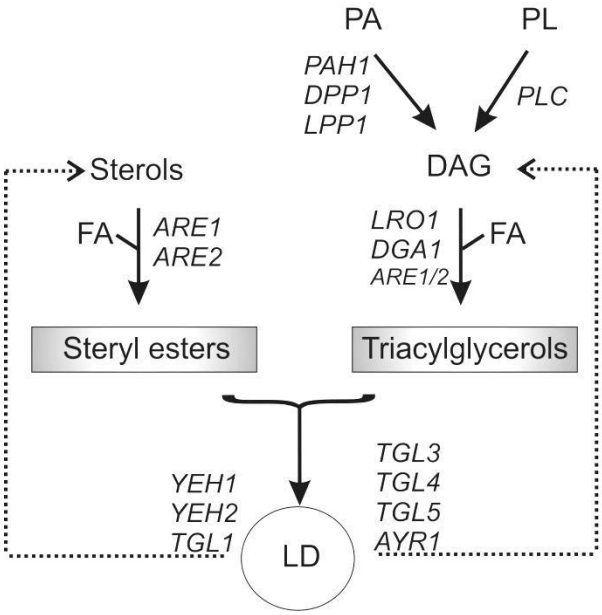

Figure 2.4: Overview of non-polar lipid metabolism in *S. cerevisiae*. For details see text. PA phosphatidic acid, PL phospholipids, DAG diacylglycerol, FA fatty acids, LD lipid droplets.

Non-polar lipid metabolism is inevitably connected to LD biology. Their biogenesis is still a matter of debate and different possible models have been published (Farese and Walther, 2009). The most widely accepted model describes its formation at special membrane

microdomains in the ER, where non-polar lipids accumulate between the two leaflets of the phospholipid bilayer until the size of the LD reaches a critical dimension (Murphy and Vance, 1999; Ploegh, 2007). At this stage, LD may bud off forming an independent organelle-like structure. Apart from their classical role as a storage compartment, it has to be noted that LD also participate in many other cellular processes (reviewed by Kohlwein et al., 2012). Connerth *et al.* (2010) described an indirect role of LD in the maintenance of membrane fluidity under environmental pressure of exogenous FA. Functions of LD unrelated to lipid turnover have also been investigated. As an example, Fei *et al.* (2009) reported that LD accumulated in yeast mutants with compromised protein glycosylation. The authors discussed a possible role of LD as a temporary safe depot for protein aggregates or incorrectly folded proteins. In recent studies LD emerged as dynamic organelles through their interaction with the ER (Fei et al., 2009; Jacquier et al., 2011; Wolinski et al., 2011), peroxisomes (Binns et al., 2006) or mitochondria (Pu et al., 2011), and novel factors influencing the biogenesis and dynamics of LD were identified (Adeyo et al., 2011).

Sphingolipids

Sphingolipids are composed of a sphingoid base, a fatty acid and a polar head group. In yeast, the sphingoid base can be dihydrosphingosine or phytosphingosine, linked through an amide bond to a very long chain fatty acid, mostly C26:0, and O-linked to the charged head group inositol. The *de novo* synthesis of sphingolipids is carried out in the ER starting with the condensation of serine and palmitoyl-CoA (Figure 2.5). This reaction is catalyzed by the serine palmitoyltransferase complex (*SPT*), which is a heterodimeric complex consisting of two major subunits, Lcb1p and Lcb2p (Nagiec et al., 1994), and one minor subunit, Tsc3p, which is necessary for full enzymatic activity (Gable et al., 2000). The product of this reaction, 3-ketodihydrosphingosine, is rapidly converted to dihydrosphingosine (also named sphinganine) by Tsc10p (Beeler et al., 1998). This product is the first sphingoid base that can be further hydroxylated by Sur2p, yielding a second sphingoid base, phytosphingosine (Grilley et al., 1998). These sphingoid bases can be either acylated to ceramides by gene products of *LIP1*, *LAG1* and *LAC1*, or phosphorylated by the sphingoid kinases encoded by *LCB4* and *LCB5* (Nagiec et al., 1998). Sphingoid base phosphates are further converted by Dpl1p to form fatty aldehydes and ethanolamine phosphates. This is the only route by which sphingolipids can exit the pathway and the link of sphingolipid metabolism to the CDP-

ethanolamine branch of the Kennedy pathway (Saba et al., 1997; Panwar and Moye-Rowley, 2006).

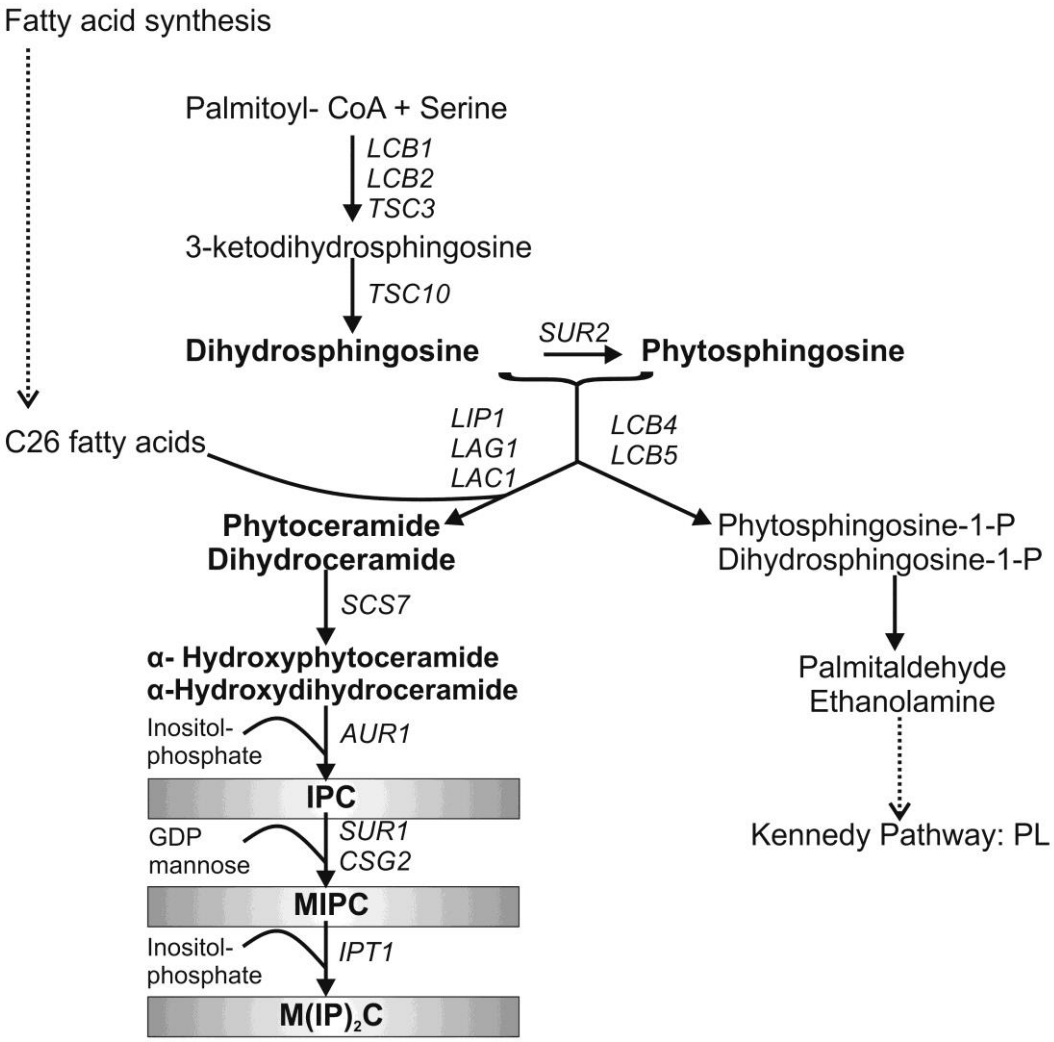

Figure 2.5: Sphingolipid synthesis in *S. cerevisiae*. For details see text. IPC inositol-P-ceramide, MIPC mannose- IPC, $M(IP)_2C$ mannose-(inositol-P)$_2$-ceramide

If sphingolipids are not phosphorylated, both sphingoid bases can be N-acylated with C26-CoA by a ceramide synthase. Ceramide synthase comprises an ER membrane protein complex consisting of Lip1p, Lag1p and Lac1p (Schorling et al., 2001; Vallée and Riezman, 2005). The two sphingoid bases and ceramides are the first products in the sphingolipid synthetic pathway. Ceramides are N-acylated sphingoid bases lacking additional head groups. They serve as substrates for the formation of complex lipids that may comprise up to 10% of total membrane lipids. Prior to the formation of the complex sphingolipids, inositol-

P-ceramide (IPC), mannose-inositol-P-ceramide (MIPC), and mannose-(inositol-P)$_2$-ceramide [M(IP)$_2$C], ceramides are α-hydroxylated by Scs7p (Haak et al., 1997; Dunn et al., 1998). Aur1p, the inositolphosphorylceramide synthase, attaches a phosphoinositol headgroup to ceramide yielding IPC (Nagiec et al., 1997), which is then mannosylated by Csg1p, Csg2p and Csh1p to MIPC. After mannosylation, another inositol phosphate group is added by Ipt1p forming M(IP)$_2$C (Beeler et al., 1997; Uemura et al., 2003; Dickson et al., 1997).

The key players of sphingolipid catabolism are encoded by *ISC1*, *YPC1* and *YDC1* (Sawai et al., 2000; Mao et al., 2000a; Mao et al., 2000b). Isc1p hydrolyzes the head groups of complex sphingolipids yielding both phyto- and dihydroceramides that can be cleaved reversibly to sphingoid bases. This reaction is catalyzed by the two ceramidases Ypc1p and Ydc1p.

Although sphingolipids fulfill many important physiological roles (Dickson et al., 2006), little is known about the regulation of cellular sphingolipid levels. Cowart and Obeid (2007) showed that there is no stringent transcriptional regulation of the key enzymes of sphingolipid metabolism. One control mechanism could be phosphorylation of the sphingoid base kinase Lcb4p by interaction of Pho85p with two of its cyclin partners, Pcl1p and Pcl2p, which leads to downregulation of Lcb4p. This effect is accompanied by a decrease of sphingoid base phosphate levels and a decrease of the cell cycle. Another study showed that ceramide synthase is regulated by casein kinase Cka2p, whose deletion resulted in a 70-75% reduction of ceramide synthase activity (Kobayashi and Nagiec, 2003). Kolaczkowski *et al.* (2004) found that the promoters of some sphingolipid metabolic enzymes contain a PDR (pleiotropic drug response) element for binding of the transcriptional activators Pdr1p and Pdr3p. Active PDR elements have been found in *LAC1*, *LCB2* and *SUR2*. A central element of sphingolipid regulation appears to be the interplay of Orm-proteins (inhibitors of *SPT*) with Ypk1p (a kinase that inactivates Orm1p and Orm2p). These links were discovered recently (Breslow et al., 2010; Roelants et al., 2011; Sun et al., 2000) and will be discussed in the section on novel key players.

Sphingolipids are mainly found in the yeast plasma membrane where they are thought to interact with sterols to form so-called lipid rafts, also described as detergent-resistant membrane domains (Bagnat et al., 2000; Guan et al., 2009; Simons and Sampaio, 2011). These domains have been proposed to constitute an important platform for certain membrane proteins, such as Pma1p, Gas1p and Gap1p (Dickson et al., 2006). The physiological role of lipid rafts has been exemplified by mis-localization of Pma1p, plasma

membrane proton pump, and Gap1p, a general amino acid permease, in strains with impaired sphingolipid metabolism (Gaigg et al., 2006; Lauwers et al., 2007). The example of mis-localization of Gap1p also illustrates a functional link between sphingolipid and amino acid metabolism. In addition to their structural role, sphingolipids and their metabolites have emerged as important signaling molecules involved in endocytosis, heat stress response and cell cycle regulation (Cowart and Obeid, 2007). Additionally, sphingolipids are necessary for the transport of GPI-anchored proteins from the ER to the Golgi (Skrzypek et al., 1997; Horvath et al., 1994). As mentioned above, they also influence the topology, localization, cell surface delivery and stability of important proteins, including the uracil permease Fur4p (Hearn et al., 2003), the plasma membrane ATPase Pma1p (Gaigg et al., 2005) and the vacuolar ATPase (Chung et al., 2003).

A SELECTION OF NOVEL KEY PLAYERS IN YEAST LIPID METABOLISM

A list of the major yeast lipid synthesizing and degrading enzymes is currently available, but a detailed understanding of lipid homeostasis and regulation of lipid metabolism still awaits clarification. In recent years, several new enzymes involved in lipid metabolism and related mechanisms have been identified. This development shows that the field of lipid research has become broader as links to other cellular processes became evident. To give the reader an impression of the complexity of lipid metabolism and its regulation, a few selected examples of novel insights into yeast lipid homeostasis covering different lipid species will be discussed in the following section.

Regulation of phospholipid synthesis: Inositol, phosphatidic acid and Opi1p

Besides acetyl-CoA, inositol is a major link of carbon metabolism to lipid metabolism. It is a carbohydrate synthesized from glucose-6-P in two steps, and is not essential under standard cultivation conditions. Inositol forms the structural component of a number of secondary messenger molecules, the inositol phosphates. In addition to its signaling role, inositol is also an important component of PI and its phosphates (PIPs), and can be regarded as the master regulator of PL biosynthesis. Inositol used for PI synthesis is either synthesized *de novo* or imported into the cell from the growth medium by inositol transporters encoded by *ITR1* and *ITR2* (Nikawa et al., 1991). The switch between these two possibilities is regulated by

phosphatidic acid (PA), which acts as an essential metabolic intermediate and a regulator of phospholipid homeostasis.

The link between inositol and PA is an effector named Opi1p (Loewen et al., 2004). As noted in a previous section, many genes involved in phospholipid biosynthesis carry a *cis*-acting, inositol-sensitive upstream activating sequence (UAS_{INO}) response element (Chen et al., 2007a). All these genes are regulated by the same transcription factors. They are activated by Ino2p and Ino4p, and repressed by Opi1p. The location of Opi1p is the key whether or not it acts as a repressor. In the absence of extracellular inositol, Opi1p is bound to the ER, together with the integral ER membrane protein Scs2p (interaction of an FFAT motif) and PA. With Opi1p in this location, genes involved in inositol synthesis are transcribed. When inositol is added to the medium, PA is consumed by conversion to PI, leading to the translocation of Opi1p to the nucleus where it represses genes carrying the UAS_{INO} element (Carman and Han, 2011). This latter process is influenced by pH, because deprotonated PA is a better ligand for Opi1p than protonated PA. The intracellular pH of yeast cells is strongly dependent on the nutritional environment. During glucose starvation, it falls rapidly compromising the binding between PA and Opi1p. This effect leads to the translocation of Opi1p to the nucleus where it acts as a repressor of phospholipid synthesis (Ktistakis, 2010).
The example described above is only one among many other regulatory aspects involved in phospholipid metabolism. As another recent example, Moir *et al.* (2012) reported that Yft2p and Scs3p, the yeast homologues of the mammalian FIT proteins (fat storage-inducing transmembrane proteins), are required for normal ER membrane biosynthesis. It is suggested that these proteins could be candidates involved in global regulation of phospholipid metabolism. For a more detailed description of phospholipid regulatory networks and interconnections with other pathways the reader is referred to a recent review (Carman and Han, 2011).

Regulation of sphingolipid metabolism

Sphingolipid metabolism is regulated by a series of factors, Orm1p, Orm2p, Ypk1p, Slm1p, Slm2p and TORC2. Orm1p and Orm2p are evolutionarily conserved proteins that act as inhibitors of serine:palmitoyl-CoA transferase (*SPT*), encoded by *LCB1* and *LCB2*, which catalyzes the first and rate-limiting step in the *de novo* synthesis of sphingolipids (Figure 2.5)

(Breslow et al., 2010). Ypk1p is a serine/threonine protein kinase that inactivates Orm1p and Orm2p by phosphorylation in response to compromised sphingolipid synthesis (Roelants et al., 2011; Sun et al., 2000). Slm1p and Slm2p are phosphoinositide binding proteins that form a complex with each other and are both phosphorylated by the TORC2 complex (Niles and Powers, 2012). The interplay of these factors can be regarded as important control mechanism for sphingolipid homeostasis, because not only do the end products of sphingolipid synthesis but also several intermediates play an essential role for the cell. The feedback loop that controls sphingolipid metabolism can be summarized as follows: Orm1p and Orm2p form a stable complex with SPT when they are dephosphorylated, repressing SPT activity. Upon sphingolipid deficiency, Orm proteins are phosphorylated by Ypk1p, which leads to their relief of SPT. Ypk1p activity is in turn controlled by phosphorylation in a TORC2-dependent manner (Raychaudhuri et al., 2012). The TORC2-dependent phosphorylation of Ypk1p requires the activation of Slm proteins. These proteins appear to sense membrane stress caused by sphingolipid depletion and react by redistribution among different membrane domains. The relocation from eisosomes is caused by the inhibition of sphingolipid synthesis, which is then followed by activation of TORC2-Ypk1 signaling (Berchtold et al., 2012).

Phosphatidate phosphatase Pah1p, a switch point in glycerolipid metabolism

PAH1 encodes the enzyme phosphatidate phosphatase which has gained more and more attention, in particular because of its homology to the mammalian lipins 1 and 2, which are involved in several lipid-associated disorders in human physiology (Han et al., 2006; Reue and Brindley, 2008; Reue and Dwyer, 2009). Pah1p catalyzes dephosphorylation of PA, yielding DAG and P_i, in a Mg^{2+} dependent manner. Since both substrate and product of this reaction are important lipid mediators, Pah1p can be regarded as a central regulator of lipid homeostasis. This enzyme is an important control point deciding whether cells produce storage lipids or phospholipids as membrane constituents (for review see Pascual and Carman, 2012). Pah1p is evolutionarily conserved, since genes encoding PAP (phosphatidic acid phosphatase) enzymes have been identified in humans, mice, flies, worms and plants. The influence of Pah1p was best studied in *pah1Δ* yeast deletion strains which were severely affected at several levels of lipid homeostasis. These strains showed defects in the synthesis

of TG and PL, elevation in cellular content of PA and decreased levels of DAG and TG (Fakas et al., 2011; Han et al., 2006). Moreover, the amounts of PL, FA and SE were also increased in these mutants. The importance of Pah1p is further underlined by the occurrence in a *pah1Δ* deletion strain of several phenotypic appearances such as slow growth, defects in the biogenesis and morphology of LD, aberrant expansion of the nuclear/ER membranes, FA induced toxicity and effects in vacuole homeostasis and membrane fusion, as well as in respiratory deficiency (O'Hara et al., 2006; Adeyo et al., 2011; Fakas et al., 2011; Sasser et al., 2012). The increased amount of PL is also typical of a *pah1Δ* mutant, which could be caused by the derepression of UAS_{INO}-containing lipid synthesis genes in response to elevated PA levels (Carman and Henry, 2007; Chirala et al., 1994). Recently, Dgk1p was found to be a cellular counterpart of Pah1p by its regulation of PA homeostasis (Han et al., 2008). Dgk1p is a CTP-dependent DAG kinase that catalyzes the reverse reaction of Pah1p and restores PA levels in a *pah1Δ* mutant. As unbalanced levels of PA and DAG result in many phenotypic consequences, the activity of Pah1p must be fine-tuned to maintain lipid homeostasis and normal cell physiology. Some regulatory mechanisms of Pah1p activity were reported, but all of them are very complex, occurring on different levels (Pascual and Carman, 2012). Pah1p expression was found to depend on various physiological conditions such as zinc depletion or different growth phases. Regulation by lipids and nucleotides was identified as another regulatory mechanism since Pah1p activity is stimulated in response to CDP-DAG, PI and CL, whereas it is inhibited by sphingosine, phytosphingosine and sphinganine and the nucleotides ATP and CTP (Wu and Carman, 1994; Wu and Carman, 1996; Wu et al., 1993). Pah1p activity and subcellular distribution are governed by the Nem1p-Spo7p protein phosphatase complex, and several kinases such as Pho85p-Pho80p, Cdc28p-cyclin B, protein kinase A and C as well as casein kinase II can act on Pah1p using phosphorylation/ dephosphorylation mechanisms, however, the fine-tuning of Pah1p still has to be examined (Choi et al., 2011; Siniossoglou et al., 1998). The action of the transmembrane protein phosphatase complex Nem1p-Spo7p is responsible for the recruitment of the phosphorylated form of Pah1p from the cytosol to the nuclear/ER membrane. The Nem1p-Spo7p complex dephosphorylates Pah1p, enabling a short amino-terminal amphipathic helix to anchor Pah1p thus allowing access to its substrate PA (Pascual and Carman, 2012).

Phosphatidylserine decarboxylase 1 (Psd1p)

Phosphatidylethanolamine (PE) belongs to the bulk PL of yeast. It can be synthesized by four different pathways, namely by (i) decarboxylation of PS through Psd1p, (ii) by decarboxylation of PS though Psd2p, (iii) by re-acylation of lyso-PE by Ale1p and Tgl3p and (iv) via the CDP-ethanolamine pathway (Henry et al., 2012; Böttinger et al., 2012). These pathways account for different proportions of cellular PE. Horvath *et al*. (2011) reported that the CDP-ethanolamine pathway preferentially contributes to TG synthesis by providing PE as co-substrate for Lro1p catalyzed TG synthesis, indicating a close interaction between TG and PE synthesis. The main source for PE, however, is the conversion of PS to PE by Psd1p. Psd1p is encoded by a nuclear gene, synthesized on free ribosomes and imported into mitochondria, where protein maturation takes place. This processing occurs in three steps, involving the action of the mitochondrial processing peptidase (MPP), the action of Oct1p (a mitochondrial peptidase that cleaves destabilizing N-terminal residues of a subset of proteins) and autocatalytic cleavage at a highly conserved LGST motif. These processing steps yield the mature form of the enzyme that contains an α-subunit, exposed to the intermembrane space, and a β-subunit anchoring the activated protein to the inner mitochondrial membrane. Correct localization is crucial for full enzymatic activity and also for maintaining lipid homeostasis (Horvath et al., 2012). Deletion of *PSD1* leads to reduced growth on glucose, morphological changes in mitochondria, ethanolamine auxotrophy and an altered pattern of PL (Birner et al., 2001). These observations underline the importance of Psd1p in lipid homeostasis. PE levels were shown to have a tremendous impact, not only on the distribution of other lipids, but also on the function and stability of mitochondrial proteins (Böttinger et al., 2012).

Ups1p, ERMES and Gem1p: Components affecting mitochondrial lipid transfer

Lipid transfer between and within organelles has been an important issue for several decades but is still under intense investigation. Import of lipids into mitochondria and interaction of mitochondria with the endoplasmic reticulum are a classical example of such studies. Recently, identification of new components provided some deeper insight into these problems.

Intramitochondrial lipid transport is important to provide substrates like PA or PS for efficient CL and PE synthesis, respectively, in the inner mitochondrial membrane. Transport of PA between the outer (OMM) and inner mitochondrial membranes (IMM) was found to be mediated by Ups1p, a protein localized to the intermembrane space. (Connerth et al., 2012; Tamura et al., 2010). PA is transported in three steps starting with the binding of PA by Ups1p at the surface of the OMM. Ups1p then associates with Mdm35p to be protected against proteases before PA is released at the IMM (Potting et al., 2010). This transport is bidirectional and independent of the acyl chain composition. Dissociation of Mdm35p from the complex is a prerequisite for PA release and facilitated by the interaction with negatively charged PL like CL. However, a very high concentration of CL prevents the detachment of Ups1p from the acceptor membrane, subsequently impairing the PA flux. This finding indicates that CL is a regulator of its own synthesis (Connerth et al., 2012). Deletion of *UPS1* leads to a decrease in Psd1p levels and causes a reduction of PE. This defect has been explained as Ups1p being responsible not only for PA transport, but also for the import of Psd1p into mitochondria. Moreover, it was shown that Ups1p also mediates the export of PE from the IMM to the OMM and promotes the conversion of PE to PC, which makes Ups1p a central regulator of phospholipid metabolism by influencing lipid traffic (Tamura et al., 2012).

A complex termed ERMES (ER mitochondria encounter structure) that tethers the ER to the OMM has been identified. This complex is composed of the five proteins Mmm1p, Mdm34p, Mdm10p, Mdm12p and Gem1p (Kornmann et al., 2009; Stroud et al., 2011). Gem1p is an OMM GTPase with a C-terminal single transmembrane segment that is exposed to the cytosol (Kornmann et al., 2011; Meisinger et al., 2007). Mmm1p, Mdm34p and Mdm12p each contain an SMP domain (synaptotagmin-like mitochondrial and lipid binding proteins) that is involved in binding hydrophobic ligands like lipids. This arrangement suggests a possible role for the ERMES complex in lipid transport between the ER and mitochondria (Kopec et al., 2010). A transport route between these two compartments is very important because the substrate of the mitochondrial Psd1p, PS, is synthesized in the ER, and PE synthesized by Psd1p in mitochondria is substrate of the ER-localized PC synthesizing machinery. How lipid transport via ERMES may happen is controversial and still a matter of debate. Kornmann *et al.* (2009) reported that strains bearing mutations in the ERMES proteins showed phenotypes related to phospholipid metabolism such as decreased CL

levels. However, ERMES and Gem1p have been shown not to play a direct role in the transport of PS from the ER to mitochondria. Rather, ERMES fulfills a structural role in maintaining the morphological integrity of mitochondria (Nguyen et al., 2012).

Squalene

Squalene is a polyunsaturated triterpene consisting of six isoprene units. It possesses several beneficial properties, e.g. as antioxidant or emollient, and has therefore become relevant for biotechnological applications (for review see Spanova and Daum, 2011). As described above, squalene is an important intermediate of the sterol biosynthetic pathway. Under normal growth conditions it is rapidly converted and therefore does not accumulate in yeast. However, under certain growth conditions or by genetic manipulations (overexpression of *HMG1/2*, *ERG1* or *ERG6*; deletion of *HEM1*) the amount of squalene can be increased (Polakowski et al., 1998; Jahnke and Klein, 1983; Lorenz et al., 1989). Spanova *et al.* (2010) showed that under squalene accumulating conditions this lipid is stored in LD. Unexpectedly, accumulation of squalene did not result in lipotoxic effects. In a yeast strain lacking TG and SE, which is unable to synthesize LD, squalene was found mainly in mitochondria and microsomes without causing deleterious effects. Recent reports (Spanova et al., 2012) described functions of squalene as a modulator of membrane properties affecting mainly membrane fluidity. It was shown that ER membranes become more rigid when enriched in squalene, whereas samples of plasma membranes became softer. Unlike sterols, squalene does not necessarily rigidify membranes, but modulates their dynamics in both directions. This effect could depend on the ratio of ergosterol to squalene.

Tgl3p, Tgl4p and Tgl5p: more than just triacylglycerol lipases?

As described in the section on non-polar lipids, Tgl3p, Tgl4p and Tgl5p are the main TG lipases of the yeast *S. cerevisiae*. Recently, Ayr1p was identified as further TG lipase with minor lipolytic activity (Ploier et al., 2013). TG lipases catalyze the cleavage of TG to DAG and FA. However, Tgl3p, Tgl4p and Tgl5p are not only responsible for mobilization of the main storage lipids but also contribute to lipid metabolism as acyltransferases and phospholipases, which makes them novel key players in lipid metabolism (Grillitsch and Daum, 2011; Rajakumari and Daum, 2010a, 2010b)

In general, lipases are a subclass of hydrolases whose catalytic activity depends on the so-called interfacial activation, which means that they act only at an aqueous/non-aqueous interface (Verger, 1997). As all other lipases, the three TG lipases of yeast,Tgl3p, Tgl4p, Tgl5p and Ayr1p, share a common consensus sequence GXSXG, where serine is the essential residue as interaction partner of the catalytic triad aspartic acid, glutamic acid and histidine (Schrag and Cygler, 1997). They also contain a patatin domain, named after a plant storage protein that possesses lipid acyl hydrolase activity (Mignery et al., 1988).

Tgl3p was the first yeast TG lipase to be identified and characterized in *S. cerevisiae* (Athenstaedt et al., 1999a; Athenstaedt and Daum, 2003). Tgl4p and Tgl5p, identified some years later, exhibit about 30% and 26% similarity with Tgl3p (Athenstaedt and Daum, 2005; Kurat et al., 2006). Localization studies have revealed that all four TG lipases are localized to LD, although none of these three lipases show hydrophobic domains (Athenstaedt and Daum, 2005; Müllner et al., 2004). *In vitro*, all three proteins possess lipolytic activity whereas *in vivo* only Tgl3p and Tgl4p mobilize TG efficiently. This finding was explained by different substrate specificities. It appears that Tgl5p accepts mainly TG containing cerotic acid (C26:0), a fatty acid of low abundance in yeast, whereas overall effects *in vivo* on bulk TG hydrolysis were not observed. The main TG lipase in yeast, Tgl3p, was shown to hydrolyze TG as well as DAG, whereas substrate specificity of Tgl4p is restricted to TG (Kurat et al., 2006). A *Δtgl3Δtgl4Δtgl5* yeast strain lacking all three TG lipases does not reveal any growth defect under standard growth conditions, although mutations in *TGL3* or *TLG4* lead to fat yeast cells that accumulate TG (Athenstaedt and Daum, 2005; Kurat et al., 2006). Moreover, deletion of *TGL4* and *TGL5* leads to decreased sporulation efficiency.

Recent characterization of TG lipases has revealed novel functions of these enzymes. Protein sequences of all three TG lipases contain additional sequence motifs besides the conserved GXSXG lipase motif. Tgl3p, Tgl4p and Tgl5p harbor an acyltransferase motif (H-$(X)_4$-D), and Tgl4p was found to have in addition a phospholipase motif (GXGXXG). Further investigations revealed decreased amounts of total PL in a *Δtgl3* deletion strain and increased amounts of PL in a *TGL3* overexpressing strain (Rajakumari et al., 2010; Rajakumari and Daum, 2010a). *In vitro* enzyme assays showed that both Tgl3p and Tgl5p act as lysophospholipid acyltransferases with different substrate specificities. Tgl3p mainly acylates lyso-phosphatidylethanolamine, whereas Tgl5p prefers lysophosphatidic acid as a substrate (Rajakumari and Daum, 2010a). The lipase activity of Tgl3p acts independently from the

acyltransferase activity and *vice versa* as demonstrated by site directed mutagenesis inactivating either one of the two motifs. Interestingly, the sporulation defect in a *Δtgl3Δtgl5* double mutant was still observed in a strain with mutated lipase motif but not when the acyltransferase activity was abolished.

Besides the conserved lipase motif, Tgl4p contains a (G/A)XGXXG Ca^{2+}-independent phospholipase A_2 domain. Phospholipase activity of Tgl4p was also established *in vitro* with PC and PE as substrates but not with PA or PS. Additionally, Tgl4p hydrolyzed SE and revealed lysophospholipid acyltransferase activity (Rajakumari and Daum, 2010b). Kurat *et al.* (2009) reported an impact of phosphorylation of Tgl4p activity. They showed that the lipolytic activity of Tgl4p was strongly reduced when phosphorylation sites were mutated, whereas the lysophospholipid acyltransferase activity was not affected (Rajakumari and Daum, 2010b). In conclusion, Tgl4p is an excellent example of a multifunctional enzyme involved in yeast lipid metabolism, which does not only hydrolyze TG and SE but also contributes to PL synthesis and membrane remodeling. Recent publications led to the conclusion that besides the currently known TG lipases, Tgl3p, Tgl4p and Tgl5p, also other TG lipases may play a role in the turnover of non-polar lipids (Debelyy et al., 2011; Thoms et al., 2011; Thoms et al., 2008; Ploier et al., 2013).

Phospholipases

Phospholipases cleave different bonds in glycerophospholipid molecules, and their physiological effects are based on the resulting products. Depending on the bond(s) cleaved, phospholipases are divided into groups A_1, A_2, B, C and D (Figure 2.6).

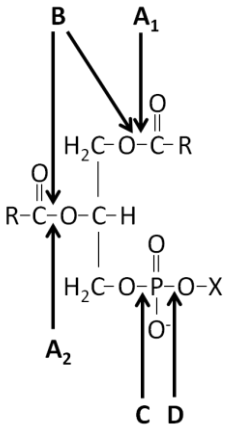

Figure 2.6: Sites of hydrolytic action of phospholipases A1, A2, B, C and D on a model phospholipid molecule.

The example of Plc1p, the canonical yeast phospholipase C, illustrates well how phospholipases activate signaling cascades by generating, in the case of Plc1p, DAG and inositol 1,4,5-triphosphate, both of which exert their intrinsic biological activity as secondary messengers (reviewed in Rebecchi and Pentyala, 2000; Strahl and Thorner, 2007; York, 2006). However, the activity of some phospholipases appears to be restricted to metabolic functions, such as that of Pgc1p, another yeast phospholipase C, which is specific for PG hydrolysis and required for its degradation (Simocková et al., 2008). The third known yeast phospholipase C is encoded by the *ISC1* gene. It accepts phosphosphingolipids (see above) as substrate and generates phytoceramide, a signaling molecule affecting several cellular processes (reviewed in Matmati and Hannun, 2008). Two phospholipases D, Spo14p and Fmp30p, have also been described in *S. cerevisiae*. The former enzyme hydrolyzes PC to choline and phosphatidic acid and is involved in several cellular processes including growth, secretion and regulation of *INO1* expression (Sreenivas et al., 1998), as well as sporulation (Rudge et al., 1998) and general transcription (García-López et al., 2011). Fmp30p, an IMM protein with sequence similarity to the mammalian N-acylethanolamine specific phospholipases D (Merkel et al., 2005), is also required for CL homeostasis (Kuroda et al., 2011).

Deacylating phospholipases in yeast include the phospholipases B, Plb1p, Plb2p, Plb3p, Nte1p and Spo1p; phospholipases A_2, Cld1p, Tgl4p, Per1p and Bst1p, and Yor022cp, a putative phospholipase A_1. A detailed understanding of the biochemical pathways leading to the specific FA composition of PL is important, among other reasons also from the perspective of yeast-based biofuel production (see below). All deacylating phospholipases could in principle be involved in acyl-chain remodeling of phospholipids, but a recent study showed that rather than phospholipases B, it is the PL:DAG acyltransferase Lro1p that provides FA for PL remodeling (Mora et al., 2012). Two other acyltransferases, Psi1p (=Cst26p) and Taz1p, play crucial roles in PL acyl-chain remodeling (reviewed by Henry et al., 2012). Plb1/2/3 proteins, on the other hand, have been proposed to be involved in biosynthesis and, together with the phospholipase D Fmp30p, in signaling through N-acylethanolamines and N-acylphosphatidylethanolamines (Merkel et al., 2005).

A special case among yeast phospholipases is Tgl4p which, as described above, is a multifunctional enzyme with reported triacylglycerol lipase, steryl ester hydrolase, and Ca^{2+}-independent phospholipase A_2 activities (Rajakumari and Daum, 2010b). Importantly,

regulation of the activity of this protein also links lipid metabolism to cell-cycle regulation (Kurat et al., 2009). Multiple cellular processes are also affected by the activity of Per1p and Bst1p, albeit the diversity of their effects stems from the fact that these phospholipases A_2 are active on GPI-protein anchors that enable specific localization of the proteins targeted to lipid raft regions of the plasma membrane (Fujita et al., 2006; Tanaka et al., 2004). *SPO1* is a meiosis-induced gene that encodes a phospholipase B with a role in distinct steps of sporulation, exhibiting epistasis with Spo14p phospholipase D, whereas the absence of Spo1p can be partially suppressed by over-expression of *PLB3* gene (Tevzadze et al., 2007). *CLD1* codes for a cardiolipin-specific deacetylase which, together with Taz1p, ensures the biosynthesis of mature cardiolipin (Beranek et al., 2009). Also Nte1p seems to play an interesting role possibly regulating transcription of PL biosynthesis genes through its PC-specific phospholipase A_2 activity and subsequent modulation of Opi1p activity (see above) (Fernández-Murray et al., 2009).

Izh proteins, zinc homeostasis, and regulation by inositol and fatty acids

As described in previous sections, regulation of inositol biosynthesis is one of the central processes in yeast lipid and general metabolism homeostasis. In this section, we will address the role of Izh (Implicated in Zinc Homeostasis) proteins, yeast homologs of the mammalian adiponectin receptors, which have recently emerged as players enabling the connection between inositol and fatty acids metabolism and zinc homeostasis. Zinc depletion in yeast activates the Zap1p transcriptional activator which affects several target genes, among them the *PIS1* encoded PI synthase, and the *DPP1* encoded DGPP phosphatase. Zn depletion thus causes a decrease in PE and an increase in PI concentration (Carman and Han, 2011; Iwanyshyn et al., 2004). In addition, zinc depletion results in a decreased concentration of PA, thus triggering the release of Opi1p from the ER membrane and its translocation into the nucleus, where it represses expression of the *CHO1* gene that encodes PS synthase by binding to and inhibition of the Ino2/4 complex (Carman and Han, 2011). There are four genes in yeast, *IZH1, IZH2, IZH3* and *IZH*4 which encode proteins with sequence similarity to adiponectin receptors. In humans, adiponectin receptors mediate the antidiabetic metabolic activity of the polypeptide hormone adiponectin (Kadowaki et al., 2006). The yeast Izh2p has been confirmed as a functional homolog of adiponectin receptors in an experiment where heterologous expression of human adiponectin receptors in yeast functionally

complemented Izh2p (Kupchak et al., 2007). *IZH1/2/3/4* genes were implicated to have a role in zinc metabolism after they had been identified in a screening for Zap1p targets, and were confirmed to have zinc-related phenotypes (Lyons et al., 2004). Expression of *IZH1* and *IZH2* is directly regulated by Zap1p, and the promoters of these genes contain zinc response elements. In addition, *IZH1, IZH2* and *IZH3* genes are regulated by exogenous FA through Oaf1p/Pip2p transcription factors that bind to oleate response elements present in their promoters (Lyons et al., 2004). Specifically, *IZH2* expression is highly induced in cells grown in the presence of saturated FA such as myristate, and strains without this gene fail to grow normally in the presence of myristate (Karpichev et al., 2002). Transcriptome analysis of *izh2*Δ cells has revealed that a number of genes encoding proteins involved in FA metabolism and in the phosphate signaling pathway are regulated by Izh2p (Karpichev et al., 2002). Three functions of Izh proteins have been proposed by Lyons et al. (2004): (i) a role in sterol metabolism by which they would influence the permeability of the plasma membrane and consequently zinc homeostasis; (ii) a role as transporters for zinc; and (iii) a role in a zinc independent signal transduction cascade with Zap1p as downstream target. The above results imply that, at least for Izh2p, the third possibility is the most likely one. Thus, Izh2p is emerging as a central component of a putative feed-back regulatory pathway leading from FA to Zap1p activation and finally to inositol and regulation of PL biosynthesis.

BIOTECHNOLOGICAL ASPECTS

Lipids and their expansive roles have become increasingly recognized, resulting in a great demand for industrial high-level production of particular valuable lipid compounds. Lipid metabolism in yeasts as described above has been studied intensively and well described. Since this process is well conserved in eukaryotic cells, yeasts are ideal host systems for the biotechnological production of industrially and pharmaceutically relevant lipid compounds. *S. cerevisiae* in particular has been successfully applied for their production. This section describes examples, selected to illustrate the importance of lipid metabolism in biotechnology. In this section some of the most important approaches are described. The reader is also referred to recent reviews on these topics (such as Beopoulos et al., 2011; de Jong et al., 2012; Ruenwai et al., 2011; Uemura, 2012; Veen and Lang, 2004) for more details.

Polyunsaturated fatty acids (PUFA)

PUFA are FA with more than 16 carbon atoms in the chain that contains more than one double bond. They have multiple positive effects on human health, such as lowering the risk of heart attacks, cardiovascular diseases and cancer, and they also have major impacts on the development and improvement of retinal and brain function and on the regulation of membrane fluidity (Uemura, 2012; Opekarová and Tanner, 2003). Since mammals are not able to synthesize essential PUFA such as linoleic acid (C18:2n-6) or the omega-3 and omega-6 PUFA, they must be taken up from the diet. Since natural sources, such as fish oils, are limited it is highly desirable to produce PUFA from alternative and sustainable sources. One promising option is *S. cerevisiae* or other yeasts. *S. cerevisiae* in particular has been shown to have a considerable potential for metabolic engineering approaches to the production of certain metabolites (Ostergaard et al., 2000).

The physiological FA composition of *S. cerevisiae* includes mainly C16:1 and C18:1 as described above in the section on fatty acids. Since *OLE1,* that encodes a Δ9-fatty acid desaturase, is the only endogenous desaturase (Stukey et al., 1990), production of PUFA in *S. cerevisiae* requires the introduction of further desaturase and elongase genes from donor organisms such as *Mucor rouxii*, *Caenorhabditis elegans*, *Arabidopsis thaliana* or *Mortiella alpina* to produce, for example, α-linolenic acid (C18:3n-3), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) (Ruenwai et al., 2011; Uemura, 2012). Combinations of multiple desaturases and elongases from various organisms were tried, but since Δ5- and Δ6-fatty acid desaturases can accept both n-3 and n-6 FA the resulting products mostly depend on the substrate fatty acid added to the medium. Most studies used a large excess of precursor FA, yet the final yield of the PUFA produced was still low and strongly depended on cultivation conditions, such as growth media, temperature and incubation time (Uemura, 2012; Misawa, 2011). Construction of the complete pathway for the production of C20-PUFA, such as DGLA (dihomogamma linoleic acid) from the endogenous oleic acid, has been described by Yazawa and co-authors (Yazawa et al., 2007). The authors cloned a Δ12-desaturase gene from *K. lactis*, and a Δ6-desaturase and the elongase *ELO1* genes from rat into *S. cerevisiae*.

One severe limitation of *S. cerevisiae* as a production host for PUFA is the low total lipid content compared to some other yeast genera. One alternative is the use of oleaginous yeasts such *Y. lipolytica* which are characterized by their ability to accumulate lipids up to

40% of their biomass (Beopoulos et al., 2011). *Y. lipolytica* has been applied successfully for the production of ω-3 and ω-6 PUFA such as docosahexaenoic acid, eicosapentaenoic acid and γ-linolenic acid. Dupont de Nemours, for example, genetically engineered *Y. lipolytica* by expressing desaturases of the oleaginous fungus *M. alpine*, and elongases of the stramenophile *Thraustochytrium aureum* (patent WO/2006/052871) (Beopoulos et al., 2011).

Isoprenoids

Isoprenoids, also referred to as terpenoids, comprise a large group of naturally occurring secondary metabolites built from isoprene units, IPP (isopentenyl diphosphate) and its isomer DMAPP (dimethylallyl diphosphate). Eukaryotes synthesize IPP via the mevalonate pathway as described in the section on sterols. Head-to-tail condensation of IPP and DMAPP yields geranyl pyrophosphate (GPP), which is then converted to farnesyl pyrophosphate (FPP) by linkage of another molecule IPP. IPP is a branching point between GGPP (geranylgeranyl diphosphate) and the sterol pathway (see Figure 2.3) (Pichler, 2005). GPP and FPP are the precursors of monoterpenoids and sesquiterpenoids, respectively, and GGPP of diterpenes. Typically, two molecules of FPP are condensed to yield squalene, the precursor of sterols, and phytoene, which can be converted to carotenoids. Steroids will be discussed in the next section while the other isoprenoids are dealt with in this section.

Terpenoids comprise over 40,000 structurally different compounds. They are the largest group of natural products and have valuable properties for medical and industrial usage, especially as constituents of plant oils such as limonene, menthol and citronellol, which are used as flavors and fragrances, in their occurrence of carotenoids and as pharmaceuticals such as taxol (Misawa, 2011; Chang and Keasling, 2006).

S. cerevisiae does not produce monoterpenoids. Due to industrial requirement of these compounds, however, metabolic engineering approaches have been accomplished to this end (Lee et al., 2009). Herrero *et al*. (2008), for example, reported a recombinant wine yeast strain of *S. cerevisiae*, that expresses the (*S*)-linalool synthase gene from the plant *Clarkia breweri,* and concomitantly overexpresses HMG-CoA reductase, resulting in efficient excretion of linalool reaching concentrations of 77 µg/L.

Sesquiterpenoids comprise the largest group of isoprenoids, and occur in plants and insects as pheromones and defensive agents. Because of their anticancer, antitumor and antibiotic

properties they are industrially important compounds (Asadollahi et al., 2010). One prominent example is artemisin, which is an effective anti-malarial drug and has been discussed as an anticancer agent (Firestone and Sundar, 2009; Chaturvedi et al., 2010).

Several pharmaceuticals belong to the group of diterpenoids, including taxol that is used as a potent anticancer agent (Wani et al., 1971). As the demand for taxol exceeds the amounts which can be isolated from its natural source *Taxus bevifolia*, heterologous production in *S. cerevisiae* by introducing parts of the 19 enzymatic step biosynthetic pathway is one alternative. Engels *et al*. (2008) described the production of a precursor of taxol, taxa-4(5),11(12)-diene, by expressing *Taxus chinensis* taxadiene synthase and truncated HMG-CoA reductase genes in *S. cerevisiae* together with a archaeal GGPP synthase gene from *Sulfolobus aciocaldarius*. These manipulations resulted in formation of 8.7 mg/L of taxadiene.

Carotenoids, such as β-carotene, astaxanthin and lycopene, are also isoprenoids. They are widely distributed as yellow, orange and red natural pigments in all phototrophic plants as well as in some bacteria, algae and fungi. In addition to their important physiological roles as components of the photosynthetic complex, precursors of phytohormones, and chromophoric compounds of animals and plants, their antioxidative and photoprotective effects were proposed (Fraser and Bramley, 2004). These effects are also beneficial for human health and carotenoids attracted attention as neutraceutical agents. Lycopene, for example, which occurs in tomatoes, is thought to prevent cardiovascular disease, UV-light aging in humans and age-related macular degeneration. Carotenoid biosynthetic pathways have been introduced into *S. cerevisiae* to produce lycopene and β-carotene. The engineered strains yielded β-carotene at 5.9 mg/g dry cell weight and lycopene at 7.8 mg/g dry cell weight (Verwaal et al., 2007; Yamano et al., 1994). For a recent review of this topic see Wriessnegger and Pichler (2013).

Steroids

Steroids comprise a large group of compounds with cyclopentanoperhydrophenanthrene as the common basic structure as described in the section on sterols. This group of components are roughly divided into sterols, which are steroid alcohols with a hydroxyl group in the 3-position of the A-ring, steroid hormones, steroid alkaloids and bile acids. Hundreds of distinct steroids are found in plants, animals and fungi, all of them sharing the mutual

precursor squalene. They have sex-determining, growth regulating and anti-inflammatory properties and are responsible for membrane fluidity and permeability (Riad et al., 2002). The chemical synthesis is very difficult and extraction from natural sources is low-yielding and unsustainable. Therefore, the production in yeast is an appreciated alternative (Heiderpriem et al., 1992).

Several sterol intermediates are of biotechnological interest and have already found applications in industry (Donova and Egorova, 2012). Lanosterol, for example, serves as an emulsifier in cosmetics, zymosterol as a precursor for cholesterol lowering substances, and ergosterol itself as provitamin D2 and as a constituent of liposomal steroids used as carriers for drugs. As of special pharmaceutical interest, they can serve as valuable precursors for the production of hydrocortisone and other steroid hormones like dehydroepiandosteroine, progesterone, testosterone and estrogens. The natural content of sterols in yeast is, however, too low for commercial applications and several attempts have therefore been made to increase the total sterol content in this microorganism (Veen et al., 2003). The most successful strategies were the concomitant overexpression of *ERG1* and *ERG11* and a truncated version of *HMG1*, and the overexpression of *ERG4* and *ARE2*. The accumulation of sterols can also be promoted by the addition of ethanol into the cultivation medium by fermentation under nitrogen limiting conditions (Sajbidor et al., 1995; Shang et al., 2006). For recent review about yeast metabolic engineering targeting sterol metabolism see (Wriessnegger and Pichler, 2013).

Biofuels

Current transportation fuels are obtained mainly from fossil sources, which are not only limited but also associated with air pollution and global warming. These developments have prompted a desire for a shift from fossil fuels to biofuels. The concept of biofuels relies on the conversion of renewable resources to fuels. It comprises not only first generation biofuels such as bioethanol and biodiesel, but also advanced biofuels such as alkanes, terpenes, short chain alcohols and fatty acyl ethyl esters. Compared to bioethanol, the latter compounds promise energy content and combustion properties similar to those of current petroleum based fuels (de Jong et al., 2012).

The most frequently employed microorganism to produce bioethanol is *S. cerevisiae* since it is able to hydrolyze sucrose from sugar cane into glucose and fructose at concentrations

over 100 g/L, which can be converted by fermentation to ethanol. However, the availability of inexpensive fermentable sugars is limited, and rededicating farmland for biofuel production causes economic and ethical problems. Another limitation for ethanol as biofuel is the difficulty to distill it from fermentation broth due to its miscibility with water and its corrosive effect to storage and distribution infrastructures. As alternatives, non-food cellulose sources, including wheat straw and forest waste, can be used for the production of biofuels but, since *S. cerevisiae* is unable to convert cellulose or efficiently ferment C-5 sugars (pentoses), metabolic engineering approaches are necessary or the employment of other yeast genera (Madhavan et al., 2012).

Biodiesel is a biodegradable, non-toxic and sulfur-free alternative form of fuel, currently produced mainly by chemical transesterification of vegetable oils. One promising alternative is to use oleaginous yeasts, such as *Yarrowia lipolytica*, *Cryptococcus curvatus* or *Lipomyces starkeyi* to produce lipids using cellulosic sugars as carbon source. These yeasts accumulate lipids at up to 40% of their biomass, under nutrient limiting conditions even up to 70% (Chen et al., 2009). The microbial lipids produced show similar composition and energy values to those of vegetable oils, comprising mainly myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1, n-7), stearic (C18:0), oleic (C18:1, n-9), linoleic (C18:2, n-6) and α- linolenic (C18:3 n-3) acids, and are therefore of great commercial value for the production of sustainable biodiesel which requires C16-C18 FA (Zhao et al., 2011; Yu et al., 2011). However, from an economic point of view, the development of yeasts that produce more than 80% lipids of their biomass would be necessary. Several engineering strategies have already been published and patented as reviewed by Beopoulos et al. (2011).

Flavor compounds

Yeast biosynthesis of flavor compounds is important in fermentations of wine, beer and sake. An important group of volatile compounds produced by yeast during fermentation, which include fusel alcohols, monoterpenoids and volatile sulphur compounds, are lipid metabolism-derived acetate esters and medium-chain fatty acid (MCFA) ethyl esters (reviewed by Cordente et al., 2012). These esters are produced intracellularly by acetyl transferases from acetyl-CoA and ethanol or complex alcohols as substrates, or by acyl transferases from MCFA-CoA and ethanol as substrates. Many such esters can pass the plasma membrane and diffuse into the medium. The best studied group from the

perspective of biosynthesis pathway are acetate esters. Their synthesis is catalyzed by acetyl transferases I and II, encoded by *ATF1* and *ATF2* genes (Fujii et al., 1994; Nagasawa et al., 1998). Atf1p has been shown to be localized to lipid droplets (Verstrepen et al., 2004). Apart from volatile esters such as ethyl acetate or isoamyl acetate, Aft1p and Aft2p are also responsible for the formation of less volatile esters which add no flavor characteristics to the fermentation products. A certain amount of acetate esters is produced also in cells deleted of both *AFT* genes, indicating that additional, as yet unknown acetyl transferases may exist in yeast. Ethyl esters are the product of Eeb1p or Eht1p catalyzed condensation reaction between acyl-CoA and ethanol (Saerens et al., 2006). These two acyl transferases differ in their specificity towards different length of the substrate molecules, and they also possess esterase activity. Similar to acetyl transferases, undiscovered acyl transferases responsible for MCFA ethyl ester biosynthesis are encoded in the yeast genome.

Understanding the physiological regulation of volatile esters biosynthesis is the prerequisite for the engineering of flavor compounds in yeast-fermented beverages. For the synthesis of acetate esters, the main regulatory step is the reaction catalyzed by acetyl transferases, whereas for MCFA ethyl ester formation, the availability of MCFA-CoA substrate is the limiting factor (Saerens et al., 2010). Amount and nature of acetyl esters could therefore be regulated by over-expression of *AFT1* or *AFT2* at different levels, possibly from different strains and therefore with different substrate specificities. The amount of MCFA ethyl esters could be controlled by modifying lipid metabolic pathways, specifically at the level of acetyl-CoA carboxylase whose activity determines the release of MCFAs from the fatty acid synthase complex (Dufour et al., 2003). Alternatively, the level of peroxisomal uptake of MCFAs may be changed, because a specific system exists for the import of this group of fatty acids towards oxidative degradation (van Roermund et al., 2001).

For more details describing the nature and properties of yeast flavor compounds the reader is referred to recent reviews (Saerens at al., 2010; Sumby et al., 2010; Cordente et al., 2012).

CONCLUSIONS AND PERSPECTIVES

Over the last few decades outstanding advancements have been made to identify the major enzymes involved in the pathways of lipid metabolism. Most of them are now known, covering the main cellular routes for synthesis, storage and degradation of lipid compounds. However, some gaps still remain. One intriguing open question is how cells can sense and manage their lipid composition under different environmental conditions. Investigations addressing such lipid sensors might also shed more light on the issue of how the different lipid compositions of different membranes within a single organism can be maintained. The situation gets even more complicated by the fact that enzymes of lipid synthesis are located in close vicinity to each other. To elucidate the topology of these enzymes in detail will be a challenge for the future. Other examples of unsolved problems are metabolic channelling and lipid trafficking that are just at the beginning to be addressed and understood. Regulation of lipid metabolism is an issue under discussion. It occurs at many different levels, and the cellular lipid composition is not only extremely dependent on growth conditions, such as nutrient availability, growth phase and pH, but also on many transcriptional control mechanisms that have been reported. Thus, the crosstalk between lipid metabolism and other cellular processes, as well as the regulatory network and interconnections of lipid metabolic pathways, will have to be studied in more detail. The elucidation of all these questions will foster the powerful role of yeast as a model organism.

ACKNOWLEDGMENTS

We thank Prof. Roger Pain and Dr. Karlheinz Grillitsch for carefully reading the manuscript. Research in G.D.'s laboratory was financially supported by the Austrian Science Fund (FWF) project W901 (DK Molecular Enzymology). The work in U.P.'s laboratory was financially supported by the Slovenian Research Agency (ARRS) grants J1—6507 and P1—0207.

REFERENCES

Adeyo, O., Horn, P.J., Lee, S., Binns, D.D., Chandrahas, A., Chapman, K.D., and Goodman, J.M. (2011). The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. J. Cell Biol. *192*, 1043–1055.

Alvarez-Vasquez, F., Riezman, H., Hannun, Y.A., and Voit, E.O. (2011). Mathematical modeling and validation of the ergosterol pathway in *Saccharomyces cerevisiae*. PLoS ONE *6*, e28344.

Anderson, M.S., Muehlbacher, M., Street, I.P., Proffitt, J., and Poulter, C.D. (1989a). Isopentenyl diphosphate:dimethylallyl diphosphate isomerase. An improved purification of the enzyme and isolation of the gene from *Saccharomyces cerevisiae*. J. Biol. Chem. *264*, 19169–19175.

Anderson, M.S., Yarger, J.G., Burck, C.L., and Poulter, C.D. (1989b). Farnesyl diphosphate synthetase. Molecular cloning, sequence, and expression of an essential gene from *Saccharomyces cerevisiae*. J. Biol. Chem. *264*, 19176–19184.

Asadollahi, M.A., Maury, J., Schalk, M., Clark, A., and Nielsen, J. (2010). Enhancement of farnesyl diphosphate pool as direct precursor of sesquiterpenes through metabolic engineering of the mevalonate pathway in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. *106*, 86–96.

Athenstaedt, K., and Daum, G. (1997). Biosynthesis of phosphatidic acid in lipid particles and endoplasmic reticulum of *Saccharomyces cerevisiae*. J. Bacteriol. *179*, 7611–7616.

Athenstaedt, K., and Daum, G. (1999). Phosphatidic acid, a key intermediate in lipid metabolism. Eur. J. Biochem. *266*, 1–16.

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

Athenstaedt, K., Weys, S., Paltauf, F., and Daum, G. (1999b). Redundant systems of phosphatidic acid biosynthesis via acylation of glycerol-3-phosphate or dihydroxyacetone phosphate in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. *181*, 1458–1463.

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 source. Proteomics *6*, 1450–1459.

Bagnat, M., Keränen, S., Shevchenko, A., Shevchenko, A., and Simons, K. (2000). Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc. Natl. Acad. Sci. U.S.A. *97*, 3254–3259.

Bard, M., and Downing, J.F. (1981). Genetic and biochemical aspects of yeast sterol regulation involving 3-hydroxy-3-methylglutaryl coenzyme A reductase. J. Gen. Microbiol. *125*, 415–420.

Beeler, T., Bacikova, D., Gable, K., Hopkins, L., Johnson, C., Slife, H., and Dunn, T. (1998). The *Saccharomyces cerevisiae TSC10*/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca2+-sensitive csg2Delta mutant. J. Biol. Chem. *273*, 30688–30694.

Beeler, T.J., Fu, D., Rivera, J., Monaghan, E., Gable, K., and Dunn, T.M. (1997). *SUR1* (CSG1/BCL21), a gene necessary for growth of *Saccharomyces cerevisiae* in the presence of high Ca^{2+} concentrations at 37 degrees C, is required for mannosylation of inositolphosphorylceramide. Mol. Gen. Genet. *255*, 570–579.

Beopoulos, A., Nicaud, J.-M., and Gaillardin, C. (2011). An overview of lipid metabolism in yeasts and its impact on biotechnological processes. Appl. Microbiol. Biotechnol. *90*, 1193–1206.

Beranek, A., Rechberger, G., Knauer, H., Wolinski, H., Kohlwein, S.D., and Leber, R. (2009). Identification of a cardiolipin-specific phospholipase encoded by the gene *CLD1* (YGR110W) in yeast. J. Biol. Chem. *284*, 11572–11578.

Berchtold, D., Piccolis, M., Chiaruttini, N., Riezman, I., Riezman, H., Roux, A., Walther, T.C., and Loewith, R. (2012). Plasma membrane stress induces relocalization of Slm proteins and activation of TORC2 to promote sphingolipid synthesis. Nat. Cell Biol. *14*, 542–547.

Binns, D., Januszewski, T., Chen, Y., Hill, J., Markin, V.S., Zhao, Y., Gilpin, C., Chapman, K.D., Anderson, R.G.W., and Goodman, J.M. (2006). An intimate collaboration between peroxisomes and lipid bodies. J. Cell Biol. *173*, 719–731.

Birner, R., Bürgermeister, 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.

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

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

Breslow, D.K., Collins, S.R., Bodenmiller, B., Aebersold, R., Simons, K., Shevchenko, A., Ejsing, C.S., and Weissman, J.S. (2010). Orm family proteins mediate sphingolipid homeostasis. Nature *463*, 1048–1053.

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

Carman, G.M., and Henry, S.A. (2007). Phosphatidic acid plays a central role in the transcriptional regulation of glycerophospholipid synthesis in *Saccharomyces cerevisiae*. J. Biol. Chem. *282*, 37293–37297.

Chambon, C., Ladeveze, V., Servouse, M., Blanchard, L., Javelot, C., Vladescu, B., and Karst, F. (1991). Sterol pathway in yeast. Identification and properties of mutant strains defective in mevalonate diphosphate decarboxylase and farnesyl diphosphate synthetase. Lipids *26*, 633–636.

Chang, M.C.Y., and Keasling, J.D. (2006). Production of isoprenoid pharmaceuticals by engineered microbes. Nat. Chem. Biol. *2*, 674–681.

Chang, S.C., Heacock, P.N., Clancey, C.J., and Dowhan, W. (1998a). The *PEL1* gene (renamed *PGS1*) encodes the phosphatidylglycero-phosphate synthase of *Saccharomyces cerevisiae*. J. Biol. Chem. *273*, 9829–9836.

Chang, S.C., Heacock, P.N., Mileykovskaya, E., Voelker, D.R., and Dowhan, W. (1998b). Isolation and characterization of the gene (*CLS1*) encoding cardiolipin synthase in *Saccharomyces cerevisiae*. J. Biol. Chem. *273*, 14933–14941.

Chaturvedi, D., Goswami, A., Saikia, P.P., Barua, N.C., and Rao, P.G. (2010). Artemisinin and its derivatives: a novel class of anti-malarial and anti-cancer agents. Chem. Soc. Rev. *39*, 435–454.

Chen, M., Hancock, L.C., and Lopes, J.M. (2007a). Transcriptional regulation of yeast phospholipid biosynthetic genes. Biochim. Biophys. Acta *1771*, 310–321.

Chen, Q., Kazachkov, M., Zheng, Z., and Zou, J. (2007b). The yeast acylglycerol acyltransferase *LCA1* is a key component of Lands cycle for phosphatidylcholine turnover. FEBS Lett. *581*, 5511–5516.

Chen, X., Li, Z., Zhang, X., Hu, F., Ryu, D.D.Y., and Bao, J. (2009). Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. Appl. Biochem. Biotechnol. *159*, 591–604.

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.

Chirala, S.S., Zhong, Q., Huang, W., and al-Feel, W. (1994). Analysis of *FAS3*/*ACC* regulatory region of *Saccharomyces cerevisiae*: identification of a functional UASINO and sequences responsible for fatty acid mediated repression. Nucleic Acids Res. *22*, 412–418.

Choi, H.-S., Su, W.-M., Morgan, J.M., Han, G.-S., Xu, Z., Karanasios, E., Siniossoglou, S., and Carman, G.M. (2011). Phosphorylation of phosphatidate phosphatase regulates its membrane association and physiological functions in *Saccharomyces cerevisiae*: identification of SER(602), THR(723), AND SER(744) as the sites phosphorylated by CDC28 (*CDK1*)-encoded cyclin-dependent kinase. J. Biol. Chem. *286*, 1486–1498.

Choudhary, V., and Schneiter, R. (2009). Monitoring sterol uptake, acetylation, and export in yeast. Methods Mol. Biol. *580*, 221–232.

Choudhary, V., and Schneiter, R. (2012). Pathogen-Related Yeast (PRY) proteins and members of the CAP superfamily are secreted sterol-binding proteins. Proc. Natl. Acad. Sci. U.S.A. *109*, 16882–16887.

Chung, J.-H., Lester, R.L., and Dickson, R.C. (2003). Sphingolipid requirement for generation of a functional v1 component of the vacuolar ATPase. J. Biol. Chem. *278*, 28872–28881.

Clancey, C.J., Chang, S.C., and Dowhan, W. (1993). Cloning of a gene (*PSD1*) encoding phosphatidylserine decarboxylase from *Saccharomyces cerevisiae* by complementation of an Escherichia coli mutant. J. Biol. Chem. *268*, 24580–24590.

Connerth, M., Czabany, T., Wagner, A., Zellnig, G., Leitner, E., Steyrer, E., and Daum, G. (2010). Oleate inhibits steryl ester synthesis and causes liposensitivity in yeast. J. Biol. Chem. *285*, 26832–26841.

Connerth, M., Tatsuta, T., Haag, M., Klecker, T., Westermann, B., and Langer, T. (2012). Intramitochondrial transport of phosphatidic acid in yeast by a lipid transfer protein. Science *338*, 815–818.

Cordente, A.G., Curtin, C.D., Varela, C., and Pretorius, I.S. (2012). Flavour-active wine yeasts. Appl. Microbiol. Biotechnol. *96*, 601–618.

Cowart, L.A., and Obeid, L.M. (2007). Yeast sphingolipids: recent developments in understanding biosynthesis, regulation, and function. Biochim. Biophys. Acta *1771*, 421–431.

Crowley, J.H., Leak, F.W., Jr, Shianna, K.V., Tove, S., and Parks, L.W. (1998). A mutation in a purported regulatory gene affects control of sterol uptake in *Saccharomyces cerevisiae*. J. Bacteriol. *180*, 4177–4183.

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

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. (1985). Lipids of mitochondria. Biochim. Biophys. Acta *822*, 1–42.

Daum, G., and Vance, J.E. (1997). Import of lipids into mitochondria. Prog. Lipid Res. *36*, 103–130.

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

Debelyy, M.O., Thoms, S., Connerth, M., Daum, G., and Erdmann, R. (2011). Involvement of the *Saccharomyces cerevisiae* hydrolase Ldh1p in lipid homeostasis. Eukaryotic Cell *10*, 776–781.

Dennis, E.A. (2009). Lipidomics joins the omics evolution. Proc. Natl. Acad. Sci. U.S.A. *106*, 2089–2090.

Dickson, R.C., Nagiec, E.E., Wells, G.B., Nagiec, M.M., and Lester, R.L. (1997). Synthesis of mannose-(inositol-P)2-ceramide, the major sphingolipid in *Saccharomyces cerevisiae*, requires the *IPT1* (YDR072c) gene. J. Biol. Chem. *272*, 29620–29625.

Dickson, R.C., Sumanasekera, C., and Lester, R.L. (2006). Functions and metabolism of sphingolipids in *Saccharomyces cerevisiae*. Prog. Lipid Res. *45*, 447–465.

Donova, M.V., and Egorova, O.V. (2012). Microbial steroid transformations: current state and prospects. Appl. Microbiol. Biotechnol. *94*, 1423–1447.

Dowhan, W., and Bogdanov, M. (2009). Lipid-dependent membrane protein topogenesis. Annu. Rev. Biochem. *78*, 515–540.

Dufour, J.-P., Malcorps, P., and Silcock, P. (2003) Control of ester synthesis during brewery fermentation. In Brewing Yeast Fermentation Performance. Smart, K. (ed.). Oxford, UK: Blackwell Publishing, 213–233.

Dunn, T.M., Haak, D., Monaghan, E., and Beeler, T.J. (1998). Synthesis of monohydroxylated inositolphosphorylceramide (IPC-C) in *Saccharomyces cerevisiae* requires Scs7p, a protein with both a cytochrome b5-like domain and a hydroxylase/desaturase domain. Yeast *14*, 311–321.

Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., Polyakova, S., Belov, V.N., Hein, B., von Middendorff, C., Schönle, A., et al. (2009). Direct observation of the nanoscale dynamics of membrane lipids in a living cell. Nature *457*, 1159–1162.

Einerhand, A.W., Voorn-Brouwer, T.M., Erdmann, R., Kunau, W.H., and Tabak, H.F. (1991). Regulation of transcription of the gene coding for peroxisomal 3-oxoacyl-CoA thiolase of *Saccharomyces cerevisiae*. Eur. J. Biochem. *200*, 113–122.

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.

Engels, B., Dahm, P., and Jennewein, S. (2008). Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab. Eng. *10*, 201–206.

Fahy, E., Subramaniam, S., Murphy, R.C., Nishijima, M., Raetz, C.R.H., Shimizu, T., Spener, F., van Meer, G., Wakelam, M.J.O., and Dennis, E.A. (2009). Update of the LIPID MAPS comprehensive classification system for lipids. J. Lipid Res. *50 Suppl*, S9–14.

Fakas, S., Qiu, Y., Dixon, J.L., Han, G.-S., Ruggles, K.V., Garbarino, J., Sturley, S.L., and Carman, G.M. (2011). Phosphatidate phosphatase activity plays key role in protection against fatty acid-induced toxicity in yeast. J. Biol. Chem. *286*, 29074–29085.

Farese, R.V., and Walther, T.C. (2009). Lipid Droplets Finally Get a Little R-E-S-P-E-C-T. Cell *139*, 855–860.

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

Fernández-Murray, J.P., Gaspard, G.J., Jesch, S.A., and McMaster, C.R. (2009). *NTE1*-encoded phosphatidylcholine phospholipase b regulates transcription of phospholipid biosynthetic genes. J. Biol. Chem. *284*, 36034–36046.

Fickers, P., Benetti, P.-H., Waché, Y., Marty, A., Mauersberger, S., Smit, M.S., and Nicaud, J.-M. (2005). Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. FEMS Yeast Res. *5*, 527–543.

Firestone, G.L., and Sundar, S.N. (2009). Anticancer activities of artemisinin and its bioactive derivatives. Expert Rev Mol Med *11*, e32.

Fischl, A.S., and Carman, G.M. (1983). Phosphatidylinositol biosynthesis in *Saccharomyces cerevisiae*: purification and properties of microsome-associated phosphatidylinositol synthase. J. Bacteriol. *154*, 304–311.

Fraser, P.D., and Bramley, P.M. (2004). The biosynthesis and nutritional uses of carotenoids. Prog. Lipid Res. *43*, 228–265.

Fujii, T., Nagasawa, N., Iwamatsu, A., Bogaki, T., Tamai, Y., and Hamachi, M. (1994). Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. Appl. Environ. Microbiol. *60*, 2786–2792.

Fujita, M., Umemura, M., Yoko-o, T., and Jigami, Y. (2006). *PER1* is required for GPI-phospholipase A2 activity and involved in lipid remodeling of GPI-anchored proteins. Mol. Biol. Cell *17*, 5253–5264.

Gable, K., Slife, H., Bacikova, D., Monaghan, E., and Dunn, T.M. (2000). Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. J. Biol. Chem. *275*, 7597–7603.

Gaigg, B., Timischl, B., Corbino, L., and Schneiter, R. (2005). Synthesis of sphingolipids with very long chain fatty acids but not ergosterol is required for routing of newly synthesized plasma membrane ATPase to the cell surface of yeast. J. Biol. Chem. *280*, 22515–22522.

Gaigg, B., Toulmay, A., and Schneiter, R. (2006). Very long-chain fatty acid-containing lipids rather than sphingolipids per se are required for raft association and stable surface transport of newly synthesized plasma membrane ATPase in yeast. J. Biol. Chem. *281*, 34135–34145.

García-López, M.C., Pelechano, V., Mirón-García, M.C., Garrido-Godino, A.I., García, A., Calvo, O., Werner, M., Pérez-Ortín, J.E., and Navarro, F. (2011). The conserved foot domain of RNA pol II associates

with proteins involved in transcriptional initiation and/or early elongation. Genetics *189*, 1235–1248.

Georgiev, A.G., Sullivan, D.P., Kersting, M.C., Dittman, J.S., Beh, C.T., and Menon, A.K. (2011). Osh proteins regulate membrane sterol organization but are not required for sterol movement between the ER and PM. Traffic *12*, 1341–1355.

Grilley, M.M., Stock, S.D., Dickson, R.C., Lester, R.L., and Takemoto, J.Y. (1998). Syringomycin action gene *SYR2* is essential for sphingolipid 4-hydroxylation in *Saccharomyces cerevisiae*. J. Biol. Chem. *273*, 11062–11068.

Grillitsch, K., and Daum, G. (2011). Triacylglycerol lipases of the yeast. Front.Biol. *6*, 219–230.

Grillitsch, K., Connerth, M., Köfeler, H., Arrey, T.N., Rietschel, B., Wagner, B., Karas, M., and Daum, G. (2011). Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim. Biophys. Acta *1811*, 1165–1176.

Guan, X.L., Souza, C.M., Pichler, H., Dewhurst, G., Schaad, O., Kajiwara, K., Wakabayashi, H., Ivanova, T., Castillon, G.A., Piccolis, M., et al. (2009). Functional Interactions between Sphingolipids and Sterols in Biological Membranes Regulating Cell Physiology. Molecular Biology of the Cell *20*, 2083–2095.

Haak, D., Gable, K., Beeler, T., and Dunn, T. (1997). Hydroxylation of *Saccharomyces cerevisiae* ceramides requires Sur2p and Scs7p. J. Biol. Chem. *272*, 29704–29710.

Han, G.-S., Wu, W.-I., and Carman, G.M. (2006). The *Saccharomyces cerevisiae* Lipin homolog is a Mg^{2+}-dependent phosphatidate phosphatase enzyme. J. Biol. Chem. *281*, 9210–9218.

Han, G.-S., O'Hara, L., Carman, G.M., and Siniossoglou, S. (2008). An unconventional diacylglycerol kinase that regulates phospholipid synthesis and nuclear membrane growth. J. Biol. Chem. *283*, 20433–20442.

Hearn, J.D., Lester, R.L., and Dickson, R.C. (2003). The uracil transporter Fur4p associates with lipid rafts. J. Biol. Chem. *278*, 3679–3686.

Heiderpriem, R.W., Livant, P.D., Parish, E.J., Barbuch, R.J., Broaddus, M.G., and Bard, M. (1992). A simple method for the isolation of zymosterol from a sterol mutant of *Saccharomyces cerevisiae*. J. Steroid Biochem. Mol. Biol. *43*, 741–743.

Henry, S.A., Kohlwein, S.D., and Carman, G.M. (2012). Metabolism and Regulation of Glycerolipids in the Yeast *Saccharomyces cerevisiae*. Genetics *190*, 317–349.

Herrero, O., Ramón, D., and Orejas, M. (2008). Engineering the *Saccharomyces cerevisiae* isoprenoid pathway for de novo production of aromatic monoterpenes in wine. Metab. Eng. *10*, 78–86.

Hiltunen, J.K., Wenzel, B., Beyer, A., Erdmann, R., Fosså, A., and Kunau, W.H. (1992). Peroxisomal multifunctional beta-oxidation protein of *Saccharomyces cerevisiae*. Molecular analysis of the *FOX2* gene and gene product. J. Biol. Chem. *267*, 6646–6653.

Hiltunen, J.K., Autio, K.J., Schonauer, M.S., Kursu, V.A.S., Dieckmann, C.L., and Kastaniotis, A.J. (2010). Mitochondrial fatty acid synthesis and respiration. Biochim. Biophys. Acta *1797*, 1195–1202.

Hjelmstad, R.H., and Bell, R.M. (1992). Choline- and ethanolaminephosphotransferases from *Saccharomyces cerevisiae*. Meth. Enzymol. *209*, 272–279.

Hoppins, S., Collins, S.R., Cassidy-Stone, A., Hummel, E., Devay, R.M., Lackner, L.L., Westermann, B., Schuldiner, M., Weissman, J.S., and Nunnari, J. (2011). A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. J. Cell Biol. *195*, 323–340.

Horvath, A., Sütterlin, C., Manning-Krieg, U., Movva, N.R., and Riezman, H. (1994). Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. EMBO J. *13*, 3687–3695.

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

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

Iwanyshyn, W.M., Han, G.-S., and Carman, G.M. (2004). Regulation of phospholipid synthesis in *Saccharomyces cerevisiae* by zinc. J. Biol. Chem. *279*, 21976–21983.

Jacquier, N., and Schneiter, R. (2012). Mechanisms of sterol uptake and transport in yeast. J. Steroid Biochem. Mol. Biol. *129*, 70–78.

Jacquier, N., Choudhary, V., Mari, M., Toulmay, A., Reggiori, F., and Schneiter, R. (2011). Lipid droplets are functionally connected to the endoplasmic reticulum in *Saccharomyces cerevisiae*. J. Cell. Sci *124*, 2424–2437.

Jahnke, L., and Klein, H.P. (1983). Oxygen requirements for formation and activity of the squalene epoxidase in *Saccharomyces cerevisiae*. J. Bacteriol. *155*, 488–492.

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.

Janssen, M.J., Koorengevel, M.C., de Kruijff, B., and de Kroon, A.I. (2000). The phosphatidylcholine to phosphatidylethanolamine ratio of *Saccharomyces cerevisiae* varies with the growth phase. Yeast *16*, 641–650.

Jiang, F., Rizavi, H.S., and Greenberg, M.L. (1997). Cardiolipin is not essential for the growth of *Saccharomyces cerevisiae* on fermentable or non-fermentable carbon sources. Mol. Microbiol. 26, 481–491.

De Jong, B., Siewers, V., and Nielsen, J. (2012). Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels. Curr. Opin. Biotechnol. *23*, 624–630.

Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K., and Tobe, K. (2006). Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J. Clin. Invest. *116*, 1784–1792.

Karpichev, I.V., Cornivelli, L., and Small, G.M. (2002). Multiple regulatory roles of a novel *Saccharomyces cerevisiae* protein, encoded by YOL002c, in lipid and phosphate metabolism. J. Biol. Chem. *277*, 19609–19617.

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

Klose, C., Surma, M.A., Gerl, M.J., Meyenhofer, F., Shevchenko, A., and Simons, K. (2012). Flexibility of a eukaryotic lipidome- insights from yeast lipidomics. PLoS ONE *7*, e35063.

Kobayashi, S.D., and Nagiec, M.M. (2003). Ceramide/long-chain base phosphate rheostat in *Saccharomyces cerevisiae*: regulation of ceramide synthesis by Elo3p and Cka2p. Eukaryotic Cell *2*, 284–294.

Köffel, 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. *285*, 15663–15667.

Kohlwein, S.D., Veenhuis, M., and van der Klei, I.J. (2012). Lipid Droplets and Peroxisomes: Key Players in Cellular Lipid Homeostasis or A Matter of Fat- Store 'em Up or Burn 'em Down. Genetics *193*, 1–50.

Kolaczkowski, M., Kolaczkowska, A., Gaigg, B., Schneiter, R., and Moye-Rowley, W.S. (2004). Differential regulation of ceramide synthase components *LAC1* and *LAG1* in *Saccharomyces cerevisiae*. Eukaryotic Cell *3*, 880–892.

Kopec, K.O., Alva, V., and Lupas, A.N. (2010). Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria. Bioinformatics *26*, 1927–1931.

Kornmann, B., Currie, E., Collins, S.R., Schuldiner, M., Nunnari, J., Weissman, J.S., and Walter, P. (2009). An ER-mitochondria tethering complex revealed by a synthetic biology screen. Science *325*, 477–481.

Kornmann, B., Osman, C., and Walter, P. (2011). The conserved GTPase Gem1 regulates endoplasmic reticulum-mitochondria connections. Proc. Natl. Acad. Sci. U.S.A. *108*, 14151–14156.

Kristan, K., and Rižner, T.L. (2012). Steroid-transforming enzymes in fungi. J. Steroid Biochem. Mol. Biol. *129*, 79–91.

Ktistakis, N.T. (2010). Lipid signaling and homeostasis: PA- is better than PA-H, but what about those PIPs? Sci Signal *3*, pe46.

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

Kupchak, B.R., Garitaonandia, I., Villa, N.Y., Mullen, M.B., Weaver, M.G., Regalla, L.M., Kendall, E.A., and Lyons, T.J. (2007). Probing the mechanism of *FET3* repression by Izh2p overexpression. Biochim. Biophys. Acta *1773*, 1124–1132.

Kuranda, K., François, J., and Palamarczyk, G. (2010). The isoprenoid pathway and transcriptional response to its inhibitors in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res. *10*, 14–27.

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.

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.

Kuroda, T., Tani, M., Moriguchi, A., Tokunaga, S., Higuchi, T., Kitada, S., and Kuge, O. (2011). FMP30 is required for the maintenance of a normal cardiolipin level and mitochondrial morphology in the absence of mitochondrial phosphatidylethanolamine synthesis. Mol. Microbiol. *80*, 248–265.

Lauwers, E., Grossmann, G., and André, B. (2007). Evidence for coupled biogenesis of yeast Gap1 permease and sphingolipids: essential role in transport activity and normal control by ubiquitination. Mol. Biol. Cell *18*, 3068–3080.

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.

Leber, R., Landl, K., Zinser, E., Ahorn, H., Spök, 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.

Lee, S.Y., Kim, H.U., Park, J.H., Park, J.M., and Kim, T.Y. (2009). Metabolic engineering of microorganisms: general strategies and drug production. Drug Discov. Today *14*, 78–88.

Lees, N.D., Skaggs, B., Kirsch, D.R., and Bard, M. (1995). Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*- a review. Lipids *30*, 221–226.

Linder, M.E., and Deschenes, R.J. (2004). Model organisms lead the way to protein palmitoyltransferases. J. Cell. Sci. *117*, 521–526.

Lingwood, D., and Simons, K. (2010). Lipid rafts as a membrane-organizing principle. Science *327*, 46–50.

Loewen, C.J.R., Gaspar, M.L., Jesch, S.A., Delon, C., Ktistakis, N.T., Henry, S.A., and Levine, T.P. (2004). Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. Science *304*, 1644–1647.

London, E., and Brown, D.A. (2000). Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). Biochim. Biophys. Acta *1508*, 182–195.

Lorenz, R.T., Casey, W.M., and Parks, L.W. (1989). Structural discrimination in the sparking function of sterols in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. *171*, 6169–6173.

Lyons, T.J., Villa, N.Y., Regalla, L.M., Kupchak, B.R., Vagstad, A., and Eide, D.J. (2004). Metalloregulation of yeast membrane steroid receptor homologs. Proc. Natl. Acad. Sci. U.S.A. *101*, 5506–5511.

Madhavan, A., Srivastava, A., Kondo, A., and Bisaria, V.S. (2012). Bioconversion of lignocellulose-derived sugars to ethanol by engineered *Saccharomyces cerevisiae*. Crit. Rev. Biotechnol. *32*, 22–48.

Mao, C., Xu, R., Bielawska, A., Szulc, Z.M., and Obeid, L.M. (2000a). Cloning and characterization of a *Saccharomyces cerevisiae* alkaline ceramidase with specificity for dihydroceramide. J. Biol. Chem. *275*, 31369–31378.

Mao, C., Xu, R., Bielawska, A., and Obeid, L.M. (2000b). Cloning of an alkaline ceramidase from *Saccharomyces cerevisiae*. An enzyme with reverse (CoA-independent) ceramide synthase activity. J. Biol. Chem. *275*, 6876–6884.

Matmati, N., and Hannun, Y.A. (2008). Thematic review series: sphingolipids. *ISC1* (inositol phosphosphingolipid-phospholipase C), the yeast homologue of neutral sphingomyelinases. J. Lipid Res. *49*, 922–928.

Meisinger, C., Pfannschmidt, S., Rissler, M., Milenkovic, D., Becker, T., Stojanovski, D., Youngman, M.J., Jensen, R.E., Chacinska, A., Guiard, B., et al. (2007). The morphology proteins Mdm12/Mmm1 function in the major beta-barrel assembly pathway of mitochondria. EMBO J. *26*, 2229–2239.

Merkel, O., Schmid, P.C., Paltauf, F., and Schmid, H.H.O. (2005). Presence and potential signaling function of N-acylethanolamines and their phospholipid precursors in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta *1734*, 215–219.

Mesmin, B., and Maxfield, F.R. (2009). Intracellular sterol dynamics. Biochimica et Biophysica Acta (BBA) *1791*, 636–645.

Mignery, G.A., Pikaard, C.S., and Park, W.D. (1988). Molecular characterization of the patatin multigene family of potato. Gene *62*, 27–44.

Misawa, N. (2011). Pathway engineering for functional isoprenoids. Curr. Opin. Biotechnol. *22*, 627–633.

Moir, R.D., Gross, D.A., Silver, D.L., and Willis, I.M. (2012). *SCS3* and *YFT2* link transcription of phospholipid biosynthetic genes to ER stress and the UPR. PLoS Genet. *8*, e1002890.

Mora, G., Scharnewski, M., and Fulda, M. (2012). Neutral Lipid Metabolism Influences Phospholipid Synthesis and Deacylation in *Saccharomyces cerevisiae*. PLoS ONE *7*, e49269.

Müllner, H., Zweytick, D., Leber, R., Turnowsky, F., and Daum, G. (2004). Targeting of proteins involved in sterol biosynthesis to lipid particles of the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta *1663*, 9–13.

Müllner, H., Deutsch, G., Leitner, E., Ingolic, E., and Daum, G. (2005). *YEH2*/YLR020c encodes a novel steryl ester hydrolase of the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. *280*, 13321–13328.

Murphy, D.J., and Vance, J. (1999). Mechanisms of lipid-body formation. Trends Biochem. Sci. *24*, 109–115.

Nadolski, M.J., and Linder, M.E. (2007). Protein lipidation. FEBS J. *274*, 5202–5210.

Nagasawa, N., Bogaki, T., Iwamatsu, A., Hamachi, M., and Kumagai, C. (1998). Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (*ATF2*) from *Saccharomyces cerevisiae* Kyokai No. 7. Biosci. Biotechnol. Biochem. *62*, 1852–1857.

Nagiec, M.M., Baltisberger, J.A., Wells, G.B., Lester, R.L., and Dickson, R.C. (1994). The *LCB2* gene of *Saccharomyces* and the related *LCB1* gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis. Proc. Natl. Acad. Sci. U.S.A. *91*, 7899–7902.

Nagiec, M.M., Nagiec, E.E., Baltisberger, J.A., Wells, G.B., Lester, R.L., and Dickson, R.C. (1997). Sphingolipid synthesis as a target for antifungal drugs. Complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the *AUR1* gene. J. Biol. Chem. *272*, 9809–9817.

Nagiec, M.M., Skrzypek, M., Nagiec, E.E., Lester, R.L., and Dickson, R.C. (1998). The *LCB4* (YOR171c) and *LCB5* (YLR260w) genes of *Saccharomyces* encode sphingoid long chain base kinases. J. Biol. Chem. *273*, 19437–19442.

Natter, K., and Kohlwein, S.D. (2012). Yeast and cancer cells - common principles in lipid metabolism. Biochim. Biophys. Acta. *1831 (2)*, 314-326

Natter, K., Leitner, P., Faschinger, A., Wolinski, H., McCraith, S., Fields, S., and Kohlwein, S.D. (2005). The spatial organization of lipid synthesis in the yeast *Saccharomyces cerevisiae* derived from large scale green fluorescent protein tagging and high resolution microscopy. Mol. Cell Proteomics *4*, 662–672.

Nes, W.D., Janssen, G.G., Crumley, F.G., Kalinowska, M., and Akihisa, T. (1993). The structural requirements of sterols for membrane function in *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. *300*, 724–733.

Nguyen, T.T., Lewandowska, A., Choi, J.-Y., Markgraf, D.F., Junker, M., Bilgin, M., Ejsing, C.S., Voelker, D.R., Rapoport, T.A., and Shaw, J.M. (2012). Gem1 and ERMES do not directly affect phosphatidylserine transport from ER to mitochondria or mitochondrial inheritance. Traffic *13*, 880–890.

Nikawa, J., Tsukagoshi, Y., and Yamashita, S. (1991). Isolation and characterization of two distinct myo-inositol transporter genes of *Saccharomyces cerevisiae*. J. Biol. Chem. *266*, 11184–11191.

Niles, B.J., and Powers, T. (2012). Plasma membrane proteins Slm1 and Slm2 mediate activation of the AGC kinase Ypk1 by TORC2 and sphingolipids in *S. cerevisiae*. Cell Cycle *11*, 3745–3749.

O'Hara, L., Han, G.-S., Peak-Chew, S., Grimsey, N., Carman, G.M., and Siniossoglou, S. (2006). Control of phospholipid synthesis by phosphorylation of the yeast lipin Pah1p/Smp2p Mg2+-dependent phosphatidate phosphatase. J. Biol. Chem. *281*, 34537–34548.

Opekarová, M., and Tanner, W. (2003). Specific lipid requirements of membrane proteins- a putative bottleneck in heterologous expression. Biochim. Biophys. Acta *1610*, 11–22.

Osman, C., Haag, M., Wieland, F.T., Brügger, B., and Langer, T. (2010). A mitochondrial phosphatase required for cardiolipin biosynthesis: the PGP phosphatase Gep4. EMBO J. *29*, 1976–1987.

Ostergaard, S., Olsson, L., and Nielsen, J. (2000). Metabolic engineering of *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. *64*, 34–50.

Panwar, S.L., and Moye-Rowley, W.S. (2006). Long chain base tolerance in *Saccharomyces cerevisiae* is induced by retrograde signals from the mitochondria. J. Biol. Chem. *281*, 6376–6384.

Pascual, F., and Carman, G.M. (2013). Phosphatidate phosphatase, a key regulator of lipid homeostasis. Biochim. Biophys. Acta. *1831 (3)*, 514-522.

Pichler, H. (2005). Modulating membrane properties-sterol cell biology in yeast. In Cell Biology and Dynamics of Yeast Lipids, Research Signpost, Trivandum, India, G. Daum, ed., 179–199.

Ploegh, H.L. (2007). A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum. Nature *448*, 435–438.

Ploier, B., Scharwey, M., Koch, B., Schmidt, C., Schatte, J., Rechberger, G., Kollroser, M., Hermetter, A., and Daum, G. (2013). Screening for hydrolytic enzymes reveals Ayr1p as a novel triacylglycerol lipase in *Saccharomyces cerevisiae*. J. Biol. Chem. *288*, 36061–36072.

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.

Polakowski, T., Stahl, U., and Lang, C. (1998). Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl. Microbiol. Biotechnol. *49*, 66–71.

Potting, C., Wilmes, C., Engmann, T., Osman, C., and Langer, T. (2010). Regulation of mitochondrial phospholipids by Ups1/PRELI-like proteins depends on proteolysis and Mdm35. EMBO J. *29*, 2888–2898.

Pu, J., Ha, C.W., Zhang, S., Jung, J.P., Huh, W.-K., and Liu, P. (2011). Interactomic study on interaction between lipid droplets and mitochondria. Protein Cell *2*, 487–496.

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. *285*, 15769–15776.

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

Rajakumari, S., Rajasekharan, R., and Daum, G. (2010). Triacylglycerol lipolysis is linked to sphingolipid and phospholipid metabolism of the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta *1801*, 1314–1322.

Raychaudhuri, S., Young, B.P., Espenshade, P.J., and Loewen, C., Jr (2012). Regulation of lipid metabolism: a tale of two yeasts. Curr. Opin. Cell Biol. *24*, 502–508.

Rebecchi, M.J., and Pentyala, S.N. (2000). Structure, function, and control of phosphoinositide-specific phospholipase C. Physiol. Rev. *80*, 1291–1335.

Reue, K., and Brindley, D.N. (2008). Thematic Review Series: Glycerolipids. Multiple roles for lipins/phosphatidate phosphatase enzymes in lipid metabolism. J. Lipid Res. *49*, 2493–2503.

Reue, K., and Dwyer, J.R. (2009). Lipin proteins and metabolic homeostasis. J. Lipid Res. *50 Suppl*, S109–114.

Riad, M., Mogos, M., Thangathurai, D., and Lumb, P.D. (2002). Steroids. Curr Opin Crit Care *8*, 281–284.

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

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.

Roelants, F.M., Breslow, D.K., Muir, A., Weissman, J.S., and Thorner, J. (2011). Protein kinase Ypk1 phosphorylates regulatory proteins Orm1 and Orm2 to control sphingolipid homeostasis in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. *108*, 19222–19227.

Rudge, S.A., Morris, A.J., and Engebrecht, J. (1998). Relocalization of phospholipase D activity mediates membrane formation during meiosis. J. Cell Biol. *140*, 81–90.

Ruenwai, R., Neiss, A., Laoteng, K., Vongsangnak, W., Dalfard, A.B., Cheevadhanarak, S., Petranovic, D., and Nielsen, J. (2011). Heterologous production of polyunsaturated fatty acids in *Saccharomyces cerevisiae* causes a global transcriptional response resulting in reduced proteasomal activity and increased oxidative stress. Biotechnol. J. *6*, 343–356.

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.

Saerens, S.M.G., Verstrepen, K.J., Van Laere, S.D.M., Voet, A.R.D., Van Dijck, 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.

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J., and Thevelein, J.M. (2010). Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. *3*, 165–177.

Sajbidor, J., Ciesarová, Z., and Smogrovicová, D. (1995). Influence of ethanol on the lipid content and fatty acid composition of *Saccharomyces cerevisiae*. Folia Microbiol (Praha) *40*, 508–510.

Santos, A.X.S., and Riezman, H. (2012). Yeast as a model system for studying lipid homeostasis and function. FEBS Lett. *586*, 2858–2867.

Sasser, T., Qiu, Q.-S., Karunakaran, S., Padolina, M., Reyes, A., Flood, B., Smith, S., Gonzales, C., and Fratti, R.A. (2012). Yeast lipin 1 orthologue Pah1p regulates vacuole homeostasis and membrane fusion. J. Biol. Chem. *287*, 2221–2236.

Sawai, H., Okamoto, Y., Luberto, C., Mao, C., Bielawska, A., Domae, N., and Hannun, Y.A. (2000). Identification of *ISC1* (YER019w) as inositol phosphosphingolipid phospholipase C in *Saccharomyces cerevisiae*. J. Biol. Chem. *275*, 39793–39798.

Schneiter, R., Brügger, B., Sandhoff, R., Zellnig, G., Leber, A., Lampl, M., Athenstaedt, K., Hrastnik, C., Eder, S., Daum, G., 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.

Schorling, S., Vallée, B., Barz, W.P., Riezman, H., and Oesterhelt, D. (2001). Lag1p and Lac1p are essential for the Acyl-CoA-dependent ceramide synthase reaction in *Saccharomyces cerevisae*. Mol. Biol. Cell *12*, 3417–3427.

Schrag, J.D., and Cygler, M. (1997). Lipases and alpha/beta hydrolase fold. Meth. Enzymol. *284*, 85–107.

Schulz, T.A., and Prinz, W.A. (2007). Sterol transport in yeast and the oxysterol binding protein homologue (OSH) family. Biochimica et Biophysica Acta (BBA) *1771*, 769–780.

Shang, F., Wen, S., Wang, X., and Tan, T. (2006). Effect of nitrogen limitation on the ergosterol production by fed-batch culture of *Saccharomyces cerevisiae*. J. Biotechnol. *122*, 285–292.

Shen, H., Heacock, P.N., Clancey, C.J., and Dowhan, W. (1996). The *CDS1* gene encoding CDP-diacylglycerol synthase in *Saccharomyces cerevisiae* is essential for cell growth. J. Biol. Chem. *271*, 789–795.

Simocková, M., Holic, R., Tahotná, D., Patton-Vogt, J., and Griac, P. (2008). Yeast Pgc1p (YPL206c) controls the amount of phosphatidylglycerol via a phospholipase C-type degradation mechanism. J. Biol. Chem. *283*, 17107–17115.

Simons, K., and Sampaio, J.L. (2011). Membrane organization and lipid rafts. Cold Spring Harb. Perspec.t Biol. *3*, a004697.

Siniossoglou, S., Santos-Rosa, H., Rappsilber, J., Mann, M., and Hurt, E. (1998). A novel complex of membrane proteins required for formation of a spherical nucleus. EMBO J. *17*, 6449–6464.

Skrzypek, M., Lester, R.L., and Dickson, R.C. (1997). Suppressor gene analysis reveals an essential role for sphingolipids in transport of glycosylphosphatidylinositol-anchored proteins in *Saccharomyces cerevisiae*. J. Bacteriol. *179*, 1513–1520.

Souza, C.M., and Pichler, H. (2007). Lipid requirements for endocytosis in yeast. Biochim. Biophys. Acta *1771*, 442–454.

Spanova, M., and Daum, G. (2011). Squalene- biochemistry, molecular biology, process biotechnology, and applications. European Journal of Lipid Science and Technology *113*, 1299–1320.

Spanova, M., Czabany, T., Zellnig, G., Leitner, E., Hapala, I., and Daum, G. (2010). Effect of lipid particle biogenesis on the subcellular distribution of squalene in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. *285*, 6127–6133.

Spanova, M., Zweytick, D., Lohner, K., Klug, L., Leitner, E., Hermetter, A., and Daum, G. (2012). Influence of squalene on lipid particle/droplet and membrane organization in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta *1821*, 647–653.

Sreenivas, A., Patton-Vogt, J.L., Bruno, V., Griac, P., and Henry, S.A. (1998). A role for phospholipase D (Pld1p) in growth, secretion, and regulation of membrane lipid synthesis in yeast. J. Biol. Chem. *273*, 16635–16638.

Strahl, T., and Thorner, J. (2007). Synthesis and function of membrane phosphoinositides in budding yeast, *Saccharomyces cerevisiae*. Biochim. Biophys. Acta *1771*, 353–404.

Stroud, D.A., Oeljeklaus, S., Wiese, S., Bohnert, M., Lewandrowski, U., Sickmann, A., Guiard, B., van der Laan, M., Warscheid, B., and Wiedemann, N. (2011). Composition and topology of the endoplasmic reticulum-mitochondria encounter structure. J. Mol. Biol. *413*, 743–750.

Stukey, J.E., McDonough, V.M., and Martin, C.E. (1990). The *OLE1* gene of *Saccharomyces cerevisiae* encodes the delta 9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. J. Biol. Chem. *265*, 20144–20149.

Sumby KM, Grbin PR and Jiranek V (2010) Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. Food chemistry. 121, 1-16

Sun, Y., Taniguchi, R., Tanoue, D., Yamaji, T., Takematsu, H., Mori, K., Fujita, T., Kawasaki, T., and Kozutsumi, Y. (2000). Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. Mol. Cell. Biol. *20*, 4411–4419.

Tamura, Y., Iijima, M., and Sesaki, H. (2010). Mdm35p imports Ups proteins into the mitochondrial intermembrane space by functional complex formation. EMBO J. *29*, 2875–2887.

Tamura, Y., Onguka, O., Hobbs, A.E.A., Jensen, R.E., Iijima, M., Claypool, S.M., and Sesaki, H. (2012). Role for two conserved intermembrane space proteins, Ups1p and Ups2p, in intra-mitochondrial phospholipid trafficking. J. Biol. Chem. *287*, 15205–15218.

Tanaka, S., Maeda, Y., Tashima, Y., and Kinoshita, T. (2004). Inositol deacylation of glycosylphosphatidylinositol-anchored proteins is mediated by mammalian PGAP1 and yeast Bst1p. J. Biol. Chem. *279*, 14256–14263.

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

Tevzadze, G.G., Pierce, J.V., and Esposito, R.E. (2007). Genetic evidence for a *SPO1*-dependent signaling pathway controlling meiotic progression in yeast. Genetics *175*, 1213–1227.

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.

Thoms, S., Debelyy, M.O., Connerth, M., Daum, G., and Erdmann, R. (2011). The putative *Saccharomyces cerevisiae* hydrolase Ldh1p is localized to lipid droplets. Eukaryotic Cell *10*, 770–775.

Tinkelenberg, A.H., Liu, Y., Alcantara, F., Khan, S., Guo, Z., Bard, M., and Sturley, S.L. (2000). Mutations in yeast ARV1 alter intracellular sterol distribution and are complemented by human *ARV1*. J. Biol. Chem. *275*, 40667–40670.

Tiwari, R., Köffel, R., and Schneiter, R. (2007). An acetylation/deacetylation cycle controls the export of sterols and steroids from *S. cerevisiae*. EMBO J. *26*, 5109–5119.

Toth, M.J., and Huwyler, L. (1996). Molecular cloning and expression of the cDNAs encoding human and yeast mevalonate pyrophosphate decarboxylase. J. Biol. Chem. *271*, 7895–7898.

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.

Tuller, G., Hrastnik, C., Achleitner, G., Schiefthaler, U., Klein, F., and Daum, G. (1998). YDL142c encodes cardiolipin synthase (Cls1p) and is non-essential for aerobic growth of *Saccharomyces cerevisiae*. FEBS Lett. *421*, 15–18.

Uemura, H. (2012). Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives. Appl. Microbiol. Biotechnol. *95*, 1–12.

Uemura, S., Kihara, A., Inokuchi, J.-I., and Igarashi, Y. (2003). Csg1p and newly identified Csh1p function in mannosylinositol phosphorylceramide synthesis by interacting with Csg2p. J. Biol. Chem. *278*, 45049–45055.

Vallée, B., and Riezman, H. (2005). Lip1p: a novel subunit of acyl-CoA ceramide synthase. EMBO J. *24*, 730–741.

Van der Laan, M., Bohnert, M., Wiedemann, N., and Pfanner, N. (2012). Role of MINOS in mitochondrial membrane architecture and biogenesis. Trends Cell Biol. *22*, 185–192.

Van Meer, G., Voelker, D.R., and Feigenson, G.W. (2008). Membrane lipids: where they are and how they behave. Nat. Rev. Mol. Cell Biol. *9*, 112–124.

Van Roermund, C.W., Elgersma, Y., Singh, N., Wanders, R.J., and Tabak, H.F. (1995). The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under *in vivo* conditions. EMBO J. *14*, 3480–3486.

Van Roermund, C.W., Drissen, R., van Den Berg, M., Ijlst, L., Hettema, E.H., Tabak, H.F., Waterham, H.R., and Wanders, R.J. (2001). Identification of a peroxisomal ATP carrier required for medium-chain fatty acid beta-oxidation and normal peroxisome proliferation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *21*, 4321–4329.

Van Roermund, C.W.T., Ijlst, L., Majczak, W., Waterham, H.R., Folkerts, H., Wanders, R.J.A., and Hellingwerf, K.J. (2012). Peroxisomal fatty acid uptake mechanism in *Saccharomyces cerevisiae*. J. Biol. Chem. *287*, 20144–20153.

Veen, M., and Lang, C. (2004). Production of lipid compounds in the yeast *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. *63*, 635–646.

Veen, M., Stahl, U., and Lang, C. (2003). Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*. FEMS Yeast Res. *4*, 87–95.

Vehring, S., and Pomorski, T. (2005). Dynamics and regulation of phospholipid distribution in the yeast. In Cell Biology and Dynamics of Yeast Lipids, Research Signpost, Trivandum, India, G. Daum, ed., 85–104.

Verger, R. (1997). “Interfacial activation” of lipases: facts and artifacts. Trends in Biotechnology *15*, 32–38.

Verstrepen, K.J., Van Laere, S.D.M., Vercammen, J., Derdelinckx, G., Dufour, J.-P., Pretorius, I.S., Winderickx, J., Thevelein, J.M., and Delvaux, F.R. (2004). The *Saccharomyces cerevisiae* alcohol acetyl transferase Atf1p is localized in lipid particles. Yeast *21*, 367–377.

Verwaal, R., Wang, J., Meijnen, J.-P., Visser, H., Sandmann, G., van den Berg, J.A., and van Ooyen, A.J.J. (2007). High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from Xanthophyllomyces dendrorhous. Appl. Environ. Microbiol. *73*, 4342–4350.

Voelker, D.R. (2003). New perspectives on the regulation of intermembrane glycerophospholipid traffic. J. Lipid Res. *44*, 441–449.

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.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., and McPhail, A.T. (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J. Am. Chem. Soc. *93*, 2325–2327.

Wilcox, L.J., Balderes, D.A., Wharton, B., Tinkelenberg, A.H., Rao, G., and Sturley, S.L. (2002). Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. J. Biol. Chem. *277*, 32466–32472.

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

Wolinski, H., Kolb, D., Hermann, S., Koning, R.I., and Kohlwein, S.D. (2011). A role for seipin in lipid droplet dynamics and inheritance in yeast. J. Cell. Sci. *124*, 3894–3904.

Wriessnegger, T., and Pichler, H. (2013). Yeast metabolic engineering - Targeting sterol metabolism and terpenoid formation. Prog. Lipid Res. *52*, 277–293.

Wu, W.I., and Carman, G.M. (1994). Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by nucleotides. J. Biol. Chem. *269*, 29495–29501.

Wu, W.I., and Carman, G.M. (1996). Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by phospholipids. Biochemistry *35*, 3790–3796.

Wu, W.I., Lin, Y.P., Wang, E., Merrill, A.H., Jr, and Carman, G.M. (1993). Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by sphingoid bases. J. Biol. Chem. *268*, 13830–13837.

Yamano, S., Ishii, T., Nakagawa, M., Ikenaga, H., and Misawa, N. (1994). Metabolic engineering for production of beta-carotene and lycopene in *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. *58*, 1112–1114.

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.

Yazawa, H., Iwahashi, H., Kamisaka, Y., Kimura, K., Aki, T., Ono, K., and Uemura, H. (2007). Heterologous production of dihomo-gamma-linolenic acid in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. *73*, 6965–6971.

York, J.D. (2006). Regulation of nuclear processes by inositol polyphosphates. Biochim. Biophys. Acta *1761*, 552–559.

Yu, C., Kennedy, N.J., Chang, C.C., and Rothblatt, J.A. (1996). Molecular cloning and characterization of two isoforms of *Saccharomyces cerevisiae* acyl-CoA:sterol acyltransferase. J. Biol. Chem. *271*, 24157–24163.

Yu, X., Zheng, Y., Dorgan, K.M., and Chen, S. (2011). Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresour. Technol. *102*, 6134–6140.

Zanghellini, J., Natter, K., Jungreuthmayer, C., Thalhammer, A., Kurat, C.F., Gogg-Fassolter, G., Kohlwein, S.D., and von Grünberg, H.-H. (2008). Quantitative modeling of triacylglycerol homeostasis in yeast--metabolic requirement for lipolysis to promote membrane lipid synthesis and cellular growth. FEBS J. *275*, 5552–5563.

Zerbes, R.M., Bohnert, M., Stroud, D.A., von der Malsburg, K., Kram, A., Oeljeklaus, S., Warscheid, B., Becker, T., Wiedemann, N., Veenhuis, M., et al. (2012). Role of MINOS in mitochondrial membrane architecture: cristae morphology and outer membrane interactions differentially depend on mitofilin domains. J. Mol. Biol. *422*, 183–191.

Zhao, X., Hu, C., Wu, S., Shen, H., and Zhao, Z.K. (2011). Lipid production by Rhodosporidium toruloides Y4 using different substrate feeding strategies. J. Ind. Microbiol. Biotechnol. *38*, 627–632.

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.

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*, 2026–2034.

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.

3

ANALYSIS OF YEAST LIPID DROPLET PROTEOME AND LIPIDOME

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Published in: *Methods in Cell Biology* (2013), 116C: 15-37

KEY WORDS

Lipid droplets/particles, yeast, non-polar lipids, phospholipids, fatty acids, mass spectrometry, thin layer chromatography, gas liquid chromatography

ABBREVIATIONS

ER, endoplasmic reticulum; FA, fatty acids; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; LD, lipid droplets; MS, mass spectrometry; nLC, nano-liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; RT, room temperature; SE, steryl esters; TG, triacylglycerols; TLC, thin layer chromatography.

ABSTRACT

Lipid droplets (LD) are in the spotlight of lipid research because of the link of lipid storage to health and disease and the just incipient understanding of their involvement in cellular processes apart from non-polar lipid metabolism. Yeast is an excellent model organism to study the lipidome and proteome of LD under different environmental conditions and to address new aspects of LD biology and chemistry. In this article, we describe a versatile protocol for the isolation of LD at high purity and address specific demands for handling different yeast species. Moreover, we discuss the analysis of the LD proteome and lipidome based on standard methods such as thin layer chromatography (TLC), gas liquid chromatography (GLC), mass spectrometry (MS) as well as GLC/MS. Finally, we point out similarities and disparities of LD proteome and lipidome from the three different yeasts *Saccharomyces cerevisiae, Yarrowia lipolytica* and *Pichia pastoris.*

INTRODUCTION

Yeast is a well-established model organism to study the synthesis and turnover of non-polar lipids, which are inevitably linked to a very specific cellular compartment named lipid droplets (LD) (Athenstaedt and Daum, 2006; Zweytick et al., 2000). They are small spherical organelles with an approximate diameter of 400 nm in *S. cerevisiae*. Increasing interest in LD biochemistry and biology is due to their universal occurrence in almost all kingdoms of life, but also to the involvement of lipid storage to health and disease. Moreover, our understanding of LD participation in cellular processes apart from non-polar lipid turnover is steadily increasing but nevertheless just at its infancy.

A recent review compares the state of the art of LD research ranging from archaea to mammals with emphasis on the yeast *S. cerevisiae* as an appropriate model system (Murphy, 2012). Importantly, a number of parallels between LD from yeast and mammalian cells have been discovered supporting this view. Examples for such parallels are the occurrence of lipid metabolizing enzymes in both types of LD, such as the lipases ATGL and Tgl3p, Tgl4p and Tgl5p, respectively (Athenstaedt and Daum, 2003, 2005; Zimmermann et al., 2004), enzymes of sterol biosynthesis (Leber et al., 1998; Milla et al., 2002; Zinser et al., 1993), or more specifically seipin in mammalian cells and Fld1p in yeast (Fei et al., 2008, 2011; Wolinski et al., 2011). Also in plant LD certain enzymes of lipid metabolism were detected (Baud and Lepiniec, 2010; Murphy, 2001). The major advantage of the yeast, however, to perform studies with LD or other organelles, is the ease of manipulation either by culture conditions or by genetic means.

The main storage lipids of the yeast are triacylglycerols (TG) and steryl esters (SE). These biologically inert forms of free fatty acids (FA) and sterols are often referred to as non-polar or neutral lipids as they lack charged groups. They mainly function as a reservoir of energy and building blocks for membrane components, but at the same time they provide an internal cell protective mechanism against possible toxic effects caused by an excess of free FA and sterols. LD consist of a highly hydrophobic core of mainly TG, which is surrounded by shells of SE and covered by a phospholipid monolayer (Czabany et al., 2008) with specific proteins embedded in the surface membrane of LD (Athenstaedt et al., 1999; Czabany et al.,

2007; Leber et al., 1994). Although LD appear to be important for yeast cells under normal growth conditions their existence is not essential (Sandager et al., 2002).

The biogenesis of LD is still a matter of discussion (Kohlwein et al., 2013). However, all biogenesis models have in common that LD are most likely formed *de novo* from the endoplasmic reticulum (ER) (Walther and Farese, 2012). The currently most accepted model of LD biogenesis proposes formation at specific membrane microdomains in the ER where non-polar lipids accumulate until the size of the LD reaches a critical dimension to bud off forming an independent organelle-like structure (Murphy and Vance, 1999; Ploegh, 2007; Wältermann et al., 2005; Zweytick et al., 2000). It has to be noted that LD do not only serve as lipid storage pool but also fulfill many other functions in lipid metabolism (Zinser et al., 1993). As an example, (Connerth et al., 2010) discussed an indirect role of LD in maintaining ideal membrane fluidity under environmental stress caused by exogenous FA. Besides the undisputed influence of LD on lipid homeostasis, functions which are unrelated to lipid turnover have emerged such as storage and degradation of protein aggregates and incorrectly folded proteins (Fei et al., 2009; Fujimoto et al., 2008). Recent research on the interaction of LD with other organelles, i.e. the ER (Fei et al., 2009; Jacquier et al., 2011; Wolinski et al., 2011), peroxisomes (Binns et al., 2006; Kohlwein et al., 2013) or mitochondria (Pu et al., 2011), as well as the identification of novel factors influencing biogenesis and dynamics of LD (Adeyo et al., 2011) accentuate LD as a central topic in cellular biology.

Although occurrence and structure of LD are similar in all eukaryotes, there are some differences of the lipid composition and the set of proteins, even in different yeast species and in strains grown on different carbon sources. Mass spectrometric analysis of lipids and proteins of LD from *S. cerevisiae* cultivated on glucose and oleate, respectively, revealed that LD proteome and lipidome can adapt to environmental changes (Grillitsch et al., 2011). When cultivated on oleate, peroxisomes proliferate which are the only organelle of the yeast where β-oxidation of FA occurs. Under these growth conditions, accumulation of non-polar lipids was observed accompanied by an altered ratio of TG to SE. Oleate stimulates the formation of TG at the expense of SE in *S. cerevisiae* which is in sharp contrast to *Y. lipolytica* (Connerth et al., 2010; Rosenberger et al., 2009). This effect is only one example of differences observed with LD from the yeasts *S. cerevisiae, P. pastoris* and *Y. lipolytica*. LD of the oleaginous yeast *Y. lipolytica* vary in size from 650 nm to 2,500 nm depending on

cultivation conditions and are markedly larger than LD from *S. cerevisiae* (Athenstaedt et al., 2006). It was also shown that not only size and abundance of LD from *Y. lipolytica* depend on the carbon source but also the lipid composition and the proteome. Further examples of such effects will be described in the Results section.

To obtain highly pure LD from *S. cerevisiae, P. pastoris* and *Y. lipolytica*, isolation protocols were established as will be described in the Methods and Notes section. Besides the protocol for the isolation of LD at high purity we will present quality control by Western Blot analysis adapted to different requirements of the different yeasts. Furthermore, we will discuss the analysis of proteins and lipids from LD based on thin layer chromatography (TLC), gas liquid chromatography (GLC), mass spectrometry (MS) as well as GLC/MS. Finally, we will briefly compare LD proteome and lipidome from the three different yeasts *S. cerevisiae, Y. lipolytica* and *P. pastoris* (Athenstaedt et al., 2006; Grillitsch et al., 2011; Ivashov et al., 2012).

MATERIALS

Equipment and Supplies

Incubator (Heraeus, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA)

Table-top centrifuge (HettichRotina 46R, Heraeus Fresco 17)

Sorvall RC6 plus centrifuge (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA)

Sorvall Untracentrifuge Combi (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA)

*Fiber*Lite® F10-6x500y rotor (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA)

Sorvall AH-629 rotor (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA)

Ultra-Clear Centrifuge Tube (Beckman Coulter Inc, Brea California, USA)

Dounce Tissue grinder set (working volume 40 mL, 15 mL and 7 mL)

nLC: ProxeonBiosystems EASY-nLC™ (Odense, Denmark) coupled to SunCollect MALDI spotting device (Sunchrom, Friedrichsdorf, Germany)

Columns (Waters X-Bridge™ BEH 180 C18 300 Å 3.5)

123x81 mm OptiTOF™ LC/MALDI Insert metal target

4800 TOF/TOF™ Analyzer (ABSciex, Darmstadt, Germany) equipped with Nd:YAG laser

Microsyringe (Hamilton)

Sample applicator (CAMAG Automatic TLC sampler III, Muttenz, Switzerland)

12 mL Pyrex glass tubes with caps

Glass tubes with ground neck

Silica gel 60 TLC plates (Merck, Darmstadt, Germany)

TLC chamber (CAMAG, Muttenz, Switzerland)

TLC Scanner (CAMAG TLC Scanner 3, Muttenz, Switzerland)

GLC-MS (Hewlett-Packard 5890 Gas-Chromatograph, Palo Alto, California, USA)

FT-ICR-MS hybrid mass spectrometer (LTQ-FT, Thermo Scientific) equipped with an IonMax ESI source

Media and Reagents

YPD (2% glucose, 2% peptone, 1% yeast extract)

SD (2% glucose, 0.67% yeast nitrogen base, amino acid mixture)

YPO (0.1% yeast extract, 0.5% peptone, 0.5% KH_2PO_4, 0.1% glucose, 0.2% Tween80, 0.1% oleic acid)

Zymolyase-20 T (Seikaguku Corporation, Japan)

Ficoll™ PM400 (GE Healthcare, Buckinhamshire, England)

SP-A: 0.1 M Tris/SO_4 [pH 9.4]

SP-B: 1.2 M sorbitol, 20 mM KH_2PO_4 [pH 7.4]

LD-A: 12% Ficoll™ PM400 in 10 mM MES/Tris [pH 6.9], 0.2 mM $Na_2EDTA.2H_2O$

LD-B: 8% Ficoll™ PM400 in 10 mM MES/Tris [pH 6.9], 0.2 mM $Na_2EDTA.2H_2O$

LD-C: 0.25 M sorbitol in 10 mM MES/Tris [pH 6.9], 0.2 mM $Na_2EDTA.2H_2O$

Phenylmethanesulfonylfluoride (PMSF): 1 M in DMSO'

Chemiluminescence solution: SuperSignal™ (Pierce Chemical Company, Rockford, IL, USA)

Trichloroacetic acid (TCA)

Sodiumdodecylsulfate (SDS)

Rabbit antibodies against Erg6p, Wbp1p, Cyb2p, GAPDH from *S. cerevisiae*

Peroxidase conjugated secondary antibody

Ammonium carbonate (NH_4CO_3)

Dithiothreitol (DTT)

Iodoacetamide

Trypsin

Trifluoroacetic acid (TFA)

Alpha-cyano-4-hydroxycinnamic acid

[Glu^1]- Fibrinopeptide B

Solvents: acetic acid, acetone, acetonitrile, chloroform, diethyl ether, formic acid, light petroleum, methanol

Washing solutions for lipid extracts: 0.034% $MgCl_2$; 2 N KCl/MeOH (4:1; v/v); artificial upper phase (chloroform/methanol/water, 3:48:47; per vol.)

Charring solution: 0.63 g $MnCl_2.4H_2O$, 60mL water, 60 mL methanol, 4 mL conc. H_2SO_4

ANSA solution: 40 g $K_2S_2O_5$, 0.63 g of 8-anilio-1-naphthalenesulfonic acid and 1.25 g of Na_2SO_3 in 250 mL of water

Databases

MASCOT Database (http://www.matrixscience.com)

Saccharomyces Genome Database (http://www.yeastgenome.org)

Swissprot Protein Database (http://www.uniprot.org/)

METHODS

Isolation of lipid droplets from yeast

LD from *S. cerevisiae* are isolated from 4-5 L of full or selective media. Cultures are inoculated from a pre-culture to an OD_{600} of 0.1 and cells are grown to the stationary phase at 30°C with shaking. Yeast cells are harvested by centrifugation at 5,000 rpm for 5 min at room temperature (RT) and washed with distilled water (see Note 1). After determining the cell wet weight cells are incubated with 0.5 g/mL SP-A (0.1 M Tris/SO_4 [pH 9.4]) and 1.54 mg DTT/mL SP-A for 10 min at 30°C with shaking. Then, cells are washed once in pre-warmed SP-B (1.2 M sorbitol, 20 mM KH_2PO_4 [pH 7.4]) and spheroplasts are generated by enzymatic digestion of the cell wall using Zymolyase-20 T (Seikaguku Corporation) at a concentration of 2 mg/g cell wet weight in 6 mL SP-B/g cell wet weight. The incubation takes 30 min to 1 h at 30°C with shaking. The resulting spheroplasts are washed twice with cold SP-B. From now on, cells must be kept on ice and all solutions must be pre-cooled. Spheroplasts are resuspended in 1 mL/g cell wet weight LD-A (12% Ficoll 400 in 10 mM MES/Tris [pH 6.9], 0.2

mM $Na_2EDTA.2H_2O$) and 1 mM PMSF followed by mechanical disruption with 30 strokes using a Dounce homogenizer with a loose fitting pestle. The resulting homogenate is diluted with a half volume of LD-A and centrifuged at 7,000 rpm for 5 min at 4°C. The supernatant is collected and the pellet is resuspended in LD-A. Spheroplast disintegration and centrifugation are repeated with the same procedure. Both resulting supernatants are combined and transferred into an Ultra-Clear Centrifuge Tube (Beckman). Each tube is filled up to 1/3 with the supernatant, which is then carefully overlaid with LD-A to the top of the tube. Ultracentrifugation at 28,000 rpm for 45 min at 4°C using a swing out rotor yields a white layer on top (crude LD) that can be removed with a spatula and transferred into a 15 mL Dounce homogenizer. The crude LD are homogenized with 8 strokes using a loose fitting pestle in the presence of 1 mM PMSF. Then, the sample is transferred to a new ultracentrifuge tube (1/4 of the total tube volume) and carefully overlaid with LD-B (8% Ficoll 400 in 10 mM MES/Tris [pH 6.9], 0.2 mM $Na_2EDTA.2H_2O$). Ultracentrifugation at 28,000 rpm for 30 min at 4°C results in a top layer containing LD. This top layer is again removed and transferred to a 15 mL Dounce homogenizer where the LD are homogenized with 8 strokes using a loose fitting pestle in the presence of 1 mM PMSF. Prior to the last ultracentrifugation step, buffer LD-C (0.25 M sorbitol in 10 mM MES/Tris [pH 6.9], 0.2 mM $Na_2EDTA.2H_2O$) is filled into a fresh ultracentrifuge tube up to 3/4 of the tube volume. The homogenized LD are loaded to the bottom of the tube with the aid of a syringe. The last ultracentrifuge step at 28,000 rpm for 30 min at 4°C yields a top layer containing highly purified LD. The top layer is collected with a pipette and transferred into a 7 mL Dounce homogenizer, and LD are mixed with 8 strokes using a loose fitting pestle. LD can then be stored at -80°C for further analysis. If required, the pellet from the last centrifugation step containing mainly vacuoles can be collected and analyzed as well. Isolation of LD from *P. pastoris and Y. lipolytica* can be performed employing the same protocol with minor modifications (see Note 2 and 3).

PROTEIN ANALYSIS OF LIPID DROPLETS

Protein determination

Prior to protein determination LD fractions have to be delipidated. Therefore, lipids are extracted with two volumes of diethyl ether with repeated vigorous shaking. After centrifugation at top speed in a table top centrifuge, the organic phase is withdrawn and residual diethyl ether is removed under a stream of nitrogen (see Note 4 and 5). Proteins are precipitated with TCA (trichloroacetic acid) at a final concentration of 10% for 1 h on ice, and the resulting pellets are solubilized in 0.1% SDS/0.1 M NaOH for protein quantification. In a typical procedure, 200 µL H_2O bidest. and 100 µL TCA (50%) are added to 200 µL of an isolated, delipidated LD fraction for precipitation. Proteins are quantified by the method of (Lowry et al., 1951), which is suitable for the quantification of membrane proteins due to the fact that detergents such as SDS can be included. Moreover, this method is more sensitive than the Biuret method (Smith et al., 1985). Bovine serum albumin is used as a standard. The expected protein concentrations for LD fractions are between 0.01-0.2 mg/mL depending on culture conditions and strain background (see Note 6).

Purity control by Western blot analysis

After precipitation of the desired amount of protein as described above, the resulting pellets are suspended in SDS-loading buffer for analysis by SDS-PAGE using 12.5% separation gels (Laemmli, 1970). Western Blot analysis for testing the purity of isolated LD is performed routinely according to the method of (Haid and Suissa, 1983) using rabbit antibodies against marker proteins representing various yeast organelles, such as Erg1p (LD, ER), Erg6p (LD, ER), Ayr1p (LD, ER), Prc1p (vacuoles), Fox1p (peroxisomes), Por1p (mitochondria), Cyb2p (mitochondria) and Wbp1p (ER). Peroxidase conjugated secondary antibody and enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce Chemical Company) are used to visualize immunoreactive bands.

Mass spectrometry of proteins

Different protocols for LD proteome analysis by mass spectrometry have been published (Athenstaedt et al., 2006; Ayciriex et al., 2012; Grillitsch et al., 2011; Ivashov et al., 2012). A typical analysis of LD proteins from *S. cerevisiae* was described by (Grillitsch et al., 2011). 100 µg of proteins are precipitated by TCA as described above, and the resulting pellets are

dissolved in 100 µL of 25 mM NH_4CO_3. To reduce disulfide bridges 45 mM DTT is added and the solution is incubated for 1 h at 60°C with shaking at 400 rpm. After cooling down to RT, cysteine residues are alkylated in the presence of 100 mM iodoacetamide for 45 min in the dark. This reaction is quenched after 45 min by adding 12.5 µL 45 mM DTT and another incubation step for 45 min. Trypsin digestion for obtaining suitable peptides for further analysis is carried out at an enzyme to protein ratio of 1:50 (w/w) for 18 h at 37°C, which is stopped by addition of 1 µL of 10% trifluoroacetic acid (TFA). Then, samples are concentrated in a Speedvac to approximately 8 µL and diluted to 15 µL with solvent 1 (8% ACN and 0.1% TFA). Separation of tryptic fragments is performed by nLC on a ProxeonBiosystems EASY- nLC™ system coupled to a SunCollect MALDI spotting device (SunChrom). This method is also referred to as shotgun proteomics. Alternatively, precipitated proteins are separated by SDS-PAGE, bands are excised and proteins are digested with trypsin. The risk of this method is that proteins present at low abundance may be lost during electrophoretic separation. It has to be noted, however, that also the shotgun proteomics method has disadvantages, such as the missing chance to detect isoforms of proteins.

For desalting, samples are loaded onto a packed 100 µm x 30 mm pre column filled with Waters X-Bridge™ BEH 180 C18300 Å 3.5 µm for 15 min with 30 µL of solvent 1. Peptides are then separated on a 100 µm x 150 mm column (Waters X-Bridge™ BEH 180 C18300 Å 3.5 µm) at a flow rate of 400 nL/min. The elution gradient is linearly increased from 8 to 45% solvent 2 (92% ACN, 0.1% TFA) within 100 min, to 90% solvent 2 within 20 min, which is held for further 10 min before switching to 8% solvent 2 within 5 min, which is held for another 5 min. The LC-eluent is then mixed with matrix solution containing 3.5 mg/mL alpha-cyano-4-hydroxycinnamic acid (BrukerDaltonics) dissolved in 70% ACN and 0.1% TFA, containing 60 fmol [Glu^1]-Fibrinopeptide B (Bachem) as internal standard. The MALDI spotting is performed every 20 s on a blank 123 x 81 mm Opti- TOF™LC/MALDI Insert metal target (ABSciex). Mass spectra are acquired on a 4800 TOF/TOFTM Analyzer (ABSciex) equipped with a Nd:YAG laser, emitting at 355 nm at a frequency of 200 Hz. All spectra are obtained in the positive reflector mode between 700 and 4,500 m/z with fixed laser intensity. 750 laser shots per spot are accumulated. Fragmentation is conducted with 1 kV collision energy using air as collision gas. Conditions for MS/MS must be optimized regarding sample consumption

and multiple selections of identical precursors. The MS/MS precursor selection is carried out via the instrument's software with a total of 6 precursors per spot with a minimum signal-to-noise-ratio of 80 for fragmentation. Matrix signals can almost be eliminated by excluding potential matrix clusters between 700 and 1,400 m/z (decimal values 0.030±0.1 m/z). For protein and peptide identification a Mascot database search engine v2.2.03 (Matrix Science Ltd.) and the Swissprot Protein Database as well as the *Saccharomyces* Genome Database (http://www.yeastgenome.org) can be used.

LIPID ANALYSIS OF LIPID DROPLETS

Lipid extraction

Lipids are extracted from LD for further analysis using the method of (Folch et al., 1957). An aliquot of LD samples is extracted in 3 mL chloroform:methanol (2:1; v/v) in a Pyrex glass tube by vortexing for 1 h at RT. Proteins are removed by adding 1.5 mL of 0.034% $MgCl_2$. After incubation for 30 min, the extract is centrifuged at 1,500 rpm for 5 min at RT. An upper aqueous phase, a protein containing interface layer and a lower organic phase are formed. The aqueous phase and the interface layer have to be removed. Alternatively, the lower organic phase can be transferred into a new Pyrex glass tube by sucking off the lower layer with a Pasteur pipette. To avoid contamination of the lipid extract with proteins the extract has to be washed several times. First, 1.5 mL of 2 N KCl/MeOH (4:1; v/v) is added to the organic phase and shaken on a Vibrax for 10 min. After centrifugation at 1,500 rpm for 5 min, the upper phase is removed. Then, another washing step with an artificial upper phase (chloroform/methanol/water; 3:48:47; per vol.) follows, which can be repeated several times depending on the required purity of the extract. After shaking on the Vibrax for 10 min and centrifugation at 1,500 rpm for 5 min followed by removal of the aqueous phase the final lipid extract is dried under a stream of nitrogen and stored at -20°C.

Thin layer chromatographic analysis of non-polar lipids

For the analysis of non-polar lipids, e.g. TG and SE, an aliquot of LD is extracted as described above and dissolved in chloroform/methanol (2:1; v/v). An equivalent to 0.2-0.5 µg protein is spotted onto a Silica Gel 60 TLC plate (Merck) with a Hamilton syringe or a TLC sample

applicator (CAMAG). Additionally, 1, 5, 10 and 15 µg of triolein, ergosterol, and cholesteryl oleate are spotted onto the plates as standards for quantification.

For efficient separation and to obtain sharp bands of non-polar lipids, a two-step separation system is used. First, lipids are separated on a TLC plate using light petroleum/diethyl ether/acetic acid (35:15:1; per vol.) as a solvent system until the front reaches 2/3 of the height of the plate. After drying the plate, the separation is continued in the same direction using light petroleum/diethyl ether (49:1; v/v) as a second solvent system until the front reaches the top of the plate. On the dried plate, ergosterol and ergosteryl esters can be quantified by densitometric scanning with a TLC scanner (CAMAG) at 275 nm. Ergosterol can be used as standard for both compounds. To quantify the amount of ergosteryl esters, the value calculated from the densitometric analysis has to be multiplied by factor 1.67 because the average molecular mass of ergosteryl esters compared to ergosterol is enhanced by this factor. For irreversibly staining of non-polar lipids, the TLC plate is incubated in a charring solution of 0.63 g $MnCl_2.4H_2O$, 60 mL water, 60 mL methanol, 4 mL conc. sulfuric acid for 10 s followed by heating at 105°C. The staining intensity depends on the heating time, which should be at least 30 min. The scanning of bands for non-polar lipid quantification should be performed shortly after charring, because the spot intensity bleaches out with time. SE and TG visualized as described above can be quantified by densitometric scanning with a TLC scanner (CAMAG) at 400 nm. Triolein is used for the quantification of TG, whereas cholesteryl oleate can serve as standard for SE.

For the analysis of diacylglyerols (DG), an aliquot of LD is extracted as described above and solved in chloroform/methanol (2:1; v/v). A 1-2 µg protein equivalent of the LD fraction and 0.5, 1, 2 and 5 µg of diolein standard are spotted onto a TLC plate. Since DG and ergosterol show similar Rf-values with solvent systems described above, they should preferentially be separated in an ascending manner using chloroform/acetone/acetic acid (45:4:0.5; per vol.) as a solvent system. After drying and irreversibly staining the lipids as described above, DG can be quantified by densitometric scanning with a TLC scanner (CAMAG) at 400 nm using diolein as standard.

Phospholipid analysis

For a separation of individual phospholipids a two dimensional TLC separation system is recommended. Therefore, a LD equivalent to 0.1-0.2 mg protein is extracted, lipids are solved in chloroform/methanol (2:1; v/v) and applied as single spot to a TLC plate (10 x 10 cm) approximately 1-1.5 cm distant from one corner of the plate. Phospholipids are separated using chloroform/methanol/25% ammonia (65:35:5; per vol.) as a first solvent system for approximately 50 min. After drying the TLC plate, lipids are further separated using chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as a solvent system for the second dimension for another 50 min. Phospholipids are visualized on the dried TLC plate by staining with iodine vapor in a saturated chamber for some seconds. Prior to phosphate determination, the iodine vapor has to be removed by heating the plate after moistening with deionized water.

Phosphate determination of individual phospholipids is carried out as described by (Broekhuyse, 1968). Spots are scrapped off and transferred into a phosphate-free glass tube with ground neck. 1, 2, 4 and 6 µL of a K_2HPO_4 solution (1 µg phosphorus/µL) serve as standard. The lipid phosphorus can be measured by subjecting samples to hydrolysis. Therefore, the samples are incubated with 0.2 mL of conc. H_2SO_4/72% $HClO_4$ (9:1; v/v) at 180°C in a heating block for 30 min. After cooling down the samples to RT, 4.8 mL of freshly prepared 0.26% ammonium molybdate/ANSA solution (500:22; v/v) is added to the tubes which are then closed with glass caps. After careful vortexing, the samples are heated to 105°C in a heating chamber for at least 30 min. Then, samples are cooled to RT and centrifuged at 1,250 rpm for 3 min to sediment the silica gel and immediately measured spectrophotometrically at a wavelength of 830 nm. A blank spot from the TLC plate should be treated in the same way to serve as background sample. Data are calculated from a standard curve obtained by measuring K_2HPO_4 samples.

Gas liquid chromatographic analysis of fatty acids

For GLC of FA, an aliquot of LD equivalent to 1.5-2 µg protein is extracted as described above. FA are analyzed by GLC after hydrolysis and conversion to methyl esters. Therefore, 1 mL of a 2.5% H_2SO_4 (v/v) in methanol solution is added to lipid extracts in a glass Pyrex tube which is carefully closed with the cap. After heating the samples in a heating chamber at 80°C for 90 min and cooling them down to RT, 1 mL H_2O and 3 mL light petroleum are added.

FA methyl esters formed are extracted by shaking the tubes on the Vibrax for 30 min. After centrifugation at 1,500 rpm for 5 min at RT, the organic phase is transferred into a new Pyrex tube and the extraction procedure is repeated with another 3 mL of light petroleum. The collected organic phases are dried under a stream of nitrogen, samples are dissolved in 100 µL light petroleum and transferred into GLC vials. FA methyl esters are separated by GLC using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-INNOWax capillary column (15 m x 0.25 mm inner diameter x 0.5 µm film thickness). Aliquots of 1 µL are injected in split mode with helium as a carrier gas at a flow rate of 1.4 mL linear velocity 30 cm/s. The following program is used: 160°C (5 min) with 7.5°C/min to 250°C (15 min). Finally, FA are identified by comparison to commercially available FA methyl ester standards (NuCheck, Inc., Elysian; MN, USA) (see Note 7).

Gas liquid chromatography/mass spectrometry of sterols

Sterol analysis is performed by the method of (Quail and Kelly, 1996). For GLC/MS analysis of sterols, an aliquot of LD equivalent to 0.5-2 µg protein is extracted and dried as described above. Then, a freshly prepared mixture of 0.6 mL methanol, 0.4 mL 0.5% (w/v) pyrogallol in methanol, and 0.4 mL 60% (w/v) aqueous KOH solution is added to each sample. After addition of 5 µL cholesterol solution (2 mg/mL in ethanol) which serves as an internal standard, samples are heated in a water or sand bath at 90°C for 2 h. Then, samples are cooled to RT and lipids are extracted with 1 mL n-heptane. After centrifugation at 1,500 rpm for 3 min at RT, the upper phase is transferred into a new Pyrex tube. Lipid extraction with 1 mL n-heptane is repeated twice. Then, the collected upper phases are dried under a stream of nitrogen. Lipid extracts are resolved in 10 µL pyridine with addition of 10 µL of N'O'-bis (trimethylsilyl)-trifluoracetamide, incubated at RT for 10 min and diluted with 30 µL ethyl acetate. GLC/MS is performed on a Hewlett-Packard 5890 gas chromatograph equipped with a mass selective detector (HP 5972) and HP5-MS capillary column (Crosslinked 5% Phenyl Methyl Siloxane) with 30 m x 0.25 mm x 0.25 µm film thickness. Aliquots of 1 µL are injected at 270°C injection temperature in the splitless mode with helium as a carrier gas at a flow rate of 0.9 mL/min (constant flow). The following temperature program can be used: 1 min at 100°C with 10°C/min to 250°C and with 3°C/min to 310°C. Mass spectra are obtained in scan mode with 3.27 scans/s using a scan range of 200-500 amu. Individual sterols are identified according to their retention time and mass fragmentation pattern.

Mass spectrometry of non-polar lipids and phospholipids

A detailed protocol for mass spectrometric analysis of non-polar lipid and phospholipid species has been described by Grillitsch et al. (2011). For this analysis, lipids extracted as described above are diluted 1:100 in acetonitrile/2-propanol (5:2; v/v), 1% ammonium acetate, 0.1% formic acid. 5 µM of TG (species 51:0) and phosphatidylcholine (species 24:0) are added serving as internal standards. Thermo Hypersil GOLD C18 column (100 x 1 mm, 1.9 mm) 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) are used for chromatographic separation of lipid species. The gradient changes from 35% to 70% solvent B within 4 min, and further to 100% solvent B in 16 min. These conditions are held constant for 10 min with a flow rate of 250 µL/min. Mass spectrometry is 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 is operated at a mass accuracy of <2 ppm with external calibration and resolution of 200,000 full width at half height at 400 *m/z*. The spray voltage is set at 5,000 V, the capillary voltage at 35 V, the tube lens at 120 V and the capillary temperature at 250°C. Peak areas are calculated by QuanBrowser for all lipid species identified according to their exact mass and retention time.

RESULTS AND DISCUSSION

SDS-PAGE and Western Blot

LD are present at low abundance in yeast cells grown under standard conditions. Nevertheless, they can be isolated at high purity without significant contamination by other organelles such as mitochondria or microsomes. Yeast LD contain a distinct and characteristic set of proteins which can be used for the quality control of isolated LD fractions. To test the enrichment of LD over the homogenate, the sterol 24-C-methyltransferase Erg6p can be employed as a suitable marker protein. Contamination of the LD fraction with other organelles is usually low as can be seen by the analysis of marker proteins characteristic of various organelles. A Western Blot analysis using a typical set of antibodies directed against marker proteins from *S. cerevisiae* is shown in Figure 3.1. Antibodies against GAPDH and Erg6p can also be used for the quality control of subcellular fractions isolated from *P. pastoris* and *Y. lipolytica*. Alternatively to Cyb2p, an antibody against the porin Por1p can be used as marker for the mitochondria fraction. Additionally, marker proteins fused to a Myc-, HA- or a green fluorescent protein (GFP)-tag can be used to check the purity of different fractions if suitable antibodies are not available.

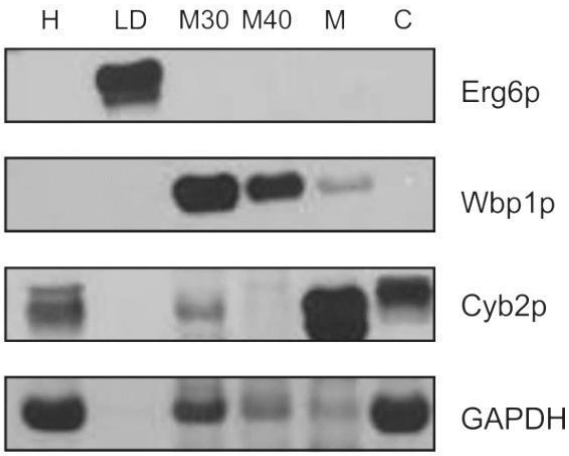

Figure 3.1: Quality control of lipid droplets isolated from *S. cerevisiae*. Cells were grown to the stationary phase on glucose medium. Western blot analysis of 10 µg protein from each fraction was performed as described in the Methods section. Homogenate (H), LD (LD), 30,000xg microsomes (M30), 40,000xg microsomes (M40), mitochondria (M) and cytosol (C). Antibodies were directed against marker proteins from *S. cerevisiae*. Erg6p (sterol 24-C-methyltransferase; LD marker), Wbp1p (dolichyl-diphosphooligosaccharide-protein glycotransferase; ER marker), Cyb2p (Cytochrome b2; L-lactate cytochrome-c oxidoreductase; mitochondrial marker), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; cytosolic marker).

However, due to the tight interaction and contacts of LD to other organelles like the ER, mitochondria and peroxisomes (Kohlwein et al., 2013) on the one hand, and slight contamination of LD with other subcellular fractions which can never be avoided on the other hand, highly sensitive MS proteome analyses do not only detect "true" LD proteins, but also contaminations. Krahmer et al. (2013) recently reported a novel methodology for LD protein identification based on MS and so-called protein correlation profiling. This profile allows the identification of LD proteins with high confidence by using quantitative, high-resolution MS and by correlating their purification profile to that of known LD proteins.

In addition to MS analysis, the presence of many proteins in LD was confirmed by fluorescence microscopy studies. SDS-PAGE revealed different protein patterns of LD fractions depending on the cultivation conditions, e.g. when glucose or oleate was used as carbon source. This effect was observed with *S. cerevisiae* and *Y. lipolytica* (Athenstaedt et al., 2006; Grillitsch et al., 2011).

Proteome analysis of lipid droplets

Proteome analysis of LD from *S. cerevisiae, P. pastoris and Y. lipolytica* revealed that LD proteins can be classified into certain functional families (Fei et al., 2011b, Grillitsch et al., 2011). Enzymes of lipid metabolism comprise the biggest group next to glycosylation and protein synthesis, cell wall organization and ER unfolded protein response. The most abundant LD proteins from *S. cerevisiae* are Ayr1p, Dga1p, Eht1p, Erg1p, Erg27p, Erg6p, Erg7p, Faa1p, Faa4p, Fat1p, Gat1p, Hfd1p, Pet10p, Pgc1p, Slc1p, Tgl1p, Tgl3p, Tgl4p, Tgl5p, Tsc10p and Vps66p. The number of LD proteins detected in *P. pastoris* (Ivashov et al., 2012) and *Y. lipolytica* (Athenstaedt et al., 2006) identified so far is low compared to *S. cerevisiae*. Different abundance of proteins in different yeast genera, but also different methods employed for proteome analysis may be the reason for this observation. For an overview of proteome analysis of the different yeasts the reader is referred to the above mentioned publications.

The proteome of *S. cerevisiae* shows an adaptive response to environmental conditions. As an example, additional LD proteins have been found in cells grown on oleate compared to growth on glucose (Grillitsch et al., 2011). (Fei et al., 2009) reported that the LD proteome is influenced by size and phospholipid composition of the droplets as shown with yeast

mutants producing “supersized” LD. Differences in the LD proteome between the investigated yeast species and caused by variation of cultivation conditions led to the speculation that a basal set of LD proteins is sufficient to maintain structure and function of this organelle.

Structural and topological investigations of LD proteins as well as targeting of proteins to this organelle are just in their infancy (Hickenbottom et al., 2004). Initial experiments to address this issue led to the conclusion that hydrophobic domains near the C-terminal end of LD proteins may play a role in their distribution between LD and the ER as demonstrated for Erg1p, Erg6p and Erg7p (Müllner et al., 2004). Another interesting feature of LD proteins seems to be that they do not harbor transmembrane spanning domains. This property can be explained by the fact that LD proteins need to be accommodated in the surface phospholipid monolayer of the organelle.

Lipid analysis of lipid droplets

Non-polar lipids of LD can be routinely analyzed by TLC and identified by comparison to standard mixtures. Figure 3.2 shows a typical analysis of non-polar lipids from LD samples and standards as mentioned in the Methods section. For the TLC shown in Figure 3.2A lipids were separated by a two-step procedure using light petroleum/diethyl ether/acetic acid (35:15:1; per vol.) as a first solvent system and light petroleum/diethyl ether (49:1; v/v) as a second solvent system. 2 µg of cholesteryl oleate, triolein and ergosterol, respectively, and 0.15 µg protein equivalent of LD sample from *S. cerevisiae* were loaded. As shown in Figure 3.2A, LD from *S. cerevisiae* contain approximately equal amounts of TG and SE. It is worth mentioning that SE and TG from yeast LD show slightly lower Rf-values than cholesteryl oleate and triolein. Additionally, small amounts of squalene on the top and free sterols on the bottom of the TLC plate can be observed. Lipids in the TLC shown in Figure 3.2B were separated using chloroform/acetone/acetic acid (45:4:0.5; per vol.) as a solvent system (see Methods section). 2.5 µg of ergosterol and diolein, respectively, and 1 µg protein equivalent of LD sample from *S. cerevisiae* were loaded. As can be seen from Figure 3.2B, LD from *S. cerevisiae* contain very low amounts of DG and free sterols. However, these two classes are well separated and can be identified by standards. Similar to TG and SE, DG show a slightly lower Rf-value than pure diolein. SE and TG are not separated in this TLC system and accumulate on the top of the TLC plate.

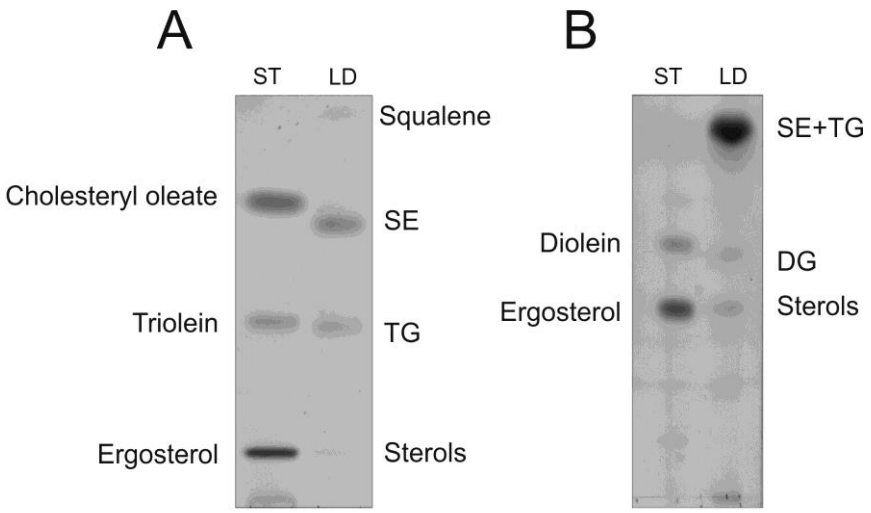

Figure 3.2: Non-polar lipid analysis of lipid droplets from *S. cerevisiae* For the TLC analysis of non-polar lipids different solvent systems were used (see Methods section). Cells were grown to the stationary phase on glucose medium. Lipids were extracted and separated using light petroleum/diethyl ether/acetic acid (35:15:1; per vol.) as the first, and light petroleum/diethyl ether (49:1; v/v) as the second solvent system in the same direction (A). For the TLC shown in Fig. B, chloroform/acetone/acetic acid (45:4:0.5; per vol.) was used as a solvent system. ST, standard mixtures.

Phospholipids of LD are usually separated by two-dimensional TLC and analyzed as described in the Methods section. Figure 3.3 shows a characteristic separation of individual phospholipids from 15 µg protein equivalent of yeast LD. Phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol are the most abundant phospholipids from *S. cerevisiae* LD.

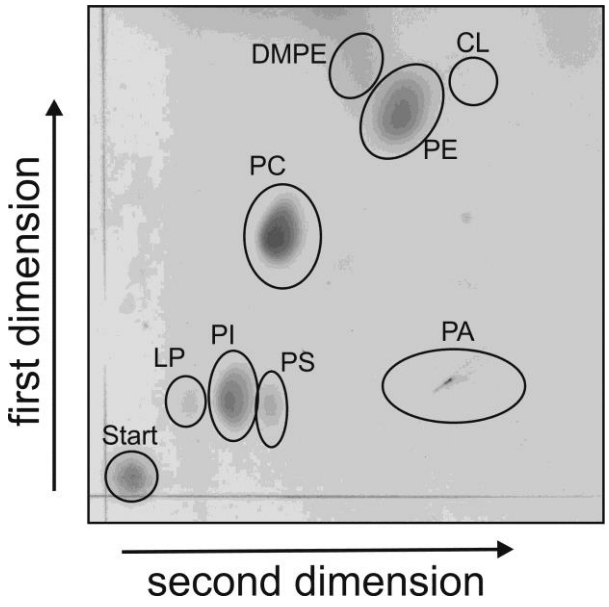

Figure 3.3: Phospholipid analysis of lipid droplets from *S. cerevisiae*. Two dimensional TLC of individual phospholipids from *S. cerevisiae* LD was performed as described in the Methods section. Cells were grown to the stationary phase on glucose medium. Lipids were extracted and separated using chloroform/methanol/25% ammonia (65:35:5; per vol.) as a solvent system for the first dimension; and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as a solvent system for the second dimension. Start, Starting point of the separation; LP, lysophospholipids; PI; phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine.

Lipid composition of lipid droplets from *Saccharomyces cerevisiae*, *Pichia pastoris* and *Yarrowia lipolytica*

As described above, TG and SE are major compounds of yeast LD. However, the lipid composition of LD from different yeast genera can vary dramatically (Athenstaedt et al., 2006; Ivashov et al., 2012; Leber et al., 1994). In contrast to *S. cerevisiae*, where nearly equal amounts of TG and SE form the hydrophobic core of LD, TG is the major and predominant non-polar lipid class in *P. pastoris* and *Y. lipolytica* (Figure 3.4). Only minor amounts of SE can be found in LD from these yeast species. The amount of phospholipids is similar in all three yeasts.

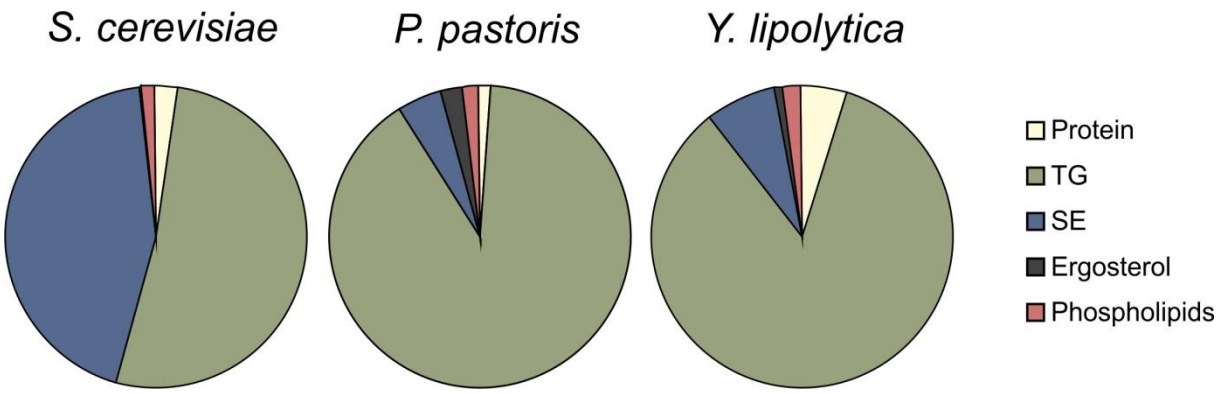

Figure 3.4: Major components of lipid droplets from *S. cerevisiae*, *P. pastoris* and *Y. lipolytica*. Data were obtained from (Athenstaedt et al., 2006; Ivashov et al., 2012; Leber et al., 1994). Relative amounts of TG (triacylglycerols), SE (steryl esters), ergosterol, phospholipids and protein were calculated according to analytical data.

The above mentioned yeast genera do not only differ in the relative distribution of non-polar lipid classes in LD, but also in the absolute amount of lipids. Table 3-1 shows total amounts of lipids per mg protein in LD isolated from *S. cerevisiae, P. pastoris* and *Y. lipolytica* grown to the stationary phase on glucose as a carbon source. These data demonstrate that the methylotrophic yeast *P. pastoris* is able to accumulate TG at much higher amount than *S. cerevisiae* cultivated under standard conditions. Total amounts of TG can be strongly increased by growing yeast cells on carbon sources different from glucose. As an example, *S. cerevisiae* grown on oleate containing medium shows a ~3 fold increase of total TG (Grillitsch et al., 2011). In *Y. lipolytica*, amounts of TG can be increased up to 40% when industrial fats or glycerol are used as carbon sources (Papanikolaou and Aggelis, 2002).

Table 3-1: Analyses of LD isolated from *Saccharomyces cerevisiae*, *Pichia pastoris* and *Yarrowia lipolytica* (Athenstaedt et al., 2006; Ivashov et al., 2012; Leber et al., 1994). Yeast strains were grown to the stationary phase on glucose media and individual components were analyzed as described in the Methods section. SE, steryl ester; TG, triacylglycerols.

	S. cerevisiae	***P. pastoris***	***Y. lipolytica***
	µg/µg protein	µg/µg protein	µg/µg protein
Protein	1	1	1
TG	19.8	59.1	16.6
SE	17.2	3.1	1.53
Ergosterol	0.1	1.6	0.12
Phospholipids	0.5	1.09	0.4

Noteworthy, LD from *S. cerevisiae, P. pastoris* and *Y. lipolytica* exhibit further differences in their lipid profiles. First, the sterol composition from yeast LD can vary significantly. Sterol analysis of *S. cerevisiae* LD revealed that ~75% of total SE are formed from ergosterol, whereas only minor amounts of zymosterol, fecosterol and episterol esters were found (Czabany et al., 2008). In contrast, SE from *P. pastoris* contain only 30% ergosterol esters, but larger amounts of esterified sterol precursors (Ivashov et al., 2012). The amount of zymosterol in SE from *P. pastoris* is similar to ergosterol (26%), and also substantial amounts of episterol, 4-methylzymosterol, fecosterol, lanosterol and 4,14-dimethylcholesta-8,24-dienol were detected. The phospholipid pattern of LD from *S. cerevisiae* and *P. pastoris* is rather similar. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol are the most abundant phospholipids forming the surface phospholipid monolayer of LD in both yeast genera (Grillitsch et al., 2011; Ivashov et al., 2012). Phosphatidylserine was also detected in LD, and cardiolipin and phosphatidic acid were found at minor quantities.

Although the major FA in LD from *S. cerevisiae, P. pastoris* and *Y. lipolytica* is oleic acid (C18:1), the total FA composition can vary notably. *S. cerevisiae* LD contain mainly unsaturated FA with nearly equal amounts of oleic acid (C18:1) and palmitoleic acid (16:1) (~40% of total fatty acid, each) (Czabany et al., 2008). Minor amounts of palmitic acid (C16:0) and stearic acid (C18:0) were detected. *P. pastoris* LD contain mono- as well as polyunsaturated fatty acids (Ivashov et al., 2012). Oleic acid (18:1), linoleic acid (18:2), linolenic acid (C18:3) and palmitic acid (C16:0) are the major FA in these LD. The FA distribution in LD from *Y. lipolytica* is in sharp contrast to *S. cerevisiae* and *P. pastoris* (Athenstaedt et al., 2006). Oleic acid (18:1) is also the most abundant FA of *Y. lipolytica* LD (50% of total FA), but palmitic acid (C16:0) is ranked second with ~22 % of total FA followed by minor amounts of C16:1, C18:0 and C18:2.

Lipidome data obtained by mass spectrometric analysis gave a detailed insight into non-polar lipid and phospholipid species (Grillitsch et al., 2011) present in LD from *S. cerevisiae*. Since major FA of *S. cerevisiae* are C16 and C18, species patterns are rather simple. The most abundant species of TG are 52:1; 52:2, 52:3 and 54:2 containing one C16 and two C18 fatty acids. These lipid species make up to 65% of total TG of LD. The remaining TG species contain two or three C16 FA, either saturated or unsaturated. Species patterns of individual phospholipids vary significantly. In phosphatidylethanolamine and phosphatidylcholine of

LD, the 32:2 (C16:1/C16:1) species is highly enriched compared to the homogenate. Additionally, the 34:2 species (C16:1/C18:1) is highly abundant in these phospholipid classes. In phosphatidylcholine 32:2 and 34:2 occur at similar levels. In contrast, 34:1 is the most abundant lipid species of phosphatidylinositol and phosphatidylserine. Two fully saturated species, namely 34:0 (C16:0/C18:0) and 36:0 (C18:0/C18:0), are found in phosphatidylserine from *S. cerevisiae* LD. Ivashov et al. (2012) demonstrated, that the species pattern of phospholipids from *S. cerevisiae* LD differs from *P. pastoris* where C36 and C34 species are the majority of all phospholipid classes. Noteworthy, phosphatidylcholine and phosphatidylethanolamine occur as several polyunsaturated species such as C36:2, C36:3, C36:4 and C36:5.

Differences in the lipid profiles of LD from different yeast genera largely reflect their overall lipid biosynthetic capacity. As example, the lack of polyunsaturated FA production in *S. cerevisiae* compared to *P. pastoris* (Grillitsch et al., 2011; Ivashov et al., 2012) also results in genera specific FA patterns of LD. Consequently, LD provide a pool of lipid components which matches the requirements of the whole cell if needed.

In summary, analytical methods described here contributed significantly to our understanding of yeast LD biology. Isolation of pure LD by the protocol presented here is the prerequisite for detailed analysis. Identification and quantification of lipids and proteins from LD by –omics approaches are a major contribution to investigate of molecular biological, cell biological and regulatory aspects of LD biogenesis. These approaches will enable researchers to develop an integrated picture of LD in cellular processes in future research.

NOTES

1. When harvesting cells cultivated on oleate, washing with 0.1% bovine serum albumin is required to remove the excess of the fatty acid on the cell surface.

2. The same buffers and protocols can be used for the isolation of LD form *Pichia pastoris*. To obtain highly pure LD it is recommended to prolong the last ultracentrifugation step to 1 h (Ivashov et al., 2012).

3. The same protocols and buffers can be used for LD isolation from *Yarrowia lipolytica*. The highest purity of LD can be achieved by adding an equal volume of 9 M urea to the recovered floating layer of the third ultracentrifugation step. The urea containing LD solution is gently agitated for 10 min at RT. The suspension is overlaid with LD-C and centrifuged at 28,000 rpm for 30 min at RT (Athenstaedt et al., 2006).

4. Delipidation of LD samples prior to TCA precipitation is not absolutely necessary, but disturbing effects during SDS-PAGE might be observed. Washing the precipitated protein pellet with ice cold acetone helps to avoid negative effects during SDS-PAGE.

5. An alternative protocol for delipidating LD and precipitation of proteins is the method of (Wessel and Flügge, 1984). LD samples of 150 µL are mixed with 600 µL methanol and 150 µL chloroform and vortexed. Then, 400 µL water is added and samples are thoroughly vortexed. After 1 min of maximum speed centrifugation in a table top centrifuge at RT, the upper organic phase is withdrawn without disturbing the interphase which contains the proteins. Then, at least 450 µL methanol are added and samples are thoroughly vortexed. After 1-2 min of centrifugation at RT at maximum speed in a table top centrifuge the supernatant can be removed and the precipitated proteins can be air dried. The precipitate can be taken up in an appropriate volume of SDS-loading buffer.

6. An average LD purification yields 0.2-0.5 mg of LD protein from 5-6 L culture volume. The average protein concentration of LD samples is 0.01-0.2 µg/µL, which can be increased by an additional centrifugation step using a table top centrifuge at maximum speed for 15 min. The excess amount of buffer below the LD layer accumulating at the top can be removed with a syringe.

7. Alternatively, FA can by hydrolyzed and converted to methyl esters by the method of (Morrison and Smith, 1964). For this purpose, 1 mL of BF_3-methanol is added to the dried lipid extract and heated to 95°C in a sand bath for 10 min. After cooling the samples down to RT, 0.86 mL of benzene are added and heated to 95°C for another 30 min. The extraction procedure is then continued with 1 mL water and 3 mL of light petroleum as described above.

ACKNOWLEDGEMENTS

We thank Dominic Baeumlisberger for methodological advice about the proteome analysis. Research in our laboratory about yeast lipid droplets was funded by the Austrian Science Fund (FWF) projects W901 (DK Molecular Enzymology) and P23029 to G. D.

REFERENCES

Adeyo, O., Horn, P.J., Lee, S., Binns, D.D., Chandrahas, A., Chapman, K.D., and Goodman, J.M. (2011). The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. J. Cell Biol. *192*, 1043–1055.

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

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.

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 source. Proteomics *6*, 1450–1459.

Ayciriex, S., Le Guédard, M., Camougrand, N., Velours, G., Schoene, M., Leone, S., Wattelet-Boyer, V., Dupuy, J.-W., Shevchenko, A., Schmitter, J.-M., et al. (2012). YPR139c/*LOA1* encodes a novel lysophosphatidic acid acyltransferase associated with lipid droplets and involved in TAG homeostasis. Mol. Biol. Cell *23*, 233–246.

Baud, S., and Lepiniec, L. (2010). Physiological and developmental regulation of seed oil production. Prog. Lipid Res. *49*, 235–249.

Binns, D., Januszewski, T., Chen, Y., Hill, J., Markin, V.S., Zhao, Y., Gilpin, C., Chapman, K.D., Anderson, R.G.W., and Goodman, J.M. (2006). An intimate collaboration between peroxisomes and lipid bodies. J. Cell Biol. *173*, 719–731.

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.

Connerth, M., Czabany, T., Wagner, A., Zellnig, G., Leitner, E., Steyrer, E., and Daum, G. (2010). Oleate inhibits steryl ester synthesis and causes liposensitivity in yeast. J. Biol. Chem *285*, 26832–26841.

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.

Fei, W., Shui, G., Gaeta, B., Du, X., Kuerschner, L., Li, P., Brown, A.J., Wenk, M.R., Parton, R.G., and Yang, H. (2008). Fld1p, a functional homologue of human seipin, regulates the size of lipid droplets in yeast. J. Cell Biol. *180*, 473–482.

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

Fei, W., Du, X., and Yang, H. (2011). Seipin, adipogenesis and lipid droplets. Trends Endocrinol. Metab. *22*, 204–210.

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

Fujimoto, T., Ohsaki, Y., Cheng, J., Suzuki, M., and Shinohara, Y. (2008). Lipid droplets: a classic organelle with new outfits. Histochem. Cell Biol. *130*, 263–279.

Grillitsch, K., Connerth, M., Köfeler, H., Arrey, T.N., Rietschel, B., Wagner, B., Karas, M., and Daum, G. (2011). Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim. Biophys. Acta *1811*, 1165–1176.

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

Hickenbottom, S.J., Kimmel, A.R., Londos, C., and Hurley, J.H. (2004). Structure of a lipid droplet protein; the PAT family member TIP47. Structure *12*, 1199–1207.

Ivashov, V.A., Grillitsch, K., Koefeler, H., Leitner, E., Baeumlisberger, D., Karas, M., and Daum, G. (2012). Lipidome and proteome of lipid droplets from the methylotrophic yeast *Pichia pastoris*. Biochim. Biophys. Acta. 1831(2), 282-90.

Jacquier, N., Choudhary, V., Mari, M., Toulmay, A., Reggiori, F., and Schneiter, R. (2011). Lipid droplets are functionally connected to the endoplasmic reticulum in *Saccharomyces cerevisiae*. J. Cell. Sci *124*, 2424–2437.

Kohlwein, S.D., Veenhuis, M., and van der Klei, I.J. (2013). Lipid Droplets and Peroxisomes: Key Players in Cellular Lipid Homeostasis or A Matter of Fat--Store 'em Up or Burn 'em Down. Genetics *193*, 1–50.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

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.

Leber, R., Landl, K., Zinser, E., Ahorn, H., Spök, 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.

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.

Milla, P., Athenstaedt, K., Viola, F., Oliaro-Bosso, S., Kohlwein, S.D., Daum, G., and Balliano, G. (2002). Yeast oxidosqualene cyclase (Erg7p) is a major component of lipid particles. J. Biol. Chem. *277*, 2406–2412.

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.

Müllner, H., Zweytick, D., Leber, R., Turnowsky, F., and Daum, G. (2004). Targeting of proteins involved in sterol biosynthesis to lipid particles of the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta *1663*, 9–13.

Murphy, D.J. (2001). The biogenesis and functions of lipid bodies in animals, plants and microorganisms. Prog. Lipid Res. *40*, 325–438.

Murphy, D.J. (2012). The dynamic roles of intracellular lipid droplets: from archaea to mammals. Protoplasma *249*, 541–585.

Murphy, D.J., and Vance, J. (1999). Mechanisms of lipid-body formation. Trends Biochem. Sci *24*, 109–115.

Papanikolaou, S., and Aggelis, G. (2002). Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. Bioresour. Technol. *82*, 43–49.

Ploegh, H.L. (2007). A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum. Nature *448*, 435–438.

Pu, J., Ha, C.W., Zhang, S., Jung, J.P., Huh, W.-K., and Liu, P. (2011). Interactomic study on interaction between lipid droplets and mitochondria. Protein Cell *2*, 487–496.

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

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 *1791*, 379–387.

Sandager, L., Gustavsson, M.H., Ståhl, 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.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. *150*, 76–85.

Wältermann, M., Hinz, A., Robenek, H., Troyer, D., Reichelt, R., Malkus, U., Galla, H.-J., Kalscheuer, R., Stöveken, T., von Landenberg, P., et al. (2005). Mechanism of lipid-body formation in prokaryotes: how bacteria fatten up. Mol. Microbiol. *55*, 750–763.

Walther, T.C., and Farese, R.V., Jr (2012). Lipid droplets and cellular lipid metabolism. Annu. Rev. Biochem. *81*, 687–714.

Wessel, D., and Flügge, U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. *138*, 141–143.

Wolinski, H., Kolb, D., Hermann, S., Koning, R.I., and Kohlwein, S.D. (2011). A role for seipin in lipid droplet dynamics and inheritance in yeast. J. Cell. Sci. *124*, 3894–3904.

Zimmermann, R., Strauss, J.G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A., et al. (2004). Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science *306*, 1383–1386.

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.

4

SCREENING FOR HYDROLYTIC ENZYMES REVEALED Ayr1p AS A NOVEL TRIACYLGLYCEROL LIPASE IN *SACCHAROMYCES CEREVISIAE*

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Published in: *The Journal of Biological Chemistry* 2013; 288(50):36061-72

KEY WORDS

Yeast, lipids, triacylglycerols, lipase, peroxisomes, lipid droplets

ABBREVIATIONS

LD, lipid droplets; MS, mass spectrometry; Px, peroxisomes; QiM, quintuple mutant; SE, steryl esters; TG, triacylglycerols; TLC, thin layer chromatography; TM, triple mutant

CAPSULE

Background: Triacylglycerols are stored in lipid droplets and can be mobilized by lipases.

Results: Ayr1p was identified as a novel triacylglycerol lipase.

Conclusion: In addition to the known lipases Tgl3p, Tgl4p and Tgl5p further hydrolytic enzymes contribute to the mobilization of non-polar lipids in yeast.

Significance: This study opens the view for a broader network of lipolytic enzymes in yeast.

ABSTRACT

Saccharomyces cerevisiae, as well as other eukaryotes, preserves fatty acids and sterols in a biologically inert form, as triacylglycerols and steryl esters. The major triacylglycerol lipases of the yeast *S. cerevisiae* identified so far are Tgl3p, Tgl4p and Tgl5p (Athenstaedt et al., 2003; Athenstaedt et al., 2005). We observed that upon cultivation on oleic acid triacylglycerol mobilization did not come to a halt in a yeast strain deficient of all currently known triacylglycerol lipases, indicating the presence of additional not yet characterized lipases/ esterases. Functional proteome analysis using lipase and esterase inhibitors revealed a subset of candidate genes for yet unknown hydrolytic enzymes on peroxisomes and lipid droplets. Based on the conserved GxSxG lipase motif, putative functions and subcellular localizations a selected number of candidates were characterized by enzyme assays *in vitro*, gene expression analysis, non-polar lipid analysis and *in vivo* triacylglycerol mobilization assays. These investigations led to the identification of Ayr1p as novel triacyl-glycerol lipase of yeast lipid droplets and confirmed the hydrolytic potential of the peroxisomal Lpx1p *in vivo*. Based on these results we discuss a possible link between lipid storage, lipid mobilization and peroxisomal utilization of fatty acids as carbon source.

INTRODUCTION

In *Saccharomyces cerevisiae* as well as in other eukaryotes, an excess of fatty acids is stored as triacylglycerols (TG) and steryl esters (SE), often referred to as non-polar lipids. Both lipids are stored in organelle-like structures called lipid droplets (LD), which are about 400 nm in diameter and consist of a highly hydrophobic core of TG, surrounded by shells of SE and a phospholipid monolayer containing a distinct set of proteins (Athenstaedt et al., 1999; Czabany et al., 2008; Grillitsch et al., 2011; Kohlwein et al., 2012). TG are synthesized by the acyltransferases Dga1p and Lro1p, and SE by the SE synthases Are1p and Are2p (Dahlqvist et al., 2000; Oelkers et al., 2000, 2002; Sorger and Daum, 2002; Yang et al., 1996; Zweytick et al., 2000). All TG and SE synthesizing enzymes are located to the ER, but Dga1p is also found on LD. TG serves as the main energy storage, and both TG and SE are depots of membrane lipid components. Upon requirement, i.e. during growth or starvation, TG and SE can be mobilized by lipases or hydrolases. Currently, three major TG lipases are known, namely Tgl3p, Tgl4p and Tgl5p, which are located on LD (Athenstaedt and Daum, 2003, 2005). The hydrolysis of SE is conducted by Tgl1p and Yeh1p localized to LD, and Yeh2p, which was found to be associated with the plasma membrane (Jandrositz et al., 2005; Köffel et al., 2005; Müllner et al., 2005; Rajakumari et al., 2008).

Tgl3p, Tgl4p and Tgl5p share a common consensus sequence GxSxG, where serine is the essential residue of the catalytic triad aspartic acid/glutamic acid and histidine (Schrag and Cygler, 1997). They also contain a patatin domain, named after a plant storage protein that possesses lipid acyl hydrolase activity (Mignery et al., 1988). *In vitro*, all three proteins exhibit lipolytic activity, whereas *in vivo* only Tgl3p and Tgl4p mobilize TG efficiently. Previous studies from our laboratory described functions for Tgl3p, Tgl4p and Tgl5p in addition to their lipase activities. Tgl3p, Tgl4p and Tgl5p harbor an acyltransferase motif (H-$(X)_4$-D), and *in vitro* enzyme assays showed that both Tgl3p and Tgl5p act as lysophospholipid acyltransferases. Besides the conserved lipase motif, Tgl4p contains a (G/A)xGxxG Ca-independent phospholipase A_2 domain. Phospholipase as well as SE hydrolase activity of Tgl4p was also established *in vitro* (Rajakumari and Daum, 2010a). A *Δtgl3Δtgl4Δtgl5* (TM) yeast strain lacking all three TG lipases does not reveal any growth defect under standard growth conditions, although mutations in *TGL3* or *TLG4* lead to fat yeast cells that

accumulate TG (Kurat et al., 2006; Rajakumari and Daum, 2010a, 2010b). Interestingly, we observed that upon cultivation on oleic acid TG mobilization did not come to a halt in the TM deficient of all currently known TG lipases, suggesting the presence of novel not yet characterized hydrolases.

S. cerevisiae grown in the presence of oleic acid proliferates peroxisomes (Px) and at the same time accumulates large LD (Rosenberger et al., 2009). Px are small ubiquitous organelles involved in the decomposition of toxic substances like H_2O_2 as well as degradation of fatty acids via β-oxidation. The mechanism of fatty acid transport to their site of degradation is not yet completely understood. In contrast to mammalian cells, the degradation of fatty acids in the yeast exclusively takes place in Px (Hiltunen et al., 2003; Kunau et al., 1988; Lazarow and De Duve, 1976). Thus, functional Px are crucial for growth of yeast cells on fatty acids as carbon source. Binns *et al.* (Binns et al., 2006) proposed a direct link between Px and LD indicating a putative pathway for lipid supply to Px. It was suggested that Px can even penetrate LD forming a structure called pexopodia and that this contact may stimulate non-polar lipid turnover.

The aim of the present study was to identify novel hydrolytic enzymes possibly involved in non-polar lipid metabolism in the yeast *S. cerevisiae*. Our data suggest that in addition to Tgl3p, Tgl4p and Tgl5p further hydrolases are responsible for the mobilization of TG. Our approach identified a novel TG lipase and shed more light on a possible link between lipid storage, lipid mobilization and peroxisomal utilization of fatty acids as carbon source.

EXPERIMENTAL PROCEDURES

Yeast strains and growth conditions

Table 4-1 gives an overview of strains used in this study. Yeast cells were either grown in YPD medium containing 1% yeast extract, 2% glucose and 2% peptone or on oleic acid supplemented medium (YPO) containing 0.3% yeast extract, 0.5% peptone, 0.1% glucose, 0.5% KH_2PO_4 and 0.1% oleic acid. For solubilizing oleic acid in YPO, 0.2% Tween 80 was added to the medium. Yeast strains bearing plasmids were cultivated in synthetic minimal medium (SD) containing 0.67% yeast nitrogen base (U.S. Biochemical Corp.), 2% glucose and the respective amino acid supplements. If not stated otherwise, all cells were cultivated in

liquid media at 30°C under vigorous shaking until early stationary phase. For gene expression studies yeast cells were grown to the mid exponential phase. Growth was monitored by measuring optical density at 600 nm (A_{600}). Expression of *CUP1* promoter-controlled genes was induced after growth for 12 h by adding $CuSO_4$ at a final concentration of 0.5 mM to the medium.

Table 4-1: Yeast strains used in this study

Strain	Genotype	Source
WT	BY4741 *Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
TM	WT; *Δtgl3*::*kanMX4; Δtgl4*::*kanMX4; Δtgl5*::*kanMX4*	Athenstaedt and Daum, 2005
QiM	*Δtgl3*::*kanMX4;Δtgl4*::*kanMX4;Δ*:*tgl5*:*kanMX4; Δare1*::*LEU2; Δare2*::kan*MX4*	Kindly provided by A. Wagner
LPX1$^+$	TM + pYEX4T-1-*LPX1*	This study
LDH1$^+$	TM + pYEX4T-1-*LDH1*	This study
YJU3$^+$	TM + pYEX4T-1-*YJU3*	This study
AYR1$^+$	TM + pYEX4T-1-*AYR1*	This study
EHT1$^+$	TM + pYEX4T-1-*EHT1*	This study
TSC10$^+$	TM + pYEX4T-1-*TSC10*	This study
YBR056w$^+$	TM + pYEX4T-1-*YBR056w*	This study
YKL050c$^+$	TM + pYEX4T-1-*YKL050c*	This study
AYR1S18A$^+$	TM + pYEX4T-1-*AYR1*^{S18A}	This study
VC	TM + pYEX4T-1 (vector control)	This study

Genetic techniques

A list of primers used for creating deletion cassettes is shown in Table 4-2. Gene deletions were performed following the PCR-mediated method described by Longtine et al. (Longtine et al., 1998). Deletion cassettes were transformed employing the high-efficiency lithium acetate transformation protocol (Gietz et al., 1995). Correct integration of the knock-out cassettes was verified by growth auxotrophy as well as colony PCR. For the expression of candidate hydrolases/lipases, the open reading frames of the respective genes were amplified from BY4741 chromosomal DNA using primers listed in Table 4-2. Restricted PCR fragments of *LPX1*, *LDH1*, *YJU3*, *AYR1*, YBR056w were inserted into the BamHI and EcoRI sites, whereas *EHT1*, *TSC10* and YKL050c were inserted into the BamHI and SalI sites of the plasmid pYEX4T-1, a vector for high-level expression of glutathione S-transferase (GST) fusion proteins in yeast under the control of a copper inducible CUP1 promoter. Point

mutations in *AYR1* were introduced by site-directed Mutagenesis. Plasmid pYEX4T-1_*AYR1*S18A was constructed by overlap extension polymerase chain reaction (OE-PCR) using the primsers listed in Table 4-2.

Table 4-2: Primers used in this study.

Name	Sequence 5'→3'
Primers used for construction of deletion mutants	
Delare1fw	GTTCAGCACGGCTTGCAGCAAGAGCGCCAAAACAGATTGCAAGACAGCTGAAGCTTCGTACGC
Delare1rev	TATATCTATCAAGGGCTTGCGAGGGACACACGTGGTATGGTGGCAGTGCATAGGCCACTAGTGGATC
Delare2fw	TATATCTATCAAGGGCTTGCGAGGGACACACGTGGTATGGTGGCAGTGCATAGGCCACTAGTGGATC
Delare2ev	AACAGACACATTACGTTAGCAAAAGCAACAATAACAAACACAACCCAGCTGAAGCTTCGTACGC
Are1fwctrl	GAAAAATGTGAGATGGTGTAGAGTG
Are1revctrl	ATGGTTCTGCCCCAGATTTACC
Are2fwctrl	CTTTCATCAATACATCTATATATTCG
Are2revctrl	GTAATTGTGGTAGCTGTGTGTTCAT
Primers for construction of overexpressing constructs	
Lpx1fw	GCGTGGATCCATGGAACAGAACAGGTTCAAGAAAG
Lpx1rev	CGGGAATTCTTACAGTTTTTGTTTAGTCGTTTTAACC
Ldh1fw	GCGTGGATCCATGAATATGGCAGAACGTGCAG
Ldh1rev	CGGGAATTCCTACAATTTGGAATTATCAATCACCTCTCG
Yju3fw	GCGTGGATCCATGGCTCCGTATCCATACAAAG
Yju3rev	CGGGAATTCTTATGGTTTAGCTTCGGTCGTG
Ayr1fw	GCGTGGATCCATGTCGGAGTTACAGTCACAAC
Ayr1rev	CGGGAATTCCTAATCGTCCTTATTCTTCTGTTTCGAC
Eht1fw	GCGTGGATCCATGTCAGAAGTTTCCAAATGGCC
Eht1rev	CGAGTCGACTCATACGACTAATTCATCAAACTTAGTG
Tsc10fw	GCGTGGATCCATGAAGTTTACGTTAGAAGACCAAGTTG
Tsc10rev	CGAGTCGACTCAGTTGGCCTTCTTGCCGTC
YBR056wfw	GCGTGGATCCATGATTGGCTCACTTAGAAACAAATTTGAG
YBR056wrev	CGGGAATTCTTAATACTTATTAAACTCATCTAACCCACGTTG
YKL050cfw	GCGTGGATCCATGTCACTAATATCTGCGTTGCAAAC
YKL050crev	CGAGTCGACTTAGATAACCTCTTTGAAAAAACTTTCCTTAGGAG
Primers for Site Directed Mutagenesis	
Ayr1_S18Afw	GTTGTTACAGGCGCCGCCGGTGGTATTGGATATG
Ayr1_S18Arev	CATATCCAATACCACCGGCGGCGGCTGTAACAACG

Isolation of organelles

Isolation of highly purified LD and Px was performed as previously described (Connerth et al., 2009; Zinser and Daum, 1995; Zinser et al., 1991) and will only be described in brief here. For the preparation of Px, cells were grown in YPO to the late exponential phase. After harvesting, washing and determining of the cell wet weight, cells were incubated with 0.5 g/mL SP-A (0.1 M Tris/SO_4, pH 9.4) and 1.54 mg DTT/mL SP-A for at least 10 min at 30°C with shaking. Cells were then washed and resuspended in pre-warmed SP-B (1.2 M sorbitol, 20 mM KH_2PO_4, pH 7.4) and spheroplasts were generated by using Zymolyase-20 T (Seikaguku Corporation) at a concentration of 2 mg/g cell wet weight in 6 mL SP-B/g cell wet weight for 1 h at 30°C with shaking. The resulting spheroplasts were then washed with cold SP-B and resuspended in breaking buffer (5 mM MES/KOH, pH 6.0, 0.6 M sorbitol, 1 mM KCl and 0.5 mM EDTA) in twice the cell volume. The cell suspension was homogenized on ice using a Dounce homogenizer with a tight fitting pestle. Nuclei, unbroken cells and cell debris were removed by centrifugation at 5,000 g for 5 min in a Sorvall SLC3000 rotor. The resulting pellet was resuspended twice, re-homogenized and centrifuged again to enhance the yield. The combined supernatants were centrifuged at 15,000 g for 15 min in an SS34 rotor. The organelle pellet, consisting of mitochondria and Px, was again suspended in breaking buffer and centrifuged at 5,000 g for 5 min to clean it from larger aggregates. The centrifugation step at 15,000 g was repeated and after suspension of the pellet in breaking buffer it was loaded onto a Nycodenz gradient (17-35%; w/v) in 5 mM MES/KOH, pH 6.0, 1 mM KCl, 0.24 M sucrose. The loaded gradient tubes were then centrifuged at 26,000 g for 90 min in a swing out rotor (Sorvall AH-629). The white Px layer was taken with a syringe, diluted with 4 volumes of breaking buffer and sedimented at 15,000 g in an SS34 rotor for 15 min at 4°C.

For the isolation of LD, cells were grown in minimal medium (SD) to the early stationary phase. After preparation of spheroplasts as described above, they were resuspended in 1 mL/g cell wet weight LD-A (12% Ficoll 400 in 10 mM MES/Tris, pH 6.9, 0.2 mM $Na_2EDTA.2H_2O$) followed by mechanical disintegration using a Dounce homogenizer with a loose fitting pestle. The resulting homogenate was diluted with a half volume of LD-A and centrifuged at 7,000 g for 5 min at 4°C. The supernatant was collected and the pellet was resuspended in LD-A. Spheroplast disintegration and centrifugation were repeated and the combined supernatants were transferred into Ultra-Clear Centrifuge Tubes (Beckman) up to

1/3 of height overlaid with LD-A to the top of the tube. Ultracentrifugation at 28,000 g for 45 min at 4°C using a swing out rotor yielded a white layer on top (crude LD) that was lifted with a spatula and transferred into a 15 mL Dounce homogenizer. The crude LD were homogenized with a loose fitting pestle. Then, the sample was transferred to a new ultracentrifuge tube (1/4 of the total tube volume) and overlaid with LD-B (8% Ficoll 400 in 10 mM MES/Tris, pH 6.9, 0.2 mM $Na_2EDTA.2H_2O$). Ultracentrifugation at 28,000 g for 30 min at 4°C resulted in a top layer containing LD which was again transferred to a 15 mL Dounce homogenizer where the LD were rehomogenized. Prior to the last ultracentrifugation step, buffer LD-C (0.25 M sorbitol in 10 mM MES/Tris, pH 6.9, 0.2 mM $Na_2EDTA.2H_2O$) was filled into a fresh ultracentrifuge tube up to 3/4 of the tube volume. The homogenized LD were loaded to the bottom of the tube with the aid of a syringe. The last ultracentrifuge step at 28,000 g for 30 min at 4°C yielded a top layer containing highly purified LD. The top layer was collected with a pipette and transferred into a 7 mL Dounce homogenizer, and LD were homogenized for further analysis.

Protein Determination

The protein concentration of isolated fractions and whole cell extracts was determined by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin as a standard. Samples of LD fractions were delipidated with 2-3 volumes of diethyl ether prior to protein analysis. The organic phase was withdrawn and residual diethyl ether was removed under a stream of nitrogen. Proteins were precipitated with trichloroacetic acid at a final concentration of 10% and solubilized in 100 µL 0.1% SDS, 0.1 M NaOH. Purified proteins were quantified by using the BIORAD protein assay based on the method of Bradford (Bradford, 1976) and bovine serum albumin as standard. 10 µg of each fraction were loaded onto SDS-gels for Western blot analysis. SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970) using 12.5% separation gels. Proteins on gels were detected by staining with Coomassie Blue. Western blot analysis was performed according to Haid and Suissa (Haid and Suissa, 1983). Samples were denatured at 37°C to avoid hydrolysis of polypeptides. Proteins were detected by a primary antibody directed against the GST-tag and a peroxidase-conjugated secondary antibody. Enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce) were used to visualize immunoreactive bands.

Lipid analysis

For lipid analysis crude homogenates were prepared by harvesting cells in the early stationary phase, resuspending the cell pellet in breaking buffer (50 mM TrisHCl, pH 7.4, 150 mM NaCl) and disintegrating by vigorous shaking in the presence of glass beads for 10 min at 4°C. After disruption, cell debris were removed at 5,000 g for 5 min. The supernatant was further used for protein determination and lipid extraction by the method of Folch *et al.* (Folch et al., 1957) using chloroform/methanol (2:1; v/v) as solvent. For the quantification of non-polar lipids, extracts were applied to Silica Gel 60 plates, and chromatograms were developed by a two-step developing system using first the solvent system light petroleum/diethyl ether/ acetic acid (35:15:1) for two thirds of the plate. Plates were dried briefly and further developed to the top of the plate using the second solvent system light petroleum/diethyl ether (49:1; v/v). Non-polar lipids were visualized by post-chromatographic charring after dipping the plates in a solution consisting of 0.63 g of $MnCl_2x4H_2O$, 60 mL of water, 60 mL of methanol, and 4 mL of concentrated sulfuric acid. The plates were then heated at 105°C for 40 min. Visualized bands were quantified by densitometric scanning (CAMAG TLC SCANNER 3) at 400 nm with authentic standards containing defined amounts of the respective lipids.

Radioactive *in vivo* TG mobilization assay

Precultures from wild type BY4741 (WT) and *Δtgl3Δtgl4Δtgl5Δare1Δ are2* (QiM) were used to inoculate a main culture to an A_{600} of 0.1 in 100 mL liquid YPO containing 10 µL [^{3}H]oleic acid (50 µCi = 110,000,000 cpm). Cells were grown for 20 h at 30°C with shaking. Then, cells were harvested and washed twice with a sterile solution containing 0.5% bovine serum albumin (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. Cells were washed with 0.5% bovine serum albumin prior to cell density measurement at 600 nm. Aliquots of 10 mL were harvested at 4,500 g for 5 min in a Hettich table top centrifuge. Cells were washed with 0.5% bovine serum albumin and cell pellets were frozen at -20°C and then used for lipid extraction as described above. Dried lipid extracts were separated by TLC as described above. Bands of TG were scraped off, and after addition of 7 mL scintillation cocktail subjected to scintillation counting using LSC Safety (Baker, Deventer, The Netherlands) with 5% water as scintillation cocktail.

Lipase/esterase inhibitor assays

Specific inhibitors were used to screen for the occurrence of TG lipases (NBD-*sn1/3*-TGP, nitrobenz-2-oxa-1,3-diazole-*sn1/3*-triacylglyceride phosphonate) and hydrolases (NBD-HE-HP,*O*-((6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)aminoethyl-*P*-(n-hexyl)phosphonic acid *p*-nitrophenyl ester) (Oskolkova et al., 2003; Schmidinger et al., 2005, 2006a, 2006b). The reactive groups of the inhibitors bind specifically to the respective enzymes. The covalently linked enzyme-inhibitor complex can be detected through the NBD-tag. For the inhibitor assay, purified LD or Px samples were incubated with 0.1% (final concentration) of the respective inhibitors and 0.6% Triton X-100 in 50 mM TrisCl, pH 7.4, at 37°C on a thermomix shaker at 600 g overnight. Proteins were precipitated with 10% (final concentration) TCA and washed in cold acetone to remove all unbound inhibitors. The remaining sample was dissolved in 10 µL SDS-sample buffer (63 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 0.0025% Bromophenol blue, 2% β-mercaptoethanol) and separated by SDS-PAGE as described above. To identify fluorescent protein-inhibitor complexes in-gel, gels were scanned at 530 nm and at an excitation wavelength of 488 nm using an Imager FXProPlus (BIORAD). For total protein staining, gels were incubated with Sypro Ruby at room temperature for 1 h, and washed in acetic acid/ethanol for 2 h. The fluorescence was scanned at 605 nm (excitation wavelength of 488 nm). LC-MS/MS analyses were performed as described by Birner-Gruenberger *et al*. (Birner-Gruenberger et al., 2005). In addition, a detailed description of the method is provided in the supplemental information. Protein quantification was carried out as described above.

Protein expression, solubilization and purification

All candidate proteins were expressed from plasmid pYEX4T-1 in *S. cerevisiae* TM. GST-fusion proteins were purified batchwise by affinity chromatography using reduced glutathione agarose. Ayr1p and Eht1p were purified from LD fractions after solubilization by Zwittergent© (Sigma) at a final concentration of 20 mM; Lpx1p was solubilized from Px fractions with Zwittergent© at 10 mM; Ldh1p and YBR056wp were obtained from homogenates after solubilization at 2 mM. Reduced glutathione agarose beads (LifeTech-Invitrogen) were washed with PBS to remove azide and equilibrated with PBS containing 1% Triton X-100 at 4°C just prior to use. Solubilized fractions were applied to GST-beads and

incubated for 2 h with moderate shaking at 4°C. After washing with PBS, fusion proteins were eluted with 20 mM reduced glutathione (Sigma) in PBS, pH 8, at 4°C for 30 min.

Esterase activity assays using p-nitrophenyl esters as substrates

p-Nitrophenylacetate (pNPA), *p*-nitrophenylbutyrate (pNPB) and *p*-nitrophenypalmitate (pNPP) were used as substrates to determine esterase activity of purified proteins. The total reaction volume was 1 mL, containing 890 µL of 1x PBS buffer, 100 µL of substrate solution (ranging from 25 µM to 10 mM final concentrations), and 10 µL of purified protein, which was added directly prior to measurement. Absorbance was measured at 405 nm for 3 min at 30°C. Enzyme activity was determined using different substrate concentrations. Michaelis-Menten kinetics was analyzed using GraphPad Prism 5.

RNA isolation and Real Time PCR

For the isolation of RNA cells were grown to the mid logarithmic phase on YPD and YPO at 30°C. RNA was isolated using RNeasy kit from Qiagen as described by the manufacturer. After DNaseI digestion, Real Time PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) by following the manufacturer's instructions. Reactions were performed in sealed MicroAmp Optical 96-Well Reaction Plates, and amplification was measured using an ABI 7500 instrument (Applied Biosystems). Samples were quantified using the *ΔΔ*Ct method described by Livak and Schmittgen (Livak and Schmittgen, 2001). Differences in mRNA expression after *ACT1* normalization relative to the control can be calculated with this method. Primers used for Real Time-PCR are listed in Table 4-3.

Table 4-3: Primers for qRT-PCR.

Primer	Sequence (5'→3')
*ACT1*_RTfw	CCAGCCTTCTACGTTTCCATCCAAG
*ACT1*_RTrev	GACGTGAGTAACACCATCACCGGA
*LPX1*_RTfw	AACTCCCTACGTTTAAAGACGTGCGACCATTTC
*LPX1*_RTrev	CAGGATTTGGCTGTGCGCATTGGT
*LDH1*_RTfw	TCATAAGATAGTGCTTGTAGGGCATTCTATGGGTT
*LDH1*_RTrev	GCGTCAACAAAACTAGCGTTTGTACCGCT
*YJU3*_RTfw	AGGAATCACATCTGATAAAGCCTATC
*YJU3*_RTrev	TCGTGTATTTGCCTAAACGACCCA
*AYR1*_RTfw	GCTGATAAAAGGCCCTTGCCTGAAACCTCAA
*AYR1*_RTrev	CAGCTGGCATGGGCTTATTGTCCTTAGC
*EHT1*_RTfw	TTCCAAATGGCTCTCTCCCCGATCA
*EHT1*_RTrev	TTCCCGGGTCGATTTAAAGCTCTTTG
*TSC10*_RTfw	CAAGTCGCTGGCCAGAGGTGATGA
*TSC10*_RTrev	AGCGGCTTTTCTTTGCGGTGAGC
YBR056w_RTfw	GGACTTTGCAGTTTGAATACGGTG
YBR056w_RTfw	AAGTCATCACCGTGAGGA
YKL050c_RTfw	GCGTGTTGGATGATGGGCCTAA
YKL050c_RTrev	CGGTGCCATCTACTGCTTGGTGCAC

In vivo TG mobilization assays

TG mobilization of strains overexpressing putative lipases/esterases was measured *in vivo* by letting cells grow until the early stationary phase in synthetic minimal medium. Then, fresh medium was inoculated with the pre-grown cultures to an A_{600} of 3. For the mobilization of TG the fatty acid synthesis inhibitor cerulenin was added to a final concentration of 10 µg/mL. At time points indicated, 10 mL aliquots were taken, cells were harvested and lipids were extracted and analyzed as described above.

Triacylglycerol lipase activity assays using [9,10-^{3}H]triolein as substrate

TG lipase activity of isolated LD and purified enzymes was determined using [9,10-^{3}H]triolein (Perkin Elmer) as substrate. TG lipase activity was measured in a final volume of 200 µL. Samples were incubated in a mixture containing 100 mM potassium phosphate buffer, pH 7.5, 125 µM [9,10-^{3}H]triolein (specific activity 50 µCi/mL), 45 µM phosphatidyl-choline/phosphatidylinositol (3:1; mol/mol), 25 mM $MgCl_2$ and 0.2% fatty acid free bovine serum albumin at 30°C for 1 h in a water bath. The reaction was stopped by adding 2 mL chloroform/methanol (2:1, v/v.), and lipids were extracted as described above. Lipids were dried under a stream of nitrogen, dissolved in 50 µL chloroform/methanol (2:1, v/v) and

separated by TLC. Chromatograms were developed using chloroform/acetone/acetic acid (45:4:0.4; per vol.) as a solvent system. Lipids were visualized by staining with iodine vapor, and bands corresponding to fatty acids were scraped off the plate. Radioactivity was measured by liquid scintillation counting using LSC Safety (Baker, Deventer, The Netherlands) with 5% water as scintillation cocktail. Enzyme activity was further determined depending on the triolein substrate concentration (0.2-250 µM) and time (1 to 200 min). Michaelis-Menten kinetics was analyzed using GraphPad Prism 5.

RESULTS

A yeast strain lacking all currently known TG lipases is able to mobilize TG *in vivo*

The mobilization of TG from LD requires the catalytic activities of TG lipases. Previous research from our laboratory identified Tgl3p as major TG lipase and the two enzymes Tgl4p as well as Tgl5p showing minor lipolytic activities (Athenstaedt and Daum, 2003, 2005). In the course of these studies, however, it became evident that the turnover of TG in a *Δtgl3Δtgl4Δtgl5* mutant (TM) did not come to a complete halt when cells were forced to mobilize TG in the presence of cerulenin, an inhibitor of fatty acid synthesis, suggesting the presence of additional TG lipases. To test this hypothesis, we constructed a mutant strain lacking both acyl-CoA:sterol acyltransferases, Are1p and Are2p, as well as the major known TG lipases Tgl3p, Tgl4p, and Tgl5p and performed a pulse chase assay monitoring the fate of TG *in vivo*. This quintuple mutant (QiM) accumulated high levels of TG but did not produce any SE ensuring that incorporation of [^{3}H]oleate occurred primarily into TG, and loss of label due to incorporation into other compounds was minimized. Cells were pulsed labeled with [^{3}H]oleic acid, and levels of ^{3}H in TG were followed during the chase on unlabeled YPO. Both wild type and QiM grew normally under the given conditions (Figure 4.1A). As expected, in wild type cells the label in TG decreased over the time indicating that TG was subject to turnover. Interestingly, in the QiM, lacking all known TG lipases, TG mobilization was observed with a slight delay but at a similar rate as wild type (Figure 4.1B). Thus, we concluded that in cells grown on oleic acid one or more additional lipases might become active accounting for TG mobilization.

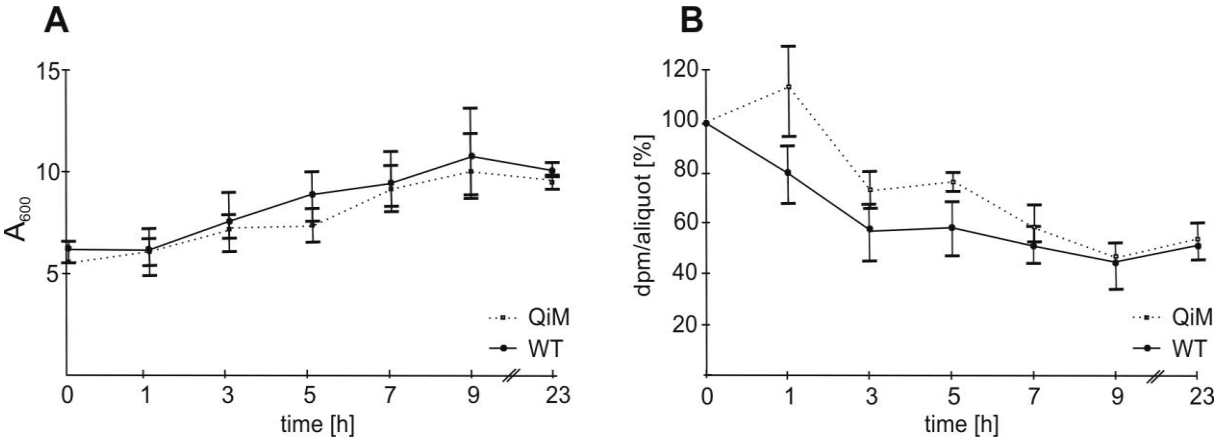

Figure 4.1: *In vivo* triacylglycerol mobilization. Cells from either wild type (WT) or the quintuple mutant (*Δtgl3Δtgl4Δtgl5Δare1Δare2*; QiM) were grown in the presence of [^{3}H]oleate for 5 h and then shifted to fresh YPO medium. At time points indicated, aliquots were withdrawn and analyzed for cellular density (A) and for the amount of [^{3}H]TG (B). Data were obtained from two independent experiments with standard errors as indicated

Screening for novel lipases and hydrolases in LD and Px

The findings described above led us to search for novel TG lipases/hydrolases in the yeast. To test whether such enzymes became active in yeast grown on oleic acid we employed a functional proteome assay making use of fluorescently labeled inhibitors of serine lipid hydrolases as probes (Schmidinger et al., 2005, 2006a). Isolated organelles from the yeast were used to screen for such novel enzymes. LD from cells grown on glucose or oleic acid, respectively, as well as Px from YPO grown cells were incubated with fluorescent phosphonic acid esters (NBD-*sn1/3*-TGP, NBD-HE-HP) which covalently bind to the active sites of serine lipid hydrolases. After incubation, proteins were separated by SDS-PAGE and labeled polypeptides were identified by the NBD-reporter tag. In LD from wild type 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 4.2A, asterisks). Moreover, bands representing possible new lipases were identified as well (Figure 4.2A, arrows). A comparison of the two growth conditions revealed that the protein pattern of cells grown on YPO was different from YPD. We concluded from these data that indeed a different subset of proteins existed when cells were grown in the presence of oleic acid compared to glucose. To follow up this idea, we analyzed LD from a TM grown on YPD or YPO, respectively. Again, we were able to detect additional protein bands which did not correspond to the already known TG lipases (Figure 4.2B, arrows).

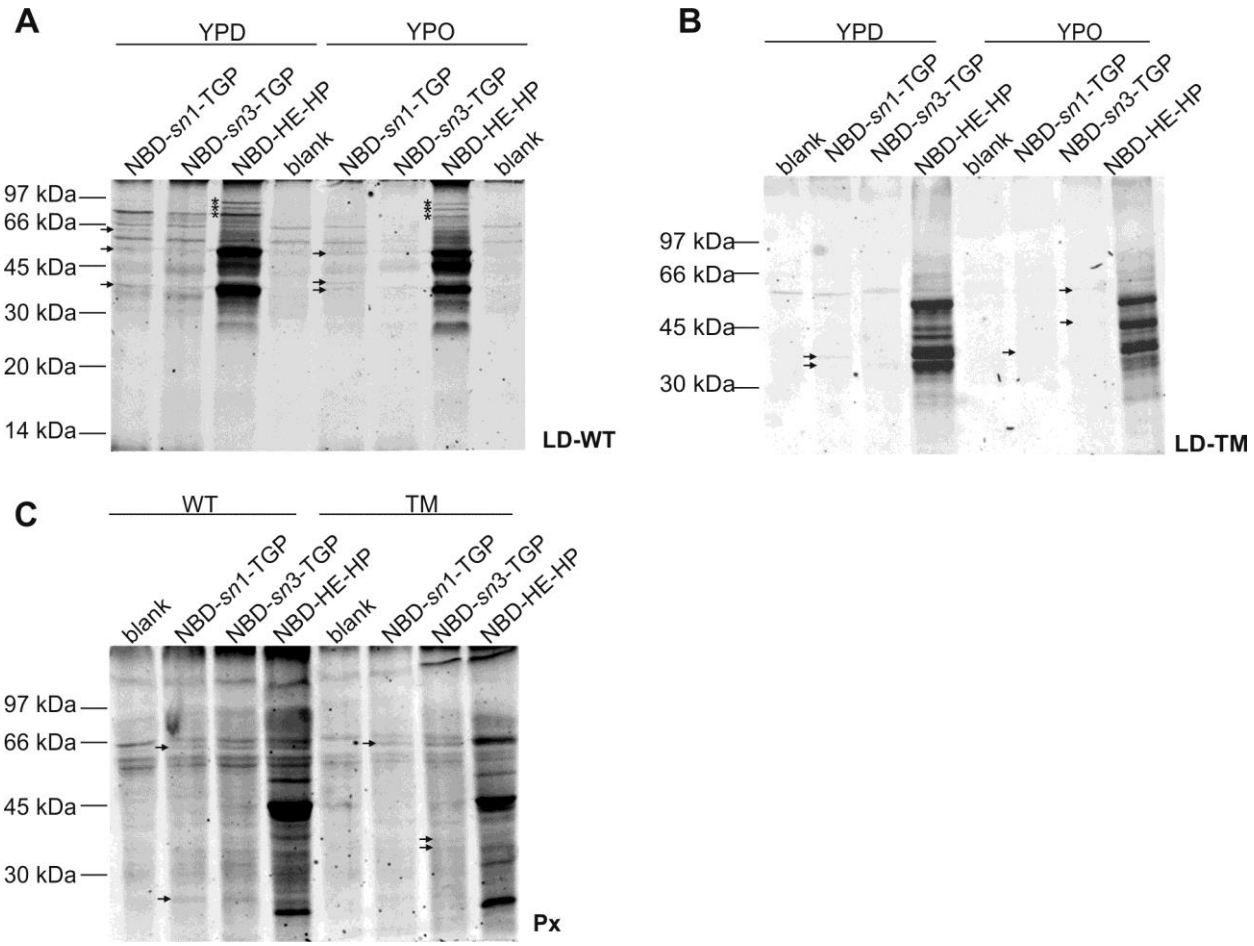

Figure 4.2: *In vitro* inhibitor assay screening for novel hydrolases from lipid droplets and peroxisomes. Lipid droplets (LD) and peroxisomes (Px) from WT and TM were isolated from cells grown on YPD or YPO. Aliquots were incubated with inhibitors as described and incubated at 37°C overnight. Proteins were then precipitated and separated by SDS-PAGE. Gels were scanned at a wavelength characteristic for NBD. (A) LD protein pattern from WT cells after incubation with inhibitors for TG lipases or hydrolases from YPD and YPO grown cells. Asterisks indicate the occurrence of the three known TG lipases Tgl3p (73 kDa), Tgl4p (102 kDa), and Tgl5p (84 kDa). Arrows show the formation of additional bands indicating the presence of additional lipases or hydrolases. Blank shows the background of proteins incubated with no inhibitor. (B) LD protein pattern from TM cells grown on either YPD or YPO. Arrows indicate the occurrence of new hydrolases/lipases. (C) Px from either WT or TM grown on YPO were incubated with a subset of inhibitors and analyzed for formation of protein bands indicating lipases or esterases (arrows). NBD-sn1(sn3)-TGP, nitrobenz-2-oxa-1,3-diazole-sn1(sn3)-triglyceride phosphonate; NBD-HE-HP, O-((6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl)aminoethyl-P-(n hexyl)phosphonic acid p-nitrophenyl ester; WT, wild type; TM, triple mutant; LD, lipid droplets; Px, peroxisomes.

Similar results were obtained when we analyzed Px from wild type and TM with the same subset of inhibitors (Figure 4.2C). Arrows indicate the occurrence of novel proteins exhibiting an esterase (NBD-HE-HP) or even lipase (NBD-*sn1/3*-TGP) activity. Proteins detected were of different molecular mass compared to those from LD. Thus, we speculated that on LD and Px

distinct hydrolases were expressed under oleic acid induced conditions. Altogether, our screening apparently identified new and not yet characterized candidates for novel hydrolases/ lipases on LD and Px. To identify these proteins reacting with NBD-HE-HP, they were separated by SDS-PAGE (Figure 4.3A), tryptically digested and subjected to MS analysis. A complete list of proteins identified by this approach is shown in the Supplementary Table S1. Proteins are listed according to their respective molecular weight as well as by peptide length and score value. All known TG lipases were identified with high abundance in LD samples. One exception was Tgl5p, which was found only on LD from cells grown on YPD. Interestingly, only a few gene products were detected in this screening in more than one organelle sample (see Supplementary Table S1).

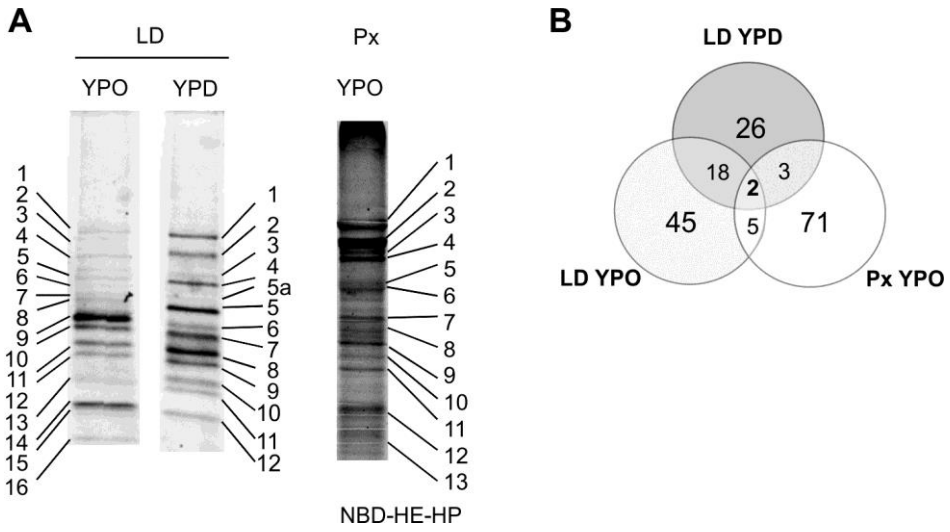

Figure 4.3: Mass spectrometric analysis of novel lipase/esterase candidates. LD and Px were prepared and incubated with NBD-HE-HP which resembles a single chain carboxylic acid ester as described in Experimental Procedures. (A) Proteins were separated by SDS-PAGE and fluorescent bands were cut from the gel, subjected to tryptic digestion and analyzed by MS/MS. (B) Venn diagram showing the number of proteins found in either LD from YPD, LD from YPO or Px from YPO indicating the overlapping hits. LD, lipid droplets; NBD-HE-HP, *O*-((6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl)aminoethyl-*P*-(n hexyl)phosphonic acid *p*-nitrophenyl ester; Px, peroxisomes.

As expected, both LD samples showed the highest overlap in protein candidates (Figure 4.3B). Eighteen candidate proteins were found on LD from cells grown on both carbon sources. These proteins represented putative new candidate hydrolases or lipases under oleic acid-induced conditions. The most promising candidates, containing a TG lipase motif (GxSxG) and showing high abundance in the performed screening were selected for further

investigations. Further analysis was therefore restricted to Lpx1p, Ldh1p, Yju3p, Ayr1p, Eht1p, Tsc10p, Ybr056wp and Ykl050cp (Table 4-4).

Table 4-4: Short list of candidate hydrolases. List of putative new lipases or hydrolases on LD from yeast cells grown on either YPD or YPO. The selection was based on motif, localization, abundance in the inhibitor assay screening and already known functions. TGL, triacylglycerol lipase motif (GxSxG); HYD, hydrolase motif (DxDxT/V); LD, lipid droplets, Mt, mitochondrion; Cyt, cytosol; ER, endoplasmic reticulum; N, nucleus.

ORF	Gene Name	Localization	Motif
YOR084W	*LPX1*	Px	TGL
YBR204C	*LDH1*	LD, Mt, Px	TGL, HYD
YKL094W	*YJU3*	LD	TGL (2x)
YIL124W	*AYR1*	ER, LD, Mt, Cyt	TGL
YBR177C	*EHT1*	LD, Mt	TGL
YBR265W	*TSC10*	Cyt, ER, Mt	TGL
YBR056W	-	Cyt	TGL
YKL050C	-	unknown	TGL

Since previous studies from our laboratory described functions of Tgl3p, Tgl4p and Tgl5p in addition to their lipase activities we speculated about dual functions and localizations of other proteins involved in non-polar lipid metabolism (Rajakumari and Daum, 2010a, 2010b). Lpx1p is an oleic acid-inducible lipase that localizes to the peroxisomal matrix and is required for normal Px morphology. However, the reaction catalyzed by Lpx1p could not be identified so far. In previous studies (Thoms et al., 2008) Lpx1p was heterologously expressed and purified from *E. coli*. The purified protein was tested for esterase, TG lipase as well as phospholipase C and D activities, but only weak lipolytic activity was measured *in vitro*. Therefore, Lpx1p was chosen as a positive control for enzymes with minor TG lipase activity *in vivo*.

Ldh1p was reported as a hydrolase with a GxSxG type motif, primarily localized to LD exerting esterase and weak TG lipase activities *in vitro* (Debelyy et al., 2011; Thoms et al., 2011). Yju3p was identified as a functional orthologue of mammalian monoacylglycerol lipase specifically hydrolyzing monoacylglycerols (Heier et al., 2010). Since TG lipase activity of Yju3p was unlikely it served as our negative control. Ayr1p has been described as NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase found on LD, in the ER, and the mitochondrial outer membrane involved in phosphatidic acid biosynthesis (Athenstaedt and

Daum, 2000). In addition, Ayr1p was shown to display 17 beta-hydroxysteroid dehydrogenase activity (Vico et al., 2002). Eht1p localizes to LD and the mitochondrial outer membrane and has been annotated as acyl-coenzymeA:ethanol *O*-acyltransferase (Athenstaedt et al., 1999; Saerens et al., 2006; Zahedi et al., 2006). It plays a minor role in medium-chain fatty acid ethyl ester biosynthesis and possesses esterase activity on short-chain substrates. Tsc10p is a ketosphinganine reductase which catalyzes the second step in phytosphingosine synthesis (Beeler et al., 1998). Ybr056wp and Ykl050cp are two proteins of currently unknown functions.

Gene expression of candidate genes in cells grown on YPO

The different protein patterns of LD from cells grown on YPD or YPO, respectively, suggested that different proteins may be active on the different carbon sources. To address this question the expression levels of the candidate proteins in wild type cultivated on YPD and YPO were compared. All candidate proteins showed an increased expression level on YPO (Table 4-5). This effect, which we called here the “YPO induction factor”, became most obvious for *LPX1*. This result can be explained by the fact that Lpx1p is a peroxisomal protein. As Px strongly proliferate in cells grown on YPO these increased mRNA levels of peroxisomal proteins were not surprising. Interestingly, the unannotated genes YBR056w and YKL050c also showed a very high expression in cells grown on YPO. *AYR1* became especially interesting since it showed a five-fold increase in the relative expression level on YPO. In a second set of experiments, we compared expression levels of candidate genes in the TM cultivated on YPD or YPO, respectively (Table 4-5). The increase of mRNA levels of *LPX1* could again be observed but was less pronounced than in wild type. *LDH1*, *EHT1*, *YKL050c* and *AYR1* also showed significantly elevated levels of mRNA in cells grown on YPO.

Table 4-5: "YPO Induction Factor" of putative hydrolases in wild type and TM. Relative gene expression of *LPX1*, *LDH1*, *YJU3*, *AYR1*, *EHT1*, *TSC10*, YBR056w and YKL050c was measured by qRT-PCR from isolated RNA of WT and TM, respectively, cultivated on YPD and YPO. Expression of the respective genes in YPD grown cells was set at 1 and values obtained with cells grown on YPO were set in relation ("YPO induction factor"). Data are mean values from three independent experiments with the respective deviations <5%. WT, wild type; TM, triple mutant (*Δtgl3Δtgl4Δtgl5*)

YPO Induction Factor		
Gene	**WT**	**TM**
LPX1	140	63
LDH1	4	2.5
YJU3	4	1
AYR1	5	3
EHT1	3	3
TSC10	2	1
YBR056w	20	2
YKL050c	40	3

In vitro esterase assays confirm hydrolytic activities of candidate proteins

The eight candidate genes described above were overexpressed in a *Δtgl3Δtgl4Δtgl5* background (TM) and purified to perform enzyme analyses. For this purpose, full-length proteins were expressed as fusion proteins with an N-terminal GST tag in *S. cerevisiae* (Figure 4.4A) and purified using affinity chromatography (Figure 4.4B). Lpx1p was successfully purified from peroxisomal fractions, Ayr1p and Eht1p from LD samples and Ldh1p and Ybr056wp were isolated from whole cell extracts. Yju3p, Tsc10p and Ykl050c could not be purified at sufficient amount and purity.

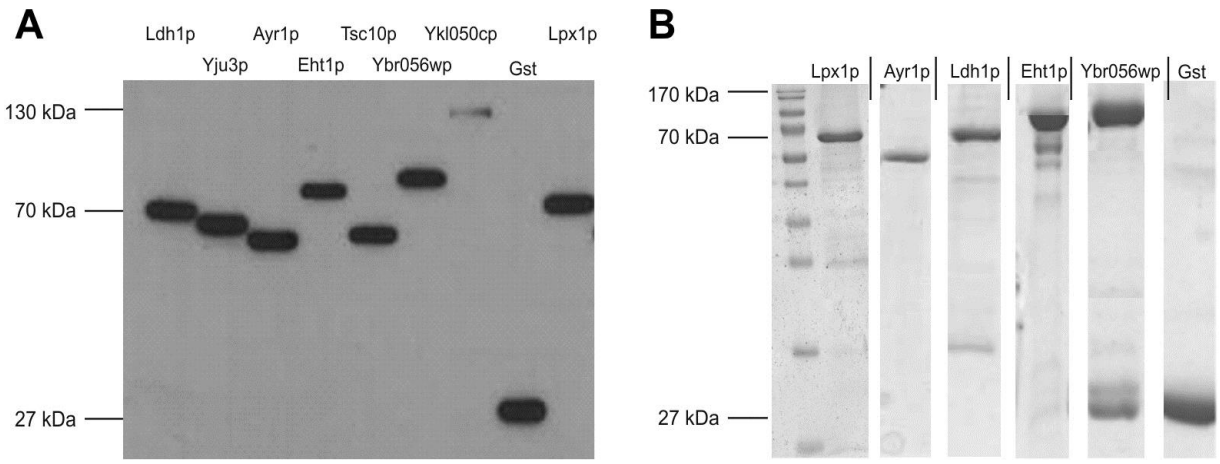

Figure 4.4: Protein expression and purification of novel triacylglycerol lipase candidates. (A) Western Blot analysis from total cell proteins from overexpressing strains grown to stationary phase after induction with $CuSO_4$ for 4 h. Putative hydrolases were expressed as fusion proteins with a GST tag and purified after induction by affinity chromatography. Western blot analyses are representative for at least two independent experiments. (B) Coomassie Blue stained SDS Gel of purified proteins: Lpx1p was purified from Px, Ayr1p and Eht1p were isolated from LD, Ybr056wp and the tag itself as a control were purified from homogenates. All other proteins could not be purified at sufficient amount or purity.

Purified enzymes were analyzed for esterase activities using *p*-nitrophenyl butyrate (pNPB) as well as *p*-nitrophenyl acetate (pNPA) as substrates (Table 4-6). Lpx1p hydrolyzed pNPB with a K_M of 0.89 mM and a v_{max} of 3.76 µmol/min/mg, which resembled values reported by Thoms *et al*. (Thoms et al., 2008). Higher activities were found with pNPA as substrate. The highest activities were found for Ayr1p, which cleaved pNPA with a K_M of 3.50 mM and a v_{max} of 18.50 µmol/min/mg; and pNPB with a K_M of 1.52 mM and a v_{max} of 14.06 µmol/min/mg. All other enzymes tested showed only minor hydrolytic activities with these substrates. *p*-Nitrophenyl palmitate was not converted by any of the enzymes (data not shown). Control assays with the GST tag showed that the tag alone did not affect our results.

Table 4-6: *In vitro* esterase activities. Esterase activity was measured from purified enzymes using pNPB (p-nitrophenyl butyrate) and pNPA (p-nitrophenylacetate) as test substrates. K_M and v_{max} values were calculated using Michaelis-Menten approximations. Data are mean values from three independent experiments with the respective standard deviations.

Enzyme	Substrate	v_{max} [µmol/min/mg]	K_M [mM]
Lpx1p	pNPB	3.76 ± 0.54	0.89 ± 0.13
	pNPA	10.94 ± 0.98	2.50 ± 0.22
Ldh1p	pNPB	0.90 ±0.15	0.89 ± 0.15
	pNPA	1.26 ± 0.18	1.78 ± 0.25
Ayr1p	pNPB	14.06 ± 2.23	1.52 ± 0.24
	pNPA	18.50 ± 2.76	3.50 ± 0.52
Eht1p	pNPB	1.01 ± 0.14	0.88 ± 0.12
	pNPA	3.44 ± 0.48	2.06 ± 0.29
Ybr056wp	pNPB	1.439 ± 0.22	0.81 ± 0.12
	pNPA	7.02 ± 1.26	3.51 ± 0.63
Control: GST	pNPB	0.35 ± 0.05	0.37 ± 0.05

Overexpression of candidate genes affects TG content of yeast cells in the stationary phase

Since TG mobilization is already strongly impaired in a TM strain, additional major lipolytic activities were not expected. To make possible effects of our candidates visible we chose overexpression of these genes in the TM background rather than additional deletions. Analysis of non-polar lipids from whole cell extracts was performed in cells grown to the stationary phase under these conditions. The TG content in the TM was increased to ~150% of wild type (Figure 4.5). Strains overexpressing *LPX1*$^+$, *LDH1*$^+$, *AYR1*$^+$, *TSC10*$^+$, YBR056w$^+$ and YKL050c$^+$ showed a reduction of TG of about 30% compared to the TM. In contrast, overexpression of *YJU3*$^+$ and *EHT1*$^+$ did not significantly change the level of TG (Figure 4.5). Lpx1p and Ldh1p had already been described as new TG lipases (Debelyy et al., 2011; Thoms et al., 2008, 2011). As expected deletion of the respective genes in addition to *TGL3*, *TGL4* and *TGL5* did not show an effect on growth in the presence of glucose or oleic acid as carbon source nor on the TG content compared to the TM (data not shown).

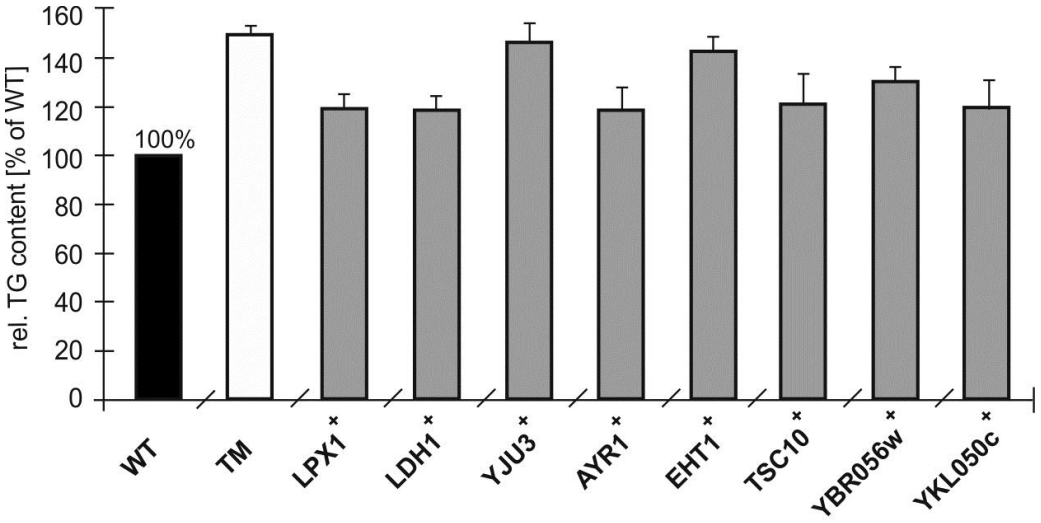

Figure 4.5: Triacylglycerol levels of strains overexpressing candidate lipases. Cells from WT, TM and strains overexpressing putative novel TG lipases in the TM background were grown in minimal medium until early stationary phase. Total cell and lipid extracts were obtained as described in Experimental Procedures. Lipids were separated by TLC and quantified densitometrically. Results shown are average from at least three independent experiments with the standard deviations indicated.

In vivo mobilization of TG identifies Ayr1p as novel TG lipase

Changes in the TG content of strains overexpressing putative TG lipases suggested that these proteins also exhibit lipolytic activities *in vivo*. Therefore, we examined the mobilization of TG in the presence of the fatty acid synthase inhibitor cerulenin with all overexpressing strains described above. Since synthesis of fatty acids is inhibited under these conditions, cells are forced to mobilize TG to ensure sufficient supply for the synthesis of membrane lipids. At the same time the synthesis of TG is blocked. Degradation of TG was compared both to the wild type and the TM. Wild type mobilized TG very quickly and showed a nearly complete depletion of TG within 7 h, whereas the TM showed poor mobilization which leveled off at 80% of the initial value after this time period. This result confirmed previous studies from our laboratory (Athenstaedt and Daum, 2005). Furthermore, we observed that *LPX1*$^{+}$ and *AYR1*$^{+}$ showed a significantly altered mobilization rate compared to the TM. The TG level of the *AYR1* overexpressing strain dropped to 53%, the strain overexpressing *LPX1* showed even enhanced mobilization to 40% of the starting value (Figure 4.6A). All other overexpression strains did not show more mobilization of TG than the TM (Table 4-7). These results demonstrated that Lpx1p acts as a TG lipase *in vivo* confirming previous results

obtained *in vitro* (Thoms et al., 2008). Additionally, the role of Ayr1p as novel TG lipase of the yeast was fostered. In parallel to the determination of the cellular TG content, growth experiments were performed to exhibit sensitivity to cerulenin. All overexpression strains showed a similar growth behavior as the TM, which was more sensitive to cerulenin than the wild type (Figure 4.6B). In the absence of cerulenin all strains grew like wild type (data not shown).

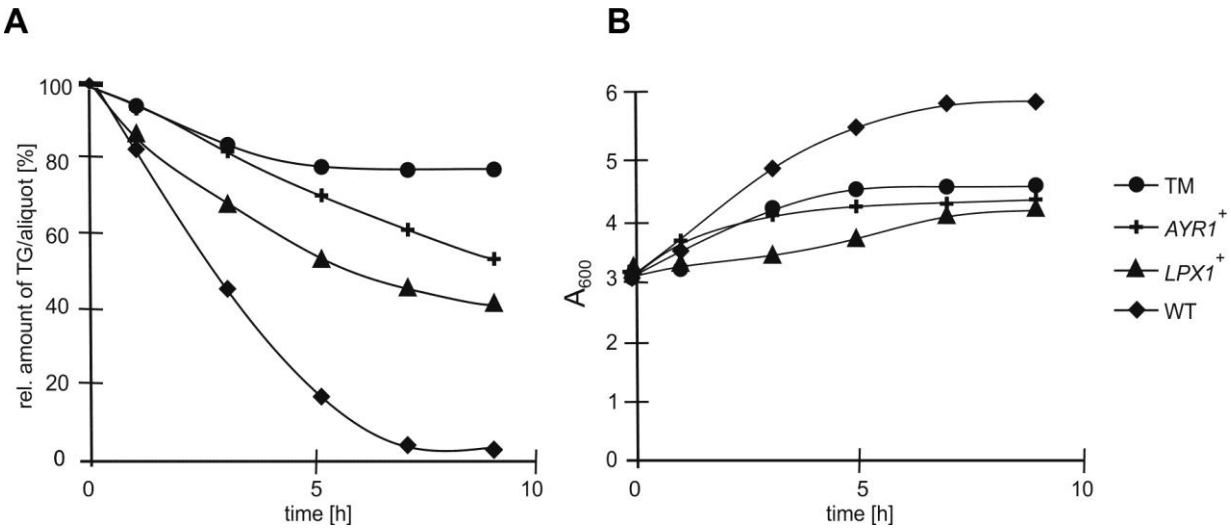

Figure 4.6*: In vivo* triacylglycerol mobilization in the presence of cerulenin. Cellular TG levels (A) and growth curves (B) of WT, TM, and the two overexpressing strains TM+*AYR1*$^+$ and TM+*LPX1*$^+$ in the presence of cerulenin (inhibitor of fatty acid synthesis) are shown. Data are expressed as relative amounts of TG per culture aliquot with the value at the time point zero set at 100%. Analyses are representative of at least four independent experiments. Data are mean values with respective standard deviations of about 10%.

Table 4-7: Triacylglycerol mobilization of overexpressing strains in the presence of cerulenin. Strains overexpressing putative new lipases as well as the TM were grown in the presence of cerulenin. Culture aliquots were taken between 0 and 7 h and analyzed for TG content as described in the Experimental Procedures. TG content at the starting point was set as 100% and followed over time. Results shown are average from at least three independent experiments. Standard deviations were <10%.

Strain	TG content after 7 h [% of starting point]
Δtgl3Δtgl4Δtgl5 **(VC)**	**80**
TM+ *LPX1*$^{+}$	40
TM+ *LDH1*$^{+}$	70
TM+ *YJU3*$^{+}$	75
TM+ *AYR1*$^{+}$	53
TM+ *EHT1*$^{+}$	70
TM+ *TSC10*$^{+}$	80
TM+ YBR056w^{+}	70
TM+ YKL050c^{+}	70

In vitro enzyme assays confirm Ayr1p as novel TG lipase

Having shown that Lpx1p and Ayr1p exhibit lipolytic activities *in vivo*, we also tested them for *in vitro* TG lipase activity. First, we tested lipase activity of purified Ayr1p and Lpx1p. It had previously been reported that Lpx1p exhibits lipase activity using 1,2-dioleoyl-3-(pyren-1-yl)decanoyl-*rac*-glycerol as substrate (Thoms et al., 2008). Therefore, Lpx1p served as a positive control. Purified Ayr1p showed an even higher TG lipase activity than Lpx1p (Figure 4.7A). To confirm lipase activity of Ayr1p, LD fractions from the *AYR1* overexpressing strain were compared to wild type and TM (Figure 4.7B). In agreement with our data of the *in vivo* TG mobilization assays as well as with results obtained with purified enzymes, LD from *AYR1*$^{+}$ showed increased TG mobilization activity compared to the TM, reaching the same level as wild type. Michalis-Menten enzyme analysis revealed Ayr1p to be an active TG lipase hydrolyzing [9,10-^{3}H]triolein with a v_{max} of 10.93 pmol/h/mg and a K_M of 61.21 µM. Linear increase of product formation was observed up to 100 min.

Ayr1p possesses the characteristic GxSxG motif. To determine the functional significance of this conserved sequence, a point mutation was introduced (S18A), and the purified mutated

protein was tested for lipase activity as described above. Replacement of serine by alanine in the lipase motif of Ayr1p completely abolished lipase activity (Figure 4.7C).

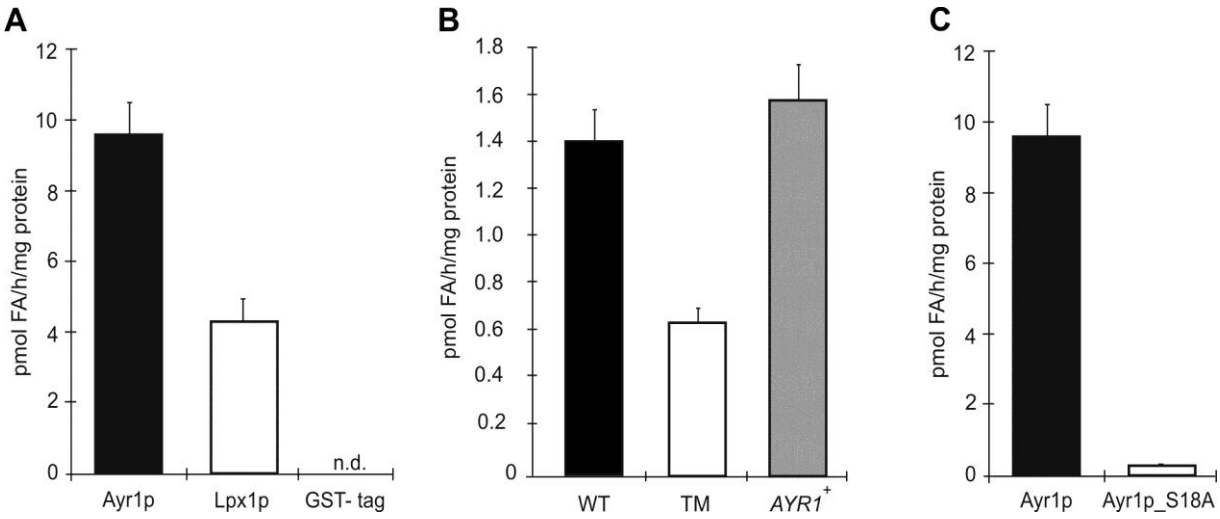

Figure 4.7: *In vitro* triacylglycerol lipase assays (A) Analysis of TG lipase activity of purified Ayr1p and Lpx1p compared to the tag control. (B) Analysis of TG lipase activity of LD fractions from WT, TM and TM+*AYR1*$^{+}$. (C) Analysis of TG lipase activity of mutated Ayr1p (S18A) compared to wild type Ayr1p. Experiments were performed in triplicate and are representative of at least two independent biological experiments. Data are mean values with the respective deviation. n.d., not detectable.

Altogether, we provide evidence for additional hydrolytic enzymes in the yeast *S. cerevisiae* involved in non-polar lipid metabolism. Moreover, we show that Lpx1p exhibits lipolytic activity not only *in vitro* as described by Thoms *et al* (Thoms et al., 2008) but also *in vivo*. Identification of Ayr1p as novel TG lipase of the yeast *S. cerevisiae* provides an additional piece of evidence in the puzzle of non-polar lipid metabolism.

DISCUSSION

Previous studies from our lab identified Tgl3p, Tgl4p and Tgl5p as major TG lipases of the yeast *S. cerevisiae* being localized to LD (Athenstaedt and Daum, 2003, 2005). In triple deletion strains lacking these major lipolytic enzymes, TG mobilization was strongly impaired but not completely abolished when cells were grown on glucose. Here, we show that the QiM lacking the three TG lipases and the sterol acyltransferases Are1p and Are2p grown on oleic acid is not only able to hydrolyze TG *in vivo*, but also to mobilize TG close to wild type level (see Figure 4.1). This result tempted us to speculate about the presence of further hydrolytic enzymes.

A major goal of the present study was to identify enzymes which might play a role in the mobilization of TG in the absence of the three major lipases. To address this question, we designed a functional proteome approach making use of specific fluorescent inhibitors of esterases and lipases as probes. This study led to the detection of a subset of proteins that showed TG hydrolase activity *in vitro* on isolated LD but also with Px (see Figure 4.2). Although these probes specifically recognized catalytic serine residues, detection of false positives could not be excluded. We were also aware that non-catalytic proteins were detected by chance due to the overlap with reactive enzyme bands on the gel. However, the sensitivity and selectivity of the assay was proven to be good enough because all known TG lipases were identified although Yeh1p, a steryl ester hydrolase of LD, was not found. Detection of all prominent LD and peroxisomal proteins in the respective samples served as a proof of purity of the isolated organelles. However, our screening detected additional, so far not annotated proteins on LD or in Px. Our findings complement a previous study from our laboratory aimed at the completion of the LD proteome (Grillitsch et al., 2011). Data provided in the present study also support the view that many proteins may have multiple localizations and functions in the cell. Regulatory mechanisms governing localization and function of proteins in different compartments have to be taken into account. One recent example of such aspects is the stability, gene expression and localization of Tgl3p depending on the availability and the presence of its substrate TG (Schmidt et al., 2013).

The list of proteins found in our screening was narrowed to a subset of proteins with putative hydrolase or lipase function (see Table 4-4) based on computational sequence

analysis, assigned functions and subcellular localizations. Nevertheless, proteins of already annotated localization and function different from non-polar lipid metabolism were included. The reason for this strategy was the discovery of a dual role for the known TG lipases Tgl3p, Tgl4p and Tgl5p, which also possess high acyltransferase activities (Rajakumari and Daum, 2010a, 2010b). Consequently, other proteins might have multiple functions, too, maybe not only on LD but also in other organelles. The short list of putative novel TG lipases included Lpx1p, Ldh1p, Yju3p, Ayr1p, Eht1p, Tsc10p, Ybr056wp and Ykl050c. All these proteins contain the consensus sequence for a classical lipase (GxSxG motif) with a classical catalytic triad containing a nucleophile serine. Lpx1p and Ldh1p had been described previously as lipases based on *in vitro* analysis, and Lpx1p was localized on Px, whereas Ldh1p was identified as a LD component (Debelyy et al., 2011; Thoms et al., 2008, 2011).

One major question, of course, was the physiological role or relevance of the newly detected hydrolytic/lipolytic enzymes. The TG content analysis of whole cell extracts from overexpression strains indeed proposed a role for Lpx1p, Ldh1p, Ayr1p, Tsc10p, Ybr056wp and Ykl050cp in TG mobilization in the absence of the major lipases at least in the stationary growth phase (see Figure 4.5). *In vivo* mobilization of TG by Lpx1p and Ayr1p confirmed the role of these two proteins as lipases in living cells, whereas all other enzymes did not exhibit lipolytic activities *in vivo* (see Figure 4.6 & Table 4-7). This result was perfectly in line with *in vitro* enzyme assays (see Figure 4.7). Moreover, these two proteins showed the highest activities when esterase substrates were used (Table 4-6). Interestingly, a yeast strain lacking not only in the major lipases Tgl3p, Tgl4p and Tgl5p but also Ayr1p, Lpx1p or Ldh1p, respectively, still showed mobilization of TG. This finding supported the view that other, most likely minor lipolytic activities set fatty acids free as supply for energy production and/or synthesis of membrane lipid components. This view was supported by gene expression studies. In these experiments, Lpx1p and Ayr1p were among the most strongly induced genes in the TM grown on oleic acid compared to cells grown on glucose containing medium (see Table 4-5). The question remains why TG are mobilized in cells suffering already from an excess of free fatty acids by supply from the medium. Currently, we can only speculate that different pools of lipids exist which are mobilized upon different requirements. One possibility might be that fatty acids mobilized from TG may serve primarily for phospholipid synthesis or β-oxidation. However, proof for this model is still

missing. In a previous study it had already been reported that growth on oleate led to accumulation of large amounts of free fatty acids (Connerth et al., 2010). From this finding it can be assumed that free fatty acids do not play an essential role in regulating the non-polar lipid turnover as TG mobilization occurred normally under these conditions.

In summary, our results suggest that TG turnover is not only catalyzed by the main lipases Tgl3p, Tgl4p and Tgl5p but is also performed by a subset of lipases and hydrolases with lower activities. Our data demonstrate that the action of such enzymes is strongly dependent on growth conditions and that they may come to the fore when the major enzymes are inactive. Finally, our investigations identified Ayr1p as a novel TG lipase and confirmed the role of Lpx1p as lipase *in vivo*. These data contribute to a more detailed knowledge of non-polar lipid turnover and set the stage to investigate regulatory aspects of non-polar lipid metabolism in more detail.

ACKNOWLEDGEMENTS

We thank the Austrian Centre of Industrial Biotechnology (ACIB) Graz for providing the ABI 7500 instrument and Karin Athenstaedt for fruitful discussions.

* This research was funded by the Austrian Science Fund (FWF) projects W901 DK Molecular Enzymology and P23029 to G. D., the Austrian Federal Ministry of Science and Research (bmwf) (GOLD project in the framework of GEN-AU/Genome Research in Austria) and the Austrian Science Funds, FWF (project F3005 SFB LIPOTOX).

REFERENCES

Athenstaedt, K., and Daum, G. (2000). 1-Acyldihydroxyacetone-phosphate reductase (Ayr1p) of the yeast *Saccharomyces cerevisiae* encoded by the open reading frame YIL124w is a major component of lipid particles. J. Biol. Chem. *275*, 235–240.

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., 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.

Beeler, T., Bacikova, D., Gable, K., Hopkins, L., Johnson, C., Slife, H., and Dunn, T. (1998). The *Saccharomyces cerevisiae TSC10*/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca^{2+}-sensitive csg2Delta mutant. J. Biol. Chem. *273*, 30688–30694.

Binns, D., Januszewski, T., Chen, Y., Hill, J., Markin, V.S., Zhao, Y., Gilpin, C., Chapman, K.D., Anderson, R.G.W., and Goodman, J.M. (2006). An intimate collaboration between peroxisomes and lipid bodies. J. Cell Biol. *173*, 719–731.

Birner-Gruenberger, R., Susani-Etzerodt, H., Waldhuber, M., Riesenhuber, G., Schmidinger, H., Rechberger, G., Kollroser, M., Strauss, J.G., Lass, A., Zimmermann, R., et al. (2005). The lipolytic proteome of mouse adipose tissue. Mol. Cell. Proteomics MCP *4*, 1710–1717.

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

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

Connerth, M., Czabany, T., Wagner, A., Zellnig, G., Leitner, E., Steyrer, E., and Daum, G. (2010). Oleate inhibits steryl ester synthesis and causes liposensitivity in yeast. J. Biol. Chem. *285*, 26832–26841.

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.

Debelyy, M.O., Thoms, S., Connerth, M., Daum, G., and Erdmann, R. (2011). Involvement of the *Saccharomyces cerevisiae* hydrolase Ldh1p in lipid homeostasis. Eukaryot. Cell *10*, 776–781.

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

Gietz, R.D., Schiestl, R.H., Willems, A.R., and Woods, R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast *11*, 355–360.

Grillitsch, K., Connerth, M., Köfeler, H., Arrey, T.N., Rietschel, B., Wagner, B., Karas, M., and Daum, G. (2011). Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim. Biophys. Acta *1811*, 1165–1176.

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

Heier, C., Taschler, U., Rengachari, S., Oberer, M., Wolinski, H., Natter, K., Kohlwein, S.D., Leber, R., and Zimmermann, R. (2010). Identification of Yju3p as functional orthologue of mammalian monoglyceride lipase in the yeast *Saccharomyces* cerevisiae. Biochim. Biophys. Acta *1801*, 1063–1071.

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.

Köffel, 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., Veenhuis, M., and van der Klei, I.J. (2012). Lipid Droplets and Peroxisomes: Key Players in Cellular Lipid Homeostasis or A Matter of Fat- Store 'em Up or Burn 'em Down. Genetics *193*, 1–50.

Kunau, W.H., Bühne, S., de la Garza, 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*, 680–685.

Lazarow, P.B., and De Duve, 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.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

Longtine, M.S., McKenzie, A., 3rd, 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*, 953–961.

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.

Mignery, G.A., Pikaard, C.S., and Park, W.D. (1988). Molecular characterization of the patatin multigene family of potato. Gene *62*, 27–44.

Müllner, H., Deutsch, G., Leitner, E., Ingolic, E., and Daum, G. (2005). *YEH2*/YLR020c encodes a novel steryl ester hydrolase of the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. *280*, 13321–13328.

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

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.

Oskolkova, O.V., Saf, R., Zenzmaier, E., and Hermetter, A. (2003). Fluorescent organophosphonates as inhibitors of microbial lipases. Chem. Phys. Lipids *125*, 103–114.

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

Rajakumari, S., and Daum, G. (2010b). Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. Mol. Biol. Cell *21*, 501–510.

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

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 *1791*, 379–387.

Saerens, S.M.G., Verstrepen, K.J., Van Laere, S.D.M., Voet, A.R.D., Van Dijck, 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.

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 Eur. J. Chem. Biol. *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 Eur. J. Chem. Biol. *7*, 527–534.

Schmidt, C., Athenstaedt, K., Koch, B., Ploier, B., and Daum, G. (2013). Regulation of the yeast triacylglycerol lipase Tgl3p by formation of non-polar lipids. J. Biol. Chem. *288* (27),19939-48.

Schrag, J.D., and Cygler, M. (1997). Lipases and alpha/beta hydrolase fold. Methods Enzymol. *284*, 85–107.

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.

Thoms, S., Debelyy, M.O., Connerth, M., Daum, G., and Erdmann, R. (2011). The putative *Saccharomyces cerevisiae* hydrolase Ldh1p is localized to lipid droplets. Eukaryot. Cell *10*, 770–775.

Vico, P., Cauet, G., Rose, K., Lathe, R., and Degryse, E. (2002). Dehydroepiandrosterone (DHEA) metabolism in *Saccharomyces cerevisiae* expressing mammalian steroid hydroxylase CYP7B: Ayr1p and Fox2p display 17beta-hydroxysteroid dehydrogenase activity. Yeast *19*, 873–886.

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.

Zahedi, R.P., Sickmann, A., Boehm, A.M., Winkler, C., Zufall, N., Schönfisch, B., Guiard, B., Pfanner, N., and Meisinger, C. (2006). Proteomic analysis of the yeast mitochondrial outer membrane reveals accumulation of a subclass of preproteins. Mol. Biol. Cell *17*, 1436–1450.

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*, 2026–2034.

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. FEBS *267*, 1075–1082.

SUPPLEMENTARY

LC-MS analysis of proteins

LC-MS/MS-analysis.

Protein spots were excised from gels and tryptically digested according to the method of Shevchenko et al. (1996). Peptide extracts were dissolved in 0.1% formic acid and separated on a nano-HPLC system (Ultimate 3000™, LC Packings, Amsterdam, Netherlands). 70 µL samples were injected and concentrated on the loading column (LC Packings C18 Pep-Map™, 5 µm, 100 Å, 300 µm inner diameter x 1mm) for 5 min using 0.1% formic acid as isocratic solvent at a flow rate of 20 µL/min. The column was then switched into the nanoflow circuit, and the sample was loaded on the nano-column (LC-Packings C18 PepMap™, 75 µm inner diameter x 150 mm) at a flow rate of 300 nL/min and separated using the following gradient: solvent A: water, 0.3% formic acid, solvent B: acetonitrile/water 80/20 (v/v), 0.3% formic acid; 0 to 5 minutes: 4% B, after 40 minutes 55% B, then for 5 minutes 90% B and 47 minutes re-equilibration at 4% B. The sample was ionized in a Finnigan nano-ESI source equipped with NanoSpray tips (PicoTip™ Emitter, New Objective, Woburn, MA, USA) and analyzed in a Thermo-Finnigan LTQ linear iontrap mass-spectrometer (Thermo, San Jose, CA, USA). The MS/MS data were analyzed by searching the "*Saccharomyces*"- subset of the National Center for Biotechnology Information (NCBI) public database (downloaded 2008-04-10) with SpectrumMill version 2.7 (Agilent, Darmstadt, GER) software. Acceptance parameters were based on the guidelines of Carr et al. (2004): two or more identified distinct peptides, a minimum peptide score of 9 and a minimum protein score of 20.

References

Carr, S., Aebersold, R., Baldwin, M., Burlingame, A., Clauser, K., and Nesvizhskii, A. (2004). The need for guidelines in publication of peptide and protein identification data. Mol.Cell. Proteomics. *3*: 531-533.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem., *68*, 850-858.

Detailed protein database search settings

Data Extraction Parameters

"Peak List Files" from RAW-data were created using the "Data Extractor" of the "Agilent - Spectrum Mill" Software (Rev. A. 03.03.078). The following settings were used:

N-terminus: Hydrogen

C-terminus: Free acid

Cys modification: Carbamidomethylation

Sequence tag length > 1

MH+: 350 – 4500 Da

Scan time: 0 – 60 min

Merge Scans +/- 1 sec, +/- 1 m/z

Find charge

Maximum z = 4

Minimum S/N: 3

Find ^{12}C

MS/MS search parameters

The database used was the "nr.gz", downloaded from "ftp://ftp.ncbi.nih.gov/blast/db/FASTA/" on 2008-04-10

Database searching was performed with the "MS/MS Search" feature of the "Agilent - Spectrum Mill" Software (Rev. 03.03.078) and the following settings were used:

Species: *Saccharomyces*

Enzyme: Trypsin

N-terminus: Hydrogen

C-terminus: free acid

Allowed missed cleavages: 2

Cys modification: carbamidomethylation

Sequence tag length > 3

Minimum detected. Peaks: 4

Minimum matched peak intensity: 50%

Precursor mass tolerance: +/- 2.5 Da

Product mass tolerance: +/- 0.7 Da

Homology search, possible multiple oxidized methionine and N-terminal pyroglutamic acid.

Acceptance parameters

Cut-off score value for accepting individual MS/MS spectra: 9

According to Spectrum Mill manual (Ref. See below) a combination of a peptide score of 9 (default 6) and a %SPI threshold of 70 /default 60) leads to an interpretation of MS/MS spectra that is likely to be valid.

Agilent G2721AA

Spectrum Mill MS

Proteomics Workbench

Application Guide

Manual Part Number

G2721-90029

Edition

Fifth edition, June 2007

Printed in USA

Agilent Technologies, Inc.

3501 Stevens Creek Blvd.

Santa Clara, CA USA 95052

Supplementary Table S1: Protein hits found in an MS screen

LD from yeast cells grown either in the presence of oleate or glucose as well as Px from cells grown on oleate were isolated as described in the Experimental Procedures. Organelle samples were incubated with the inhibitor NBD-HE-HP at 37°C overnight. 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 from the gel, subjected to trypsin digestion and analyzed by MS analysis. Proteins found in the respective bands including the number of identified distinct peptides, the SpectrumMill protein score and the NCBI- accession number are given in the Table below. Ei, eiosome; End, endosome; ER, endoplasmic reticulum; G, Golgi apparatus; LD, lipid droplets; Mem, membrane; Mt, mitochondria; N, nucleus; PLM, plasma membrane; Px, peroxisome; R, ribosome; TG, triacylglycerol; V, vacuole; Ves, vesicles

LD YPD								
Band No.	**Band size**	**Gene Name**	**Function**	**Localization**	**kDa**	**Peptides**	**Score**	**NCBI number**
1	77	*FAA1*	LCFA- CoA ligase activity	LD. Mt	77.9	13	214.38	6324893
		KAR2	ATPase	ER	74.4	8	125.48	6322426
		FAT1	FA transporter. VLCFA-CoA synthetase	LD.PX. PLM	77.1	5	67.13	41629676
2	70	*SEC63*	ATPase activator	ER. Mt	75.3	3	42.15	6324828
3	60	*VAC8*	Required for the cytoplasm-to-vacuole targeting (Cvt) pathway	V	63.2	5	65.31	6320824
4	60	*HFD1*	Putative fatty aldehyde dehydrogenase	LD. Mt	60.0	2	31.39	6323757
		TGL4	TG lipase	LD	102.7	2	29.83	6322942
5	55	*TGL5*	TG lipase	LD. C	84.7	6	88.39	6324655
		ERG1	Squalene monooxygenase	ER. C. LD	55.1	6	83.99	6321614
		TGL4	TG lipase	LD	102.7	3	43.68	6322942
6	50	*TGL1*	Sterol esterase and TG lipase	LD. C	63.0	8	132.71	6322709
		ERG6	Sterol-24-c methyltransferase	LD. Mt. C	43.4	6	106.27	6323635
7	45	*ERG6*	Sterol-24-c methyltransferase	LD. Mt. C	43.4	8	121.25	6323635
		PDR16	Phosphatidylinositol bonding/transferring	LD. ER. C. PLM	40.7	8	114.94	6324098
		QCR2	Ubiquitin-cytochrome C reductase	Mt	40.5	3	37.31	6325449
8	40	*AYR1*	Ketoreductase	LD. Mt. C	32.8	9	129.76	6322067
		TDH2	Glyceraldehyde-3-phosphate dehydrogenase	LD. C. N	35.8	3	42.91	6322468
		YJU3	Serine hydrolase	LD. ER. C. Mt	35.6	2	30.20	6322756
10	30	*YKL107w*	Putative protein of unknown function	C	34.5	12	176.06	6322742
		ERG27	3-Keto sterol reductase	LD. Mt. ER	39.7	8	132.71	6323129
		VPS66	Protein of unknown function. acyltransferase	C	33.8	4	66.61	6325397
		YNL115c	Unknown function	V	74.0	3	44.34	6324214
		YOR059c	Putative serine esterase	unknown	51.1	2	29.54	6324633
11	25	*YKL107w*	Putative protein of unknown function	C. V	34.5	5	67.12	6322742
		UBX2	Involved in ER-associated protein degradation	ER. Mt	66.7	4	52.51	6323628
		EHT1	Acyltransferase. Esterase	LD. Mt. ER. C	51.3	3	41.70	6319655
		PRB1	Peptidase. Hydrolase	C. V	69.6	2	27.11	6320775
		RPL8a	Unknown function		28.2	2	23.09	4396
		PET10	Unknown function	LD	31.2	2	26.03	6322899

12	20	*GTT1*	Glutathione-s-transferase	Mt. C. ER	26.8	6	89.40	6322229
		YDR196c	Putative dephospho-CoA kinase	Mt. N. ER. C	27.3	4	62.21	6320402
		YPL206c	Contains glycerophosphodiester phosphodiesterase motifs	Mt. ER	37.1	3	49.60	6325050

LD YPO								
Band No.	**Band size**	**Gene Name**	**Function**	**Localization**	**kDa**	**peptides**	**Score**	**NCBI number**
1	80	*FAA1*	LCFA-CoA synthetase	LD. Mt	77.9	10	145.09	6324839
		FAT1	Fatty acid transporter and VLCFA-CoA synthetase	LD.PLM. ER. Px	77.1	10	143.66	41629676
		ERG7	Lanosterol synthase	ER.LD.PLM	83.5	9	129.96	6321863
		SEC63	channel protein for targeting and import into the ER	ER. Mt	75.3	5	77.32	6324828
		ERG1	Squalene epoxidase	ER. LD	55.1	3	36.86	6321614
		IML2	Protein of unknown function	C. Mt. N	82.5	2	30.33	6322379
2	75	*TGL3*	TG lipase	LD	73.6	3	44.79	6323973
		FAA1	LCFA- CoA ligase	LD. Mt	77.9	3	42.92	6324893
		TGL4	TG lipase	LD	102.7	3	41.37	6322942
		FAT1	Fatty acid transporter and VLCFA-CoA synthetase	LD.PLM. ER. Px	77.1	3	39.16	41629676
		ERG7	Lanosterol synthase	ER.LD. PLM	83.5	3	37.09	6321863
		SDH1	Flavoprotein subunit of succinate dehydrogenase	Mt	70.2	2	32.73	6322701
		ERG1	Squalene epoxidase	ER. LD	55.1	2	29.02	6321614
		IML2	Protein of unknown function	C. Mt. N	82.5	2	26.59	6322379
		VPS1	Dynamin-like GTPase required for vacuolar sorting	C. Px. Mt	78.7	2	24.89	4528
3	70	*HSP60*	Tetradecameric mitochondrial chaperonin	Mt	60.8	4	50.61	6323288
		GUT2	Mitochondrial glycerol-3-phosphate dehydrogenase	Mt	72.4	3	39.16	51013185
		CAT2	Carnitine acetyl-CoA transferase	Mt. Px	77.2	2	32.24	3462
		FAA1	LCFA-CoA synthetase	LD. Mt	77.9	2	29.36	6324839
		URA2	Bifunctional carbamoylphosphate synthetase-aspartate transcarbamylase (ATCase)	C. Mt	245.1	2	22.59	173146
4	65	*HFD1*	Putative fatty aldehyde dehydrogenase	End. Mt. LD	59.9	7	99.28	6323757
		LPD1	Dihydrolipoamide dehydrogenase	Mt	54.0	3	49.57	14318501
		ERG1	Squalene epoxidase	ER. LD	55.1	2	30.39	6321614
		YBR056w	Putative cytoplasmic protein of unknown function	C	57.8	2	23.20	6319530
5	60	*ERG1*	Squalene epoxidase	ER. LD	55.1	4	54.38	6321614
		ATP1	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase	Mt	58.6	3	39.00	2447013
		ALG11	Alpha-1.2-mannosyltransferase	ER	63.1	2	24.59	6324280
		MIP1	Catalytic subunit of the mitochondrial DNA polymerase	Mt	143.5	2	20.98	1420721
6	60	*TEF2*	Translational elongation factor EF-1 alpha	R	50.0	5	70.03	6319594
		GDI1	GDP dissociation inhibitor. regulates vesicle traffic in secretory pathways	Mem	51.2	4	54.36	6320983
		ERG1	Squalene epoxidase	ER. LD	55.1	3	39.11	6321614
		CSR1	Phosphatidylinositol transfer protein	C. LD. ER. Mt	47.5	3	37.20	51013181
7	55	*ERG1*	Squalene epoxidase	ER. LD	55.1	2	25.40	6321614
8	55	*ERG6*	Sterol-24-c methyltransferase	LD. Mt. C	43.4	2	27.90	6323635
9	35	*ERG6*	Sterol-24-c methyltransferase	LD. Mt. C	43.4	15	217.23	6323635
		PDR16	Phosphatidylinositol bonding /transferring	LD. ER. C. PLM	40.7	6	98.46	6324098
		YBR204c	Uncharacterized serine hydrolase	LD. Px	43.3	4	68.27	6319681
10	45	*ERG6*	Sterol-24-c methyltransferase	LD. Mt. C	43.4	20	327.40	6323635
		ERG1	Squalene epoxidase	ER. LD	55.1	10	169.07	6321614

		AYR1	Ketoreductase	LD. Mt. C	32.8	9	151.65	6322067
		YJU3	Serine hydrolase	LD. ER. C. Mt	35.6	8	121.37	6322756
		HFD1	Putative fatty aldehyde dehydrogenase	End. Mt. LD	59.9	7	116.45	6323757
		KES1	Member of the oxysterol binding protein family	C. G	49.5	4	70.23	6325112
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	3	41.46	6319594
		Yim1	Protein of unknown function	LD. C. Mt	41.6	2	36.91	234847
11	40	*ERG6*	Sterol-24-c methyltransferase	LD. Mt. C	43.4	8	124.35	6323635
		ERG1	Squalene epoxidase	ER. LD	55.1	4	56.78	6321614
		HFD1	Putative fatty aldehyde dehydrogenase	End. Mt. LD	59.9	4	55.08	6323757
		AYR1	Ketoreductase	LD. Mt. C	32.8	3	40.33	6322067
		TSC10	3-Ketosphinganine reductase	C. ER. Mt	36.0	2	24.03	6319742
		YSP1	Mitochondrial protein; potential role in promoting mitochondrial fragmentation death	Mt	143.6	2	21.62	6321949
12	35	*POR1*	Mitochondrial porin (volTGe-dependent anion channel)	Mt	30.4	5	69.36	4195
		PET9	Major ADP/ATP carrier of the mitochondrial inner membrane	Mt	34.4	4	58.61	6319441
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	3	38.35	6319594
		ERG1	Squalene epoxidase	ER. LD	55.1	2	29.93	6321614
		ERG27	3-Keto sterol reductase	ER. Mt	39.7	2	27.05	6323129
		YKL050w	Protein of unknown function	Unknown	103.1	2	23.59	6322801
		YOR1	Plasma membrane ATP-binding cassette (ABC) transporter	PLM. Mem	166.7	2	21.10	6321720
13	30	*PET10*	Protein of unknown function	LD	31.2	5	67.91	6322899
		ERG1	Squalene monooxygenase	ER. C. LD	55.1	3	39.18	6321614
14	25	*GTT1*	Glutathione-s-transferase	Mt. C. ER	26.8	7	103.76	6322229
		PET10	Protein of unknown function	LD	31.2	5	69.54	6322899
		EHT1	Acyltransferase. Esterase	LD. Mt. ER. C	51.3	5	67.98	6319655
		ERG27	3-Keto sterol reductase	LD. Mt. ER	39.7	3	38.72	6323129
		YDR196c	Putative dephospho-CoA kinase	Mt. N. ER. C	27.3	3	41.05	6320402
		YPL206c	Contains glycerophosphodiester phosphodiesterase motifs	Mt. ER	37.1	2	29.82	6325050
15	20	*RPS3*	Apurinic/apyrimidinic (AP) endonuclease activity	R	26.5	3	37.37	6324151
		PET10	Protein of unknown function	LD	31.2	2	31.06	6322899
		HEF3	Translational elongation factor EF-3	R	116	2	23.94	6324314
16	14	*PET10*	Protein of unknown function	LD	31.2	3	33.98	6322899

PX YPO								
Band No.	**Band size**	**Gene Name**	**Function**	**Localization**	**kDa**	**peptides**	**Score**	**NCBI number**
2	75	*POX1*	Fatty-acyl coenzyme A oxidase	Px	84.0	10	124.11	6321233
		FAA2	LCFA-CoA synthetase	Px. Mt	83.4	8	99.85	6320852
		VPS13	Protein of unknown function	End. ext Mem. Mt	357.8	2	26.97	6322988
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase	Px	98.7	2	24.18	6322861
3	70	*POX1*	Fatty-acyl coenzyme A oxidase	Px	84.0	2	22.68	6321233
4	65	*POX1*	Fatty-acyl coenzyme A oxidase	Px	84.0	8	94.93	6321233
		FAA2	LCFA-CoA synthetase	Px. Mt	83.4	4	54.43	6320852
		CAT2	Carnitine acetyl-CoA transferase	Mt. Px	77.2	2	28.44	3462
		PMA2	Plasma membrane H+-ATPase	PLM. Mt	102.2	2	25.61	6325221
5	60	*PCS60*	Peroxisomal AMP-binding protein	Px. C	60.5	6	74.08	6319699
		CTA1	Catalase A	Mt. Px	58.6	4	55.96	6320462
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	4	55.25	6321233
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase	Px	98.7	2	30.92	6322861

		ALD4	Mitochondrial aldehyde dehydrogenase	Mt	56.7	2	27.02	6324950
		EFT2	Elongation factor 2 (EF-2)	R	93.3	2	23.79	6320593
		SMC1	Subunit of the multiprotein cohesin complex	N	141.3	2	22.09	14318514
6	55	*FOX2*	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	4	45.79	6322861
		ADE13	Adenylosuccinate lyase	unknown	54.5	3	38.36	6323391
		PCS60	Peroxisomal AMP-binding protein	Px. C	60.5	3	35.52	6319699
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	2	20.70	6321233
7	50	*POX1*	Fatty-acyl coenzyme A oxidase	Px	84.0	6	80.39	6321233
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	5	77.19	6319594
		CIT1	Citrate synthase	Mt	53.4	5	76.47	3603
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	4	73.78	6322861
		TFP1	Vacuolar ATPase V1 domain subunit A containing the catalytic nucleotide binding sites	V	118.6	6	69.61	6320016
		CIT2	Citrate synthase	Px. Mt	51.4	4	66.21	6319850
		PMA1	Plasma membrane H+-ATPase	PLM. Mt. Mem	99.6	3	43.41	6321430
		SMC4	Subunit of the condensin complex	N	162.2	3	32.63	6323115
		PEX25	Peripheral peroxisomal membrane peroxin	Px	44.9	2	27.30	6325145
		WBP1	Beta subunit of the oligosaccharyl transferase (OST) glycoprotein complex	ER. N	49.4	2	27.25	6320835
		POT1	3-Ketoacyl-CoA thiolase	Px	44.7	2	25.54	6322031
		POP1	Subunit of both RNase MRP. which cleaves pre-rRNA	R	100.4	2	24.76	6324108
8	45	*IDP3*	Peroxisomal NADP-dependent isocitrate dehydrogenase	C. Mt. Px	47.9	17	278.85	6324319
		CIT2	Citrate synthase	Px. Mt	51.4	12	181.75	6319850
		POT1	3-Ketoacyl-CoA thiolase	Px	44.7	8	139.07	6322031
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	7	103.16	6321233
		MDH2	Cytoplasmic malate dehydrogenase	C	40.7	6	99.06	1420009
		FOX2	Multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway	Px	98.7	6	94.24	6322861
		PGK1	3-Phosphoglycerate kinase	Mt. C	44.7	6	90.89	14588927
		PMA1	Plasma membrane H+-ATPase	PLM. Mt. Mem	99.6	5	76.89	6321430
		MLS1	Malate synthase	Px. C	62.8	5	72.00	125863561
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	4	67.75	6319594
		Pil1	Primary component of eisosomes	Ei. C. Mt	38.3	3	52.80	6321523
		CTA1	Catalase A	Mt. Px	58.6	3	52.41	6320462
		PEX3	Peroxisomal membrane protein (PMP)	ER. Px	50.7	2	32.75	6320536
9	40	*IDP3*	Peroxisomal NADP-dependent isocitrate dehydrogenase	C. Px. Mt	47.9	15	242.24	398365479
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	12	189.77	6322861
		LPX1	Oleic acid-inducible. peroxisomal matrix localized lipase	Px	43.7	11	168.93	6324658
		MDH2	Cytoplasmic malate dehydrogenase	C	40.7	8	135.32	1420009
		PMA1	Plasma membrane H+-ATPase	PLM. Mt. Mem	99.6	8	131.90	6321430
		POT1	3-Ketoacyl-CoA thiolase	Px	44.7	8	127.77	6322031
		LYS1	Saccharopine dehydrogenase	C	41.5	9	124.97	557840
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	7	103.37	6321233
		MLS1	Malate synthase	Px. C	62.8	6	90.96	125863561
		CIT2	Citrate synthase	Px	51.4	5	77.54	6319850
		FAA2	Long chain fatty acyl-CoA synthetase	Px. Mt	83.4	5	67.01	6320852
		AAT2	Cytosolic aspartate aminotransferase	C. Px	56.0	4	58.01	1360338

		ILV5x	Acetohydroxyacid reductoisomerase	Mt	44.4	4	53.83	957238
		ADH2	Glucose-repressible alcohol dehydrogenase II	C	36.7	3	45.39	6323961
		AGX1	Alanine:glyoxylate aminotransferase (AGT)	Mt	41.9	3	45.31	14318489
		ERG9	Farnesyl-diphosphate farnesyl transferase (squalene synthase)	ER. Mt	51.7	2	27.32	3686
10	35	*TES1*	Peroxisomal acyl-CoA thioesterase	Px. Mt	40.3	13	201.33	6322480
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	11	183.34	6322861
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	10	157.96	6321233
		ZTA1	Zeta-crystallin homolog	C. N	37.0	6	98.38	6319520
		LPX1	Oleic acid-inducible. peroxisomal matrix localized lipase	Px	43.7	5	72.99	6324658
		MDH3	Peroxisomal malate dehydrogenase	Px	37.2	5	69.80	6320125
		PMA1	Plasma membrane H+-ATPase	PLM. Mt. Mem	99.6	4	66.63	6321430
		LSP1	Primary component of eisosomes	C. Mt	38.1	4	56.78	6325253
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	3	53.22	6319594
		SEC61	channel protein for targeting and import into the ER	ER	52.9	3	52.29	6323411
		IDH2	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	Mt	39.7	3	46.26	6324709
		IDH1	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	Mt	39.3	3	43.94	6324291
		MLS1	Malate synthase	Px. C	62.8	3	42.05	125863561
		KAR2	ATPase involved in protein import into the ER	ER	74.5	3	41.80	6322426
		ILV5	Acetohydroxyacid reductoisomerase	Mt	44.4	3	37.97	957238
		PEX3	Peroxisomal membrane protein (PMP)	ER. Px	50.7	2	31.45	6320536
		AAT2	Cytosolic aspartate aminotransferase	C. Px	56.0	2	29.94	1360338
		AGX1	Alanine:glyoxylate aminotransferase (AGT)	Mt	41.9	2	26.08	14318489
11	30	*MDH3*	Peroxisomal malate dehydrogenase	Px	37.2	14	220.28	6320125
		TDH3	Glyceraldehyde-3-phosphate dehydrogenase	C. LD. Mt	35.7	8	120.79	6321631
		PCS60	Peroxisomal AMP-binding protein	Px. C	60.5	6	102.61	6319699
		ZTA1	Zeta-crystallin homolog	C. N	37.0	6	99.74	6319520
		TEF2	Translational elongation factor EF-1 alpha	R	50.0	3	51.45	6319594
		PIL1	Primary component of eisosomes	Ei. C. Mt	38.3	3	48.32	6321523
		LSP1	Primary component of eisosomes	C. Mt	38.1	2	31.21	6325253
		LPX1	Oleic acid-inducible. peroxisomal matrix localized lipase	Px	43.7	2	28.05	6324658
		YJU3	Serine hydrolase with sequence similarity to monoglyceride lipase (MGL)	LD. ER. C. Mt	35.6	2	27.54	6322756
12	25	*ECI1*	Peroxisomal delta3.delta2-enoyl-CoA isomerase	Px	31.7	12	204.20	6323314
		PET9	Major ADP/ATP carrier of the mitochondrial inner membrane	Mt	34.4	12	197.64	6319441
		FOX2	3-Hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities	Px	98.7	10	169.59	6322861
		SPS19	Peroxisomal 2.4-dienoyl-CoA reductase	Px	31.1	9	167.13	600063
		LSP1	Oleic acid-inducible. peroxisomal matrix localized lipase	Px	43.7	9	161.33	6324658
		MIR1	Mitochondrial phosphate carrier. imports inorganic phosphate into mitochondria	Mt	32.8	9	141.05	1015764
		TEF2	Translational elongation factor EF-1 alpha; also encoded by TEF1	R	50.0	7	108.71	6319594
		DPM1	Dolichol phosphate mannose (Dol-P-Man) synthase	ER. Mt	30.4	5	80.17	6325441
		BBC1	Protein possibly involved in assembly of actin patches	Ct	128.3	5	77.65	14627173

		CBR1	Microsomal cytochrome b reductase	ER. Mt	31.5	5	75.73	585542
		ATP3	Gamma subunit of the F1 sector of mitochondrial F1F0 ATP synthase	Mt	34.4	4	69.42	536260
		MDH2	Cytoplasmic malate dehydrogenase	C	40.7	5	68.13	1420009
		YOR280C	Serine hydrolase; similar to FSH1. FSH2 and FSH3		30.4	2	30.87	6324854
		YJR088c	Putative protein of unknown function	ER	33.9	2	30.82	6322547
13	20	*FOX2*	3-Hydroxyacyl-CoA dehydrogenase and Enoyl-CoA hydratase activities	Px	98.7	10	188.27	6322861
		PET9	Major ADP/ATP carrier of the mitochondrial inner membrane	Mt	34.4	9	156.62	6319441
		POX1	Fatty-acyl coenzyme A oxidase	Px	84.0	8	136.52	6321233
		LSP1	Oleic acid-inducible. peroxisomal matrix localized lipase	Px	43.7	6	112.53	6324658
		SEC4	Secretory vesicle-associated Rab GTPase essential for exocytosis	Ves. Mt	23.5	6	103.90	134394
		DCI1	Peroxisomal delta(3.5)-delta(2.4)-dienoyl-CoA isomerase	Px	30.1	6	98.68	6324754
		ATP4	Subunit b of the stator stalk of mitochondrial F1F0 ATP synthase	Mt	26.9	6	97.67	6325179
		ECI1	Peroxisomal delta3.delta2-enoyl-CoA isomerase	Px	31.7	6	94.53	6323314
		YPT32	GTPase of the Ypt/Rab family. very similar to Ypt31p	End. G. Mt	24.5	5	79.36	6321228
		GTT1	ER associated glutathione S-transferase capable of homodimerization	ER. Mt	26.8	4	71.67	6322229
		LPX1	Oleic acid-inducible. peroxisomal matrix localized lipase	Px	43.7	4	67.28	6324658
		SPS19	Peroxisomal 2.4-dienoyl-CoA reductase	Px	31.41	3	51.86	600063
		PCS60	Peroxisomal AMP-binding protein	Px. C	60.5	3	48.74	6319699
		PDI1	Protein disulfide isomerase	ER	58.2	3	47.64	4120
		FMP52	Protein of unknown function	ER. Mt	25.1	3	44.42	6320840
		MDH3	Peroxisomal malate dehydrogenase	Px	37.2	2	34.78	6320125
		ERP1	Protein that forms a heterotrimeric complex with Erp2p. Emp24p. and Erv25p	Ves. Mt	41479	2	30.98	45269311

5

REGULATORY LINK BETWEEN STERYL ESTER FORMATION AND HYDROLYSIS IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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Manuscript submitted

KEY WORDS

Yeast, lipids, steryl ester(s), sterols, hydrolase, lipid droplets

ABBREVIATIONS

LD, lipid droplets; SE, steryl esters; TG, triacylglycerols; TM, triple mutant; WT, wild type; GLC, gas liquid chromatography

ABSTRACT

Steryl esters and triacylglycerols are the major storage lipids of the yeast *Saccharomyces cerevisiae*. Steryl ester formation is carried out in the endoplasmic reticulum by the two acyl-CoA:sterol acyltransferases Are1p and Are2p, whereas steryl ester hydrolysis is catalyzed by the three steryl ester hydrolases Yeh1p, Yeh2p and Tgl1p. To shed light on the regulatory link between steryl ester formation and hydrolysis in the maintenance of cellular sterol and free fatty acid levels we employed yeast mutants which lacked the enzymes catalyzing the degradation of steryl esters. These studies revealed feedback regulation of steryl ester formation by steryl ester hydrolysis although in a *Δtgl1Δyeh1Δyeh2* triple mutant the gene expression levels of *ARE1* and *ARE2* as well as the protein levels were not altered. Nevertheless, the capacity of the triple mutant to synthesize steryl esters was significantly reduced as shown by enzyme analyses *in vitro* and *in vivo* labeling of lipids with [^{14}C]oleic and [^{14}C]acetate. As the amounts and the formation of sterols and fatty acids were also decreased in the triple mutant we concluded that defects in steryl ester hydrolysis also caused feedback inhibition on the formation of sterols and fatty acids which serve as precursors for steryl ester formation. In summary, this study demonstrates a regulatory link within the steryl ester metabolic network which contributes to non-polar lipid homeostasis in yeast cells.

INTRODUCTION

Sterol homeostasis is essential to maintain cellular membrane permeability and fluidity and to avoid harmful effects on membranes by an excess of free sterols. Yeast cells are unable to degrade sterols, and therefore mechanisms of sterol detoxification are needed. In *Saccharomyces cerevisiae*, three such mechanisms are known, namely (i) esterification of free sterols with fatty acids by the two acyltransferases Are1p and Are2p (Yang et al., 1996; Yu et al., 1996; Zweytick et al., 2000); (ii) down regulation of sterol biosynthesis; and (iii) sterol acetylation by Aft2p and secretion by Pry-proteins (Tiwari et al., 2007; Choudhary et al., 2009; Choudhary et al., 2012). Steryl esters (SE), formed by CoA dependent acylation of sterols with fatty acids are stored in organelle-like structures called lipid droplets (LD) (Kohlwein et al., 2013; Grillitsch et al., 2011)

Are1p and Are2p are acyl-CoA:cholesterol acyltransferase related enzymes (ACAT), both located to the endoplasmic reticulum (Yang et al., 1996; Yu et al., 1996). Although they share 49% of sequence identity they have different substrate specificities. Are2p, which is regarded as the major acyltransferase accounting for more than 70% of total SE synthase activity, utilizes preferentially ergosterol as a substrate. Are1p was found to esterify mainly ergosterol precursors with a slight preference for lanosterol. Thus, Are1p may prevent harmful accumulation of sterol intermediates and form a depot of sterol intermediates which can rapidly re-enter the sterol biosynthetic pathway upon requirement (Zweytick et al., 2000). A single deletion of *ARE2* decreases the cellular SE content to about 30% of wild type, whereas deletion of *ARE1* hardly affects the overall amount of SE. A mutant lacking both *ARE1* and *ARE2* completely lacks SE, but does not show any growth defects. It was demonstrated, however, that such a strain is synthetically lethal with *ARV1* (*ARE2* required for viability). Arv1p was found to play a role in sterol trafficking to the plasma membrane. Yeast cells lacking Arv1p showed altered intracellular sterol distribution and were defective in sterol uptake (Tinkelenberg et al., 2000).

SE are besides triacylglycerols (TG) the major non-polar lipids of yeast and preserve chemical energy and membrane building blocks. Under standard growth conditions TG are mainly synthesized by the diacylglycerol acyltransferase Dga1p and with a minor contribution by the second TG synthase Lro1p, both localized to the ER (Oelkers et al., 2000; Dahlqvist et al., 2000; Sorger et al., 2002; Oelkers et al., 2002). Additionally, the two SE synthases appear to

contribute to TG synthesis, although to a small extent (Sandager et al., 2000; Sandager et al., 2002).Both, TG and SE accumulate during the stationary growth phase of yeast cultures and are stored in LD. LD consist of a hydrophobic core of TG, surrounded by several shells of SE and a phospholipid monolayer with a distinct amount of proteins embedded (Kohlwein et al., 2013; Grillitsch et al., 2011; Czabany et al., 2008). Recently, the proteome of LD was investigated in more detail leading to a novel view of this organelle (Grillitsch et al., 2011; Fei et al., 2011). Nowadays, LD are no longer seen just as storage compartment but as dynamic organelles which are also involved in the storage and degradation of protein aggregates or incorrectly folded proteins (Fujimoto et al., 2008; Fujimoto et al., 2011).

Non-polar lipids can be mobilized from LD upon requirement, for example during growth or starvation [Grillitsch et al., 2011; Czabany et al., 2008; Brasaemle et al., 2012; Leber et al., 1994; Athenstaedt et al., 2006). Mobilization of TG is carried out by TG lipases. In the yeast, three major TG lipases were identified, namely Tgl3p, Tgl4p and Tgl5p (Athenstaedt and Daum 2003; 2005; Kurat et al., 2006). Recently, Ayr1p was also shown to serve as a further TG lipase although with minor lipolytic activity *in vivo* (Ploier et al., 2013). SE hydrolysis plays an important role in maintaining a balanced cellular sterol level (Wagner et al., 2009). In the yeast *S. cerevisiae*, the three SE hydrolases Yeh1p, Yeh2p and Tgl1p were identified (Köffel et al., 2005; Müllner et al., 2005), which are most likely the only SE hydrolases in this microorganism. Tgl1p and Yeh1p were localized to LD, whereas the highest activity of Yeh2p was detected in the plasma membrane. Tgl1p and Yeh1p were identified as paralogues of mammalian acid lipases, but their *in vitro* SE hydrolase activity was low. Yeh1p exhibits enhanced activity under heme deficiency mimicking anaerobiosis compared to standard growth conditions (Köffel et al, 2005; Jandrositz et al., 2005; Köffel et al., 2006). The three hydrolases have different substrate specificities. All three enzymes accept ergosteryl esters as substrate, but Tgl1p and Yeh2p show a slight preference for zymosteryl esters whereas Yeh1p efficiently hydrolyses fecosteryl esters (Wagner et al., 2009).

In addition, Tgl1p was shown to degrade TG *in vitro*, although TG mobilization by this enzyme *in vivo* was minor (Jandrositz et al., 2005).

SE synthesizing and degrading enzymes have been identified some years ago, but little is known about their regulation. Valachovic *et al*. (2002) showed that the regulation of *ARE1* and *ARE2* is oxygen-dependent, and Are1p becomes especially important under hypoxic conditions (Valachovic et al., 2002). Connerth *et al.* (2010) showed that Are2p is regulated by

oleate which acts as a competitive inhibitior of the enzyme (Connerth et al., 2010). Moreover, it was demonstrated that deletion of *ARE1* and *ARE2* influenced sterol biosynthesis by changing the expression levels of Erg-genes, e.g. *ERG1* encoding squalene epoxidase, and *ERG3* encoding a C-5 sterol desaturase (Sorger et al., 2004; Arthington-Skaggs et al., 1996). More evidence about regulatory aspects between catabolism and anabolism of non-polar lipids is still missing.

The present study is focused on the regulation of SE formation by SE mobilization. For this purpose, we analyzed gene expression and protein levels of the two acyltransferases Are1p and Are2p as well as their enzymatic activities in strains lacking the three SE hydrolases. These data were supplemented by lipid profiling of strains lacking either all or individual SE hydrolyzing enzymes. We demonstrate a marked effect on the activity of the two acyltransferases in a strain unable to mobilize SE, and discuss possible modes of feedback regulation on Are1p and Are2p at the enzyme level and on the formation of sterols and fatty acids.

MATERIALS AND METHODS

Yeast strains and culture conditions

The list of strains used in this study is shown in Table 5-1. Yeast cells were either grown in YPD medium containing 1% yeast extract, 2% glucose and 2% peptone; or in synthetic minimal medium (SD) containing 0.67% yeast nitrogen base (U.S. Biochemical Corp.), 2% glucose and the respective amino acid supplements. All cells were cultivated in liquid medium at 30°C under vigorous shaking to the exponential or to the early stationary phase as stated below. For gene expression studies yeast cells were grown to the mid exponential phase. Growth was monitored by measuring the optical density at 600 nm (A_{600}).

Genetic techniques

Chromosomal tagging of *ARE1* and *ARE2* was performed according to the PCR-mediated method of Longtine *et al*. (Longtine et al., 1998). In brief, inserts of Are1-Myc or Are2-Myc constructs were obtained by PCR from plasmid pFA6a-13Myc-HIS3MX6. Primers used for amplification of the respective DNA-fragments are listed in Table 5-2. Furthermore, 500 ng DNA were used for transformation of yeast strains employing the high-efficiency lithium acetate transformation protocol (Gietz et al., 1995). After transformation, cells were plated

on minimal medium lacking histidine for selection and incubated for 2 to 3 days at 30°C. Positive transformants were verified by colony PCR of whole yeast cell extracts with primers listed in Table 5-2.

Table 5-1: Yeast strains used throughout this study.

Strain	Genotype	Source
Wild type	BY4741 Mat a; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
TM	BY4741; *Δtgl1::kanMX4; Δyeh1::kanMX4; Δyeh2::kanMX4*	Wagner *et al*. (2009)
Δare1Δare2	BY4741; *Δare1::kanMX4; Δare2::kanMX4*	Wagner *et al*. (2009)
Δtgl1	BY4741; *Δtgl1::kanMX4*	Euroscarf
Δyeh1	BY4741; *Δyeh1::kanMX4*	Euroscarf
Δyeh2	BY4741; *Δyeh2::kanMX4*	Euroscarf
WT *Are1*-Myc	BY4741; *ARE1-13Myc::HIS3MX6*	This study
WT *Are2*-Myc	BY4741; *ARE2-13Myc::HIS3MX6*	This study
TM *Are1*-Myc	TM; *ARE1-13Myc::HIS3MX6*	This study
TM *Are2*-Myc	TM; *ARE2-13Myc::HIS3MX6*	This study

Table 5-2: Primers used throughout this study.

Primer	Sequence (5' → 3')
are1fw	TTGGTGTCTGTTCAGGGCCCAGTATCATTATGACGTTGTACCTGACCTTACGGA
are1rev	TTGTATATCTATCAAGGGCTTGCGAGGGACACACGTGGTATGGTGGCAGTATCG
are2fw	TCGGTATCTGCATGGGACCAAGTGTCATGTGTACGTTGTACTTGACATTCCGGA
are2rev	AAAATTTACTATAAAGATTTAATAGCTCCACAGAACAGTTGCACGATGCCATCG
are1fw_cPCR	GACCGCAGTTGTCCAACG
are2fw_cPCR	CAGAACCATAATCGGAAATGTTAT
13Mycrev_cPCR	TATTTAGAAGTGGCGCGAATTCAC

Isolation and characterization of microsomes

Isolation of highly pure microsomes from cells grown to the early stationary phase was performed as described previously (Zinser et al., 1995; 1991; Connerth et al., 2009). The protein concentration was analyzed by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Microsomal proteins were separated by SDS-PAGE using 12.5% polyacrylamide gels according to the method of Laemmli (Laemmli, 1970). Western Blot analysis was performed as described by Haid and Suissa (Haid and Suissa, 1983). Proteins were detected using mouse or rabbit antisera, respectively, as primary antibody directed against Myc-tag and Wbp1p (ER-marker). Primary antibodies were detected with

peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, respectively, as second antibody and SuperSignal® West Pico Chemiluminescent substrate solution.

Lipid extraction and analysis

Non-polar lipid analysis was performed from total yeast cells. Crude homogenates were prepared from cells grown to the stationary phase. Cells were resuspended in breaking buffer (50 mM TrisHCl, pH 7.4, 150 mM NaCl) and disintegrated by vigorous shaking in the presence of glass beads for 10 min at 4°C. Cell debris were removed by centrifugation at 5,000 x g for 5 min, and the supernatant was used for protein determination and lipid extraction using chloroform/methanol (2:1; v/v) as solvent (Folch et al., 1957). For the quantification of non-polar lipids, lipid extracts were applied to Silica Gel 60 plates, and chromatograms were developed by a two-step developing system using first the solvent system light petroleum/diethyl ether/acetic acid (35:15:1 per volume) for two thirds of the plate. After drying the plates, they were further developed to the top using the second solvent system light petroleum/diethyl ether (49:1; v/v). Non-polar lipids were visualized by post-chromatographic charring after dipping the plates in a solution consisting of 0.63 g of $MnCl_2x4H_2O$, 60 mL water, 60 mL methanol and 4 mL concentrated sulfuric acid. Plates were then heated at 105°C for 40 min. Bands were quantified by densitometric scanning (CAMAG TLC SCANNER 3) at 400 nm by comparing to authentic standards containing defined amounts of the respective lipids. For quantification of total phospholipids, a lipid extract from total cells (800 µg protein) was analyzed by the method of Broekhuyse (Broekhuyse, 1968).

Sterols were analyzed as described previously (Quail et al., 1996). In brief, homogenate corresponding to 1 mg protein was incubated for 2 h at 90°C with 600 µL methanol, 400 µL 0.5% pyrogallol dissolved in methanol, 400 µL 60% aqueous KOH, and 10 µg cholesterol dissolved in ethanol as internal standard. After cooling to room temperature, lipids were extracted three times with 1 mL n-heptane, and combined extracts were dried under a stream of nitrogen. The extracted sterols were then dissolved in 10 µL pyridine and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamid (Sigma). After an incubation time of 15 min samples were diluted with 80 µL ethyl acetate. GLC/MS was performed on a Hewlett-Packard HP 5890 Series II gas chromatograph (Palo Alto, CA), equipped with an HP 5972 mass selective detector, and HP 5-MS column (cross-linked with 5% phenyl methyl siloxane;

dimensions 30 m x 0.25 mm x 0.25 µm film thickness). Aliquots of 1 µL were injected in the splitless mode at 270°C injection temperature with helium as a carrier gas at a flow rate of 0.9 mL/min in constant flow mode. The temperature program used was 1 min at 100°C, 10°C/min to 250°C, and 3°C/min to 300°C. Mass spectra were acquired in scan mode (scan range 200-259 amu) with 3.27 scans per second. Sterols were identified by their mass fragmentation pattern.

Fatty acids were analyzed by gas liquid chromatography (GLC). Lipid extracts prepared as described above were subjected to methanolysis using 2.5% H_2SO_4 in methanol and converted to methyl esters. Fatty acid methyl esters were separated using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-INNOWax capillary column (15 m × 0.25 mm inner diameter × 0.50 µm film thickness) and helium as carrier gas. Fatty acids were identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN) and quantified by using pentadecanoic acid as internal standard.

Quantitative Real Time-PCR

Total RNA isolation from cells grown to the mid-logarithmic phase was performed as described by the manufacturer using the RNeasy kit from Qiagen. After DNaseI digestion, quantitative Real Time PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. Reactions were performed in sealed MicroAmp Optical 96-Well Reaction Plates, and amplification was measured using an ABI 7500 instrument (Applied Biosystems). Samples were quantified using the ΔΔCt method described by Livak and Schmittgen (Livak and Schmittgen, 2001). Primers used for Real Time-PCR are listed in Table 5-3.

Table 5-3: Primers used for qRT-PCR

Primer	Sequence (5' → 3')
Are1_RTFw	CTTCACTGTTCTGTACATGCTCACGTTTTACATG
Are1_RTRev	CGAAACGCAATTCCACCAGTCGCCGTAG
Are2_RTFw	GATGTATCCTGTAGCAATGAGAGCATTGGCTGTG
Are2_RTRev	CTGGGACGATATCAACGAGCAATCCAAC
ACT1_RTFw	CCAGCCTTCTACGTTTCCATCCAAG
ACT1_RTRev	GACGTGAGTAACACCATCACCGGA

Enzymatic analysis

Acyl-CoA:ergosterol acyltransferase activity was measured using 70 µg protein from microsomal fractions, or 250 µg protein from homogenate as enzyme source in a final volume of 200 µL. The assay mixture contained 100 mM potassium phosphate buffer, pH 7.4, 25 µM ergosterol, 0.5 mM CHAPS, 10 mM DTT, 0.7 nmol [^{14}C]oleoyl-CoA (58.6 mCi/mmol; PerkinElmer Life Sciences) and 20 µg unlabeled oleoyl-CoA (Sigma). Samples were incubated at 30°C for 15 min in a water bath. The reaction was stopped by adding 3 mL chloroform/methanol (2:1, v/v), and lipids were extracted by vortexing. Chromatograms were developed by the two step system as described above. After reversible staining with iodine vapor bands of SE were scraped off the plates and radioactivity was measured by liquid scintillation counting using 8 mL LSC Safety Cocktail (Baker, Deventer, The Netherlands) containing 5% water as scintillation cocktail.

In vivo labeling of lipids

To analyze the incorporation of fatty acids and acetate into complex lipids *in vivo*, wild type and *Δtgl1Δyeh1Δyeh2* were inoculated to an A_{600} of 0.1 in 500 mL YPD and grown to the early stationary phase. An aliquot of 20 mg cells in a total volume of 10 mL YPD was pre-incubated with stirring for 15 min at 30°C in a water bath. Cells were then incubated for 20 min at 30°C in the presence of 10 µL [$^{1\text{-}14}$C]oleic acid (0.1 mCi/mL; PerkinElmer Life Sciences) or 20 µL [$^{1,2\text{-}14}$C]acetate (0.1 mCi/mL; PerkinElmer Life Sciences), respectively. Aliquots of 2 mL were harvested by centrifugation after addition of 1 mL 2% BSA. The cell pellet was washed once with pre-cooled BSA solution (0.1%) and twice with cold distilled water. Then, cells were resuspended in ice-cold water and disintegrated for 10 min at 4°C by vigorous shaking in the presence of glass beads. Lipids were extracted and separated by TLC as described above. Bands of TG, SE and phospholipids were scraped off the plates, and radioactivity was measured as described above.

RESULTS

Lipid analysis of strains bearing defects in steryl ester metabolism

Yeh1p, Yeh2p and Tgl1p are the major SE hydrolyzing enzymes of the yeast *S. cerevisiae*, whereas synthesis of SE is catalyzed by the two acyltransferases Are1p and Are2p (Yang et al., 1996; Yu et al., 1996; Zweytick et al., 2000;Köffel et al., 2005; Müllner et al., 2005). To investigate the interplay between SE hydrolysis and SE formation, we first analyzed the amount of SE in strains bearing defects in SE metabolism (Figure 5.1A). We compared wild type (WT) to strains lacking either one or all three steryl ester hydrolases (TM; Δ*tgl1Δyeh1Δyeh2*), and a strain deficient in the two acyltransferase Are1p and Are2p. In the stationary growth phase, where cells accumulate non-polar lipids, the TM showed about 30% more SE than WT. Interestingly, the *Δtgl1* strain accumulated SE at the same amount as the TM, whereas *Δyeh1* and *Δyeh2* single mutants showed SE levels similar to WT. These data confirmed previous studies of our laboratory (Wagner et al., 2009) using YPD or MMGal (minimal media with 2% galactose) as cultivation media. The TM as well as a strain lacking the two acyltransferases Are1p and Are2p showed also increased amounts of TG (Figure 5.1B) This metabolic link between TG and SE synthesis and mobilization confirmed recent findings by Schmidt *et al*. (manuscript submitted).

To get a broader view of possible regulatory aspects of SE metabolism, we also analyzed phospholipids in the TM, the double deletion strain *Δare1Δare2* and WT (Figure 5.1C). The total amount of phospholipids was not altered in the TM, whereas the *Δare1Δare2* double mutation led to elevated amounts of phospholipids. The latter result confirmed a link of non-polar and phospholipid metabolism as also shown previously (Horvath et al., 2011; Rajakumari et al., 2010).

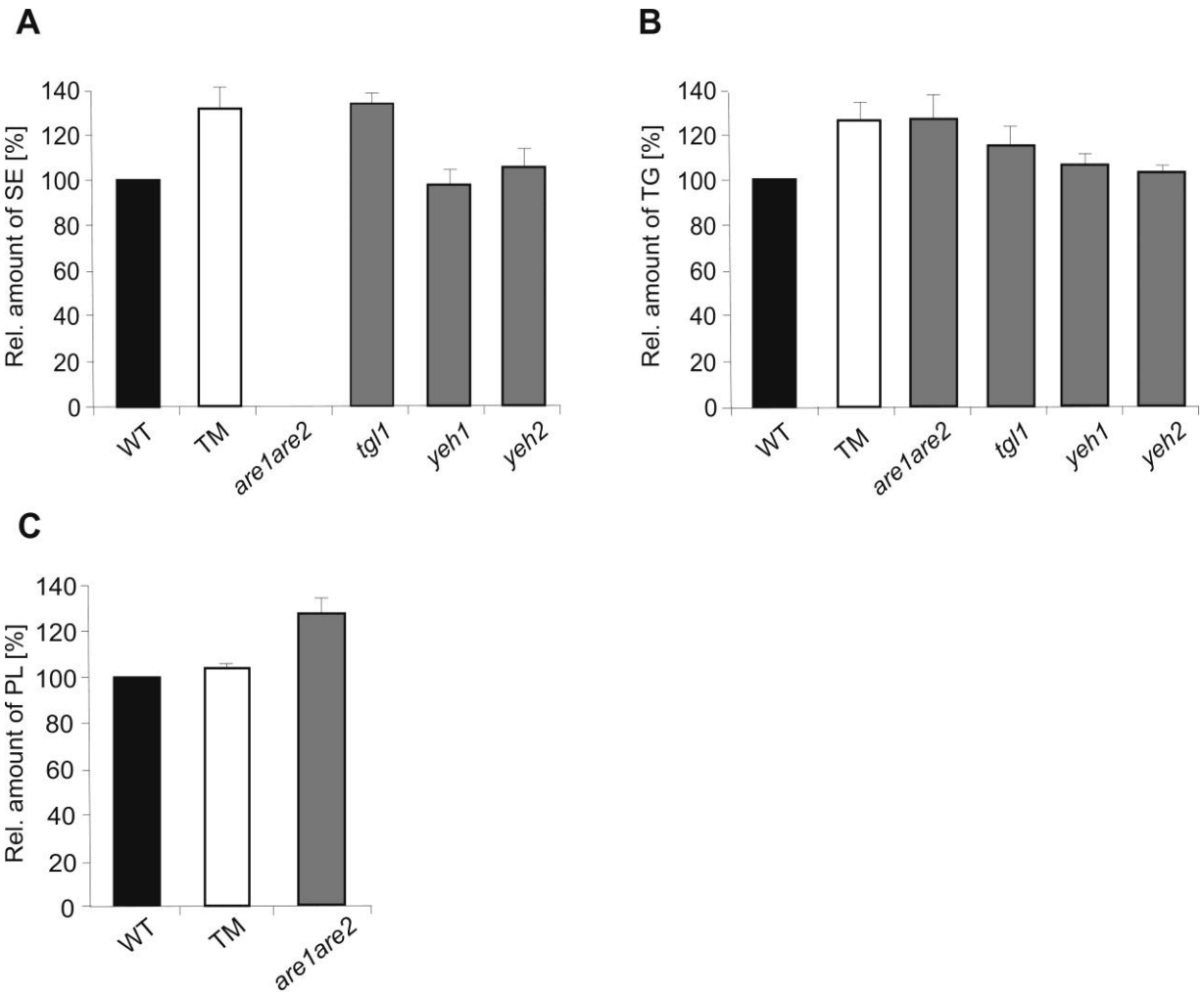

Figure 5.1 Lipid analysis of yeast strains bearing mutations in steryl ester metabolism. Data of wild type (WT), *Δtgl1Δyeh1Δyeh2* (TM), *Δare1Δare2*, *Δtgl1*, *Δyeh1*, and *Δyeh2* are shown. (A) Relative amount of steryl esters (SE). (B) Relative amount of triacylglycerols (TG). (C) Relative amount of phospholipids (PL). The amounts of SE, TG and PL from WT were set at 100%. Data are mean values of at least three independent experiments performed in duplicate with the respective standard deviations as indicated.

A strain lacking all three steryl ester hydrolases shows decreased formation of steryl esters, sterols and fatty acids

To shed more light on the synthesis of SE when their hydrolysis is blocked we performed *in vivo* labeling of lipids in wild type and *Δtgl1Δyeh1Δyeh2*. We followed the incorporation of [^{14}C]oleic acid into the main lipid classes in cultures which have reached the stationary growth phase. Figure 5.2A shows the incorporation of [^{14}C]oleic acid into phospholipids, TG and SE. In wild type, more than half of the label was found in phospholipids, whereas the other half was distributed among non-polar lipids. SE and TG were formed at nearly equal amounts. The TM deficient in all three SE hydrolases incorporated the same amount of [^{14}C]oleic acid into phospholipids as WT, but showed a significant shift of the label from SE to

TG. This result indicated that TG formation was enhanced, whereas the formation of SE was decreased in cells lacking SE hydrolyzing enzymes. Thus, a feedback regulation on Are1p and Are2p occurred when the hydrolysis of SE was blocked.

A similar experiment was performed with [^{14}C]acetate as lipid precursor to get some additional information about fatty acid biosynthesis in the TM. Figure 5.2B shows that also [^{14}C]acetic acid incorporation into SE was reduced in the TM. Most interestingly, the overall incorporation of [^{14}C]acetate into lipids was decreased in the TM (14,000 ± 500 cpm/mg cells) compared to WT (19,800 ± 1050 cpm/mg cells) suggesting a reduced overall lipid formation in the mutant strain.

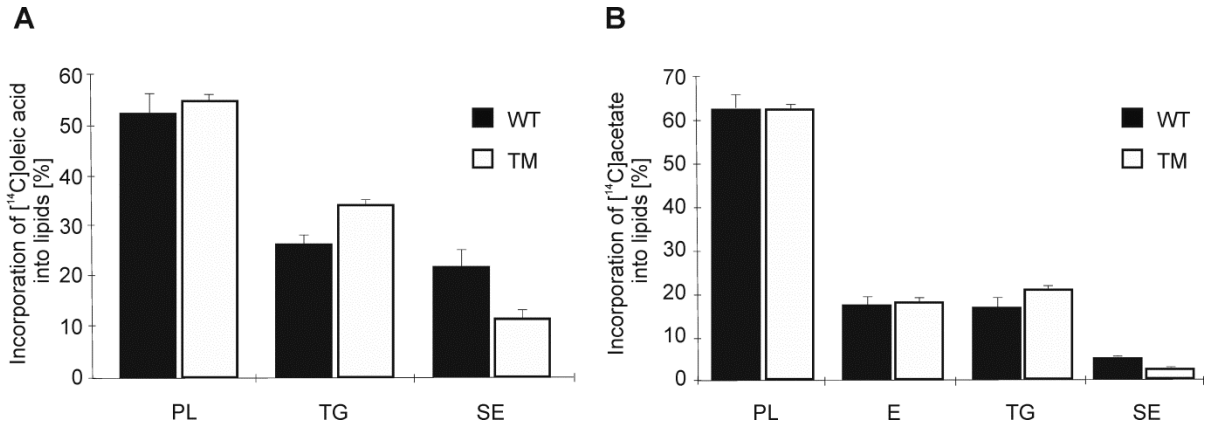

Figure 5.2 *In vivo* labeling of lipids. *In vivo* labeling with [^{14}C]oleic acid (A), and [^{14}C]acetate (B) of lipids from cells lacking the three steryl ester hydrolase (TM) compared to wild type (WT). Relative incorporation of labels into phospholipids (PL), triacylglycerols (TG) and steryl esters (SE) was analyzed. Data are mean values of two independent biological experiments analyzed in duplicate with the respective standard deviations as indicated.

To substantiate a possible feedback control to fatty acid and sterol synthesis we quantified the amounts of sterols and fatty acids in wild type and TM. Sterol quantification by GC/MS (Table 5-4) showed that deletion of the three SE hydrolases led to a decreased level of total cellular sterols to about 65% of WT. Noteworthy, these values included amounts of free sterols and sterols in the esterified form of SE and thus represented the overall sterol production in the respective strains. Quantification of free sterols can only be seen in Figure 5.3A. These results indicate that changes in SE hydrolysis had a marked influence on total sterol biosynthesis. However, the lack of SE hydrolases did not only change the total sterol content, but also the sterol pattern (see Table 5-4).

Table 5-4: Sterol analysis by GC/MS from wild type (WT) and *Δtgl1Δyeh1Δyeh2* (TM). Data are mean values from three independent experiments performed in duplicate.

	WT	TM
Total sterols (mg/g CDW)	14.85 ± 1.19	9.89 ± 0.91
	% of total sterols	
Ergosterol	70.06 ± 2.52	64.94 ± 0.38
Zymosterol	7.08 ± 0.05	12.24 ± 0.04
Lanosterol	7.90 ± 1.09	10.01 ± 0.67
Fecosterol	2.98 ± 0.77	1.05 ± 0.12
Others	11.98 ± 0.25	11.76 ± 0.48

The amount of the end product of the sterol biosynthetic pathway, ergosterol, was markedly decreased in the TM, whereas some precursors such as zymosterol and lanosterol accumulated. These results suggested that certain steps of the ergosterol biosynthetic pathway may be constricted in the TM. Indeed, expression levels of some *ERG* genes were found to be slightly decreased, e.g. *ERG2* and *ERG6* to approx. 85% of the control, thus confirming the feedback control of SE hydrolysis to sterol formation. Labeling experiments with [^{14}C]acetate described above suggested that also formation of the second substrate of SE formation, the fatty acids, was reduced in the TM. Thus, it was not surprising that the steady state level of total fatty acids in the TM was lower than in WT (Figure 5.3B). This result fostered our idea that a block in SE hydrolysis leads to substrate limitation for SE synthases.

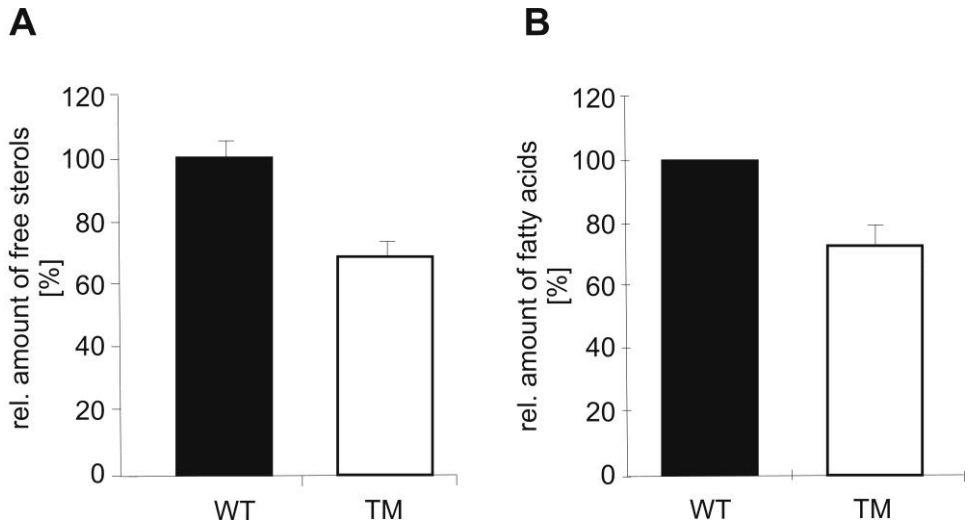

Figure 5.3. Quantification of free sterols and fatty acids. Relative amounts of (A) free sterols and (B) fatty acids in wild type (WT) and *Δtgl1Δyeh1Δyeh2* (TM) were analyzed. Free sterols were quantified by thin layer chromatography, and fatty acids were determined by GLC. Data are mean values of two independent experiments perfomed in duplicated with the respective standard deviations as indicated.

A block in steryl ester hydrolysis does not affect gene expression or protein levels but the activity of steryl ester synthesizing enzymes

Alterations in SE amounts as a consequence of defects in SE hydrolysis raised the question as to the regulation of SE synthesis. The major question addressed in the present study was how Are1p and Are2p behave when SE hydrolysis is blocked. We hypothesized that the synthesis of SE was down regulated in a yeast strain lacking all three SE hydrolases. This feedback inhibition could either be on the gene expression level, the protein level or the enzymatic level of the two SE synthesizing enzymes Are1p and Are2p. First, we investigated the expression levels of *ARE1* and *ARE2* in WT and in the TM by Real-Time PCR. As can be seen from Figure 5.4A and Fig. Figure 5.4C, the gene expression of neither *ARE1* nor *ARE2* was significantly altered in the *Δtgl1Δyeh1Δyeh2* mutant. This result excluded a regulation at the expression level. Next, we investigated whether the protein levels and stabilities of the two acyltransferases were affected. Western Blot analysis of tagged version of these enzymes performed with microsomal fractions indicated no changes of Are1p and Are2p (Figure 5.4B and Figure 5.4D).

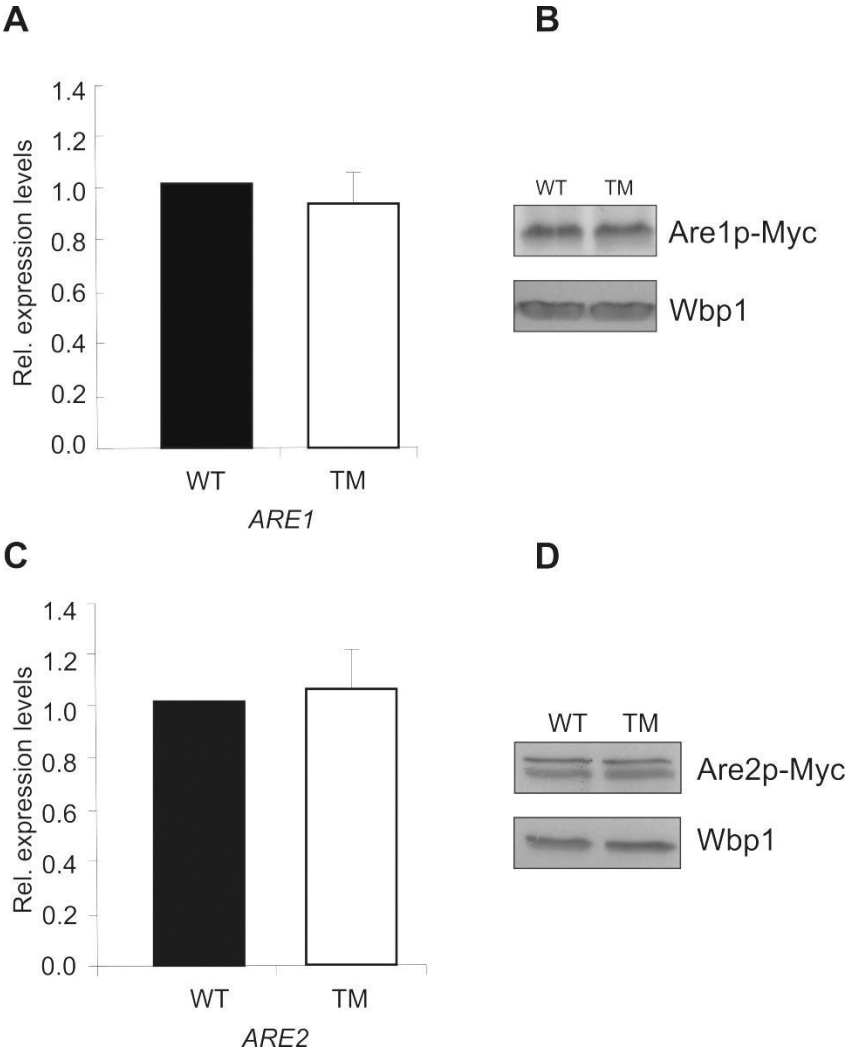

Figure 5.4: Gene expression and protein levels of Are1p and Are2p. Relative gene expression of *ARE1* (A) and *ARE2* (C) in wild type (WT) (black bar) and TM (white bar) measured by RT-PCR. Wild type was set at 1. Data are mean values from three independent experiments with the respective deviation. Protein analyses of Are1p-Myc (B) and Are2p-Myc (D) of microsomal fractions from WT and TM. Western blot analyses are representative of at least two independent experiments. Wbp1p (ER marker) was used as loading control.

Negative results obtained by Real-Time PCR and Western Blot analysis suggested that a block of SE hydrolysis may affect the activity of SE synthesizing enzymes at the enzymatic level. To test this hypothesis we performed *in vitro* enzyme assays of Are1p and Are2p. Isolated ER fractions from wild type and *Δtgl1Δyeh1Δyeh2* were used as enzyme sources. As can be seen in Figure 5.5(A,B), the acyltransferase activity was dramatically decreased in both homogenate and microsomal fractions from the TM. To exclude an influence of assay conditions on the obtained results, we varied the substrate and the detergent in the acyltransferase assays. However, use of palmitoyl-CoA instead of oleoyl-CoA as substrate, as well as replacement of CHAPS by Tyloxapol did not change the effect of the TM. To

investigate the contribution of individual SE hydrolases to the reduced enzyme activity of the two acyltransferases we also performed *in vitro* assays with single deletion strains. Figure 5.5C shows that each of the three SE hydrolases contributed partially to the reduced activity of Are1p/Are2p with the most pronounced effect of Tgl1p. Enzyme assays with tagged variants of Are1p and Are2p which were used for expression studies described above yielded identical results (data not shown), indicating that the tag did not influence the activity of the enzymes.

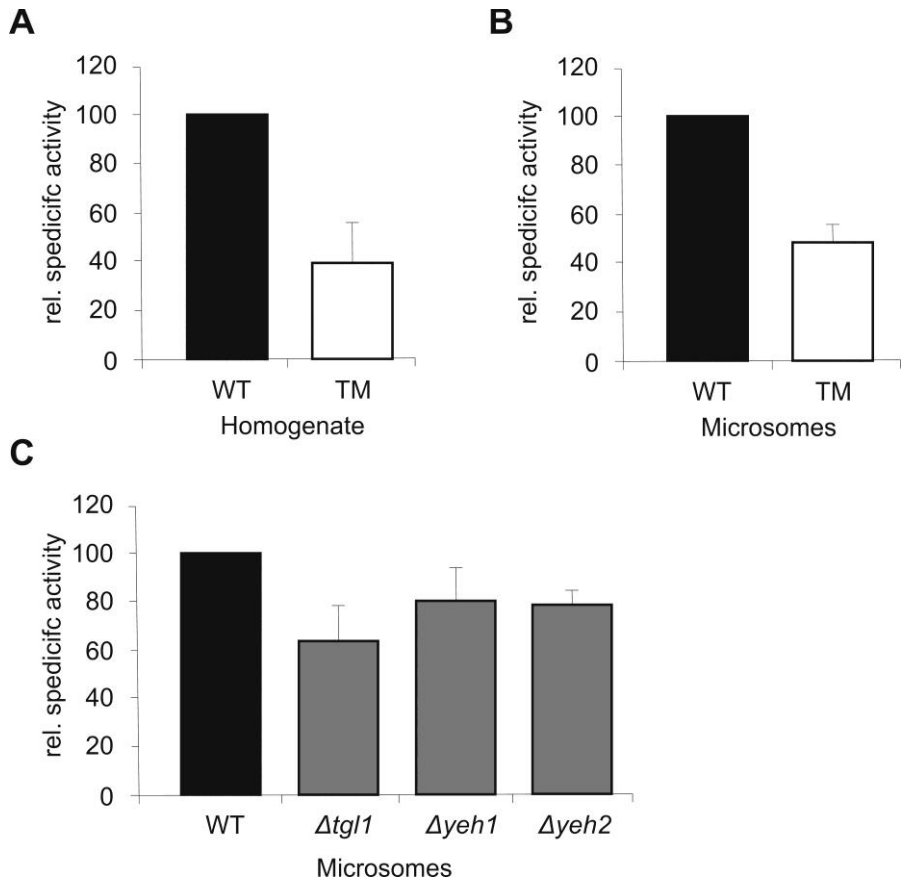

Figure 5.5: Acyl-CoA:ergosterol acyltransferase activity of Are1p/Are2p in strains bearing a defect in SE hydrolysis. (A) Homogenate was used as enzyme source. Specific activity of acyl-coA:ergosterol acyltransferase activity from wild type (WT) (1100 pmol SE/mg protein/h) was set at 100% and compared with *Δtgl1Δyeh1Δyeh2* (TM). (B) Microsomal fractions were used as enzyme source. Specific activity of acyl-coA:ergosterol acyltransferase activity from wild type (WT) (8300 pmol SE/mg protein/h) was set at 100% and compared with *Δtgl1Δyeh1Δyeh2* (TM). (C) Microsomes from single deletion mutants were compared with microsomes from WT. Specific activity of acyl-coA:ergosterol acyltransferase activity of wild type (WT) (8300 pmol SE/mg protein/h) was set at 100%. Data shown are mean values of at least three independent experiments performed in duplicate with the respective standard deviations as indicated.

DISCUSSION

Storage of the non-polar lipids SE and TG in lipid droplets is conserved from yeast to man (Reue, 2011). Although the functional characterizations as well as the substrate specificities of steps involved in non-polar lipid metabolism were studied in some detail, little is known about the regulation (Valachovic et al., 2002; Connerth et al., 2010; Sorger et al., 2004). Here, we provide some insight into regulatory aspects of the SE metabolic network in *S. cerevisiae.* We have chosen to study effects on the formation of SE in cells deleted of *TGL1*, *YEH1* and *YEH2* which encode the three known SE hydrolases. We analyzed transcriptional and translational control, protein stability as well as substrate availability as possible mechanisms regulating the activity of the two SE synthesizing enzymes Are1p and Are2p. In addition, we provide evidence of a metabolic link between SE metabolism and the biosynthesis of sterols and fatty acids.

In this study, we show that regulation of sterol acyltransferase activity in a TM lacking *TGL1*, *YEH1* and *YEH2* does not occur on the gene expression level of *ARE1* and *ARE2* (see Figure 5.4A,C). Also the protein levels of Are1p and Are2p were not significantly altered in the TM compared to wild type (see Figure 5.4B,C). Nevertheless, lipid formation analyzed by *in vivo* labeling experiments showed that a block of SE hydrolysis led to reduced SE formation whereas TG formation was enhanced. *In vitro* enzyme assays revealed that the activity of Are1p and Are2p was markedly reduced in the TM (see Figure 5.2 and Figure 5.5). These results indicated that SE synthases were subject to regulation on the enzyme level. Moreover, decreased amounts of sterols and fatty acids formed in the TM may contribute to the reduced synthesis of SE (see Table 5-4).

Based on these results we concluded that deletions of *TGL1*, *YEH1* and *YEH2* cause multiple effects of feedback regulation. The reduced formation of ergosterol in the TM deserved our special attention. Previous studies have already provided evidence for such a related feedback mechanism of SE metabolism (Sorger et al., 2004; Arthington-Skaggs et al., 1996). Sorger *et al*. (2004) reported that the amount of the squalene epoxidase Erg1p was decreased in the Δ*are1*Δ*are2* double mutant. This effect was caused by low stability of Erg1p in this strain. Arthington-Skaggs *et al*. (1996) found that the expression of *ERG3* was down-regulated in the absence of sterol esterification. These authors provided the first evidence for direct transcriptional regulation of a sterol biosynthetic gene in response to sterol

esterification. Our data presented here revealed that the expression of *ERG2* and *ERG6* was slightly decreased in the TM compromised in SE hydrolysis. Taken together, these data provide strong evidence of a regulatory link between SE metabolism and *de novo* biosynthesis of sterols.

The enhanced formation of TG in the TM indicates a link between TG formation and SE metabolism. Evidence of a "cross-talk" between SE and TG metabolism was recently also observed in another study from our lab (Schmidt *et al.*, manuscript submitted). These authors showed that yeast strains defective in the three major TG lipases Tgl3p, Tgl4p and Tgl5p produce more SE than WT. Although a balanced distribution between TG and SE seems to be a logical way to get rid of the excess of fatty acids, the question as to the specific regulation within the non-polar lipid metabolic network remains. The fact that fatty acids were not randomly distributed over all lipid classes was demonstrated by the finding that phospholipids in the Δ*tgl1*Δ*yeh1*Δ*yeh2* triple mutant were not overproduced. A novel facet of SE and TG metabolism was the identification of novel hydrolases and lipases which appear to come to the fore when the main TG lipolytic activities are blocked (Ploier et al., 2013; Thoms et al., 2011; Debelyy et al., 2011). It has to be considered that such enzymes may compensate for deletions of the major hydrolytic enzymes. Another interesting aspect which has to be taken into account is the subcellular localization of the different SE hydrolases. Although the natural substrates of these enzymes, SE, which are stored in LD, the major SE hydrolase Yeh2p was localized to the cell periphery (Köffel et al., 2005). How this enzyme gets access to its substrate and what its possible role at the cell periphery may be has to be clarified.

Thus, despite the findings mentioned above several questions regarding non-polar lipid metabolism remain open. Results presented here contribute to our knowledge of non-polar lipid metabolism by the finding that SE hydrolases in the yeast cause a feedback control to upstream enzymatic steps. These links within the SE metabolic network appear to be important for a balanced non-polar lipid metabolism and may have a broader impact on interaction of lipids as storage and membrane forming components.

ACKNOWLEDGMENTS

We thank the Austrian Centre of Industrial Biotechnology (ACIB) Graz for providing the ABI 7500 instrument and Karin Athenstaedt for fruitful discussions. This research was funded by the Austrian Science Fund (FWF) projects W901 DK Molecular Enzymology and P23029 to G. D.

REFERENCES

Arthington-Skaggs B.A., Crowell D.N., Yang H., Sturley S.L., Bard M. (1996). Positive and negative regulation of a sterol biosynthetic gene (*ERG3*) in the post-squalene portion of the yeast ergosterol pathway. FEBS Lett., *392,* 161–165.

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

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

Brasaemle D.L., Wolins N.E. (2012). Packaging of fat: an evolving model of lipid droplet assembly and expansion. J. Biol. Chem., *287*, 2273–2279.

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.

Choudhary V., Schneiter R. (2009). Monitoring sterol uptake, acetylation, and export in yeast. Methods Mol. Biol. Clifton NJ, *580*, 221–232.

Choudhary V., Schneiter R. (2012). Pathogen-Related Yeast (PRY) proteins and members of the CAP superfamily are secreted sterol-binding proteins. Proc. Natl. Acad. Sci. U. S. A., *109*, 16882–16887.

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

Connerth M., Czabany T., Wagner A., Zellnig G., Leitner E., Steyrer E., Daum G. (2010). Oleate inhibits steryl ester synthesis and causes liposensitivity in yeast. J. Biol. Chem., *285*, 26832–26841.

Czabany T., Wagner A., Zweytick D., Lohner K., Leitner E., Ingolic E., 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., Stymne S., 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 (2000) 6487–6492.

Debelyy M.O., Thoms S., Connerth M., Daum G., Erdmann R. (2011). Involvement of the *Saccharomyces cerevisiae* hydrolase Ldh1p in lipid homeostasis. Eukaryot. Cell, *10*, 776–781.

Fei W., Zhong L., Ta M.T., Shui G., Wenk M.R., Yang H. (2011). The size and phospholipid composition of lipid droplets can influence their proteome. Biochem. Biophys. Res. Commun., *415*, 455–462.

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

Fujimoto T., Parton R.G. (2011). Not just fat: the structure and function of the lipid droplet. Cold Spring Harb. Perspect. Biol., *3*, a004838

Fujimoto T., Ohsaki Y., Cheng J., Suzuki M., Shinohara Y. (2008). Lipid droplets: a classic organelle with new outfits. Histochem. Cell Biol., *130*. 263–279.

Gietz R.D., Schiestl R.H., Willems A.R., Woods R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast, *11*, 355–360.

Grillitsch K., Connerth M., Köfeler H., Arrey T.N., Rietschel B., Wagner B., Karas M., Daum G. (2011). Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim. Biophys. Acta, *1811*, 1165–1176.

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

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

Jandrositz A., Petschnigg J., Zimmermann R., Natter K., Scholze H., Hermetter A., Kohlwein S.D., 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.

Köffel R., Schneiter R. (2006). Yeh1 constitutes the major steryl ester hydrolase under heme-deficient conditions in *Saccharomyces cerevisiae.* Eukaryot. Cell, *5*, 1018–1025.

Köffel R., Tiwari R., Falquet L., 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., Veenhuis M., van der Klei I.J. (2013). Lipid droplets and peroxisomes: key players in cellular lipid homeostasis or a matter of fat- store 'em up or burn 'em down. Genetics, *193*, 1–50.

Kurat C.F., Natter K., Petschnigg J., Wolinski H., Scheuringer K., Scholz H., Zimmermann R., Leber R., Zechner R., 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*, 680–685.

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

Livak K.J., Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, *25*, 402–408.

Longtine M.S., McKenzie A. 3rd, Demarini D.J., Shah N.G., Wach A., Brachat A., Philippsen P., Pringle J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast, *14*, 953–961.

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

Müllner H., Deutsch G., Leitner E., Ingolic E., Daum G. (2005). *YEH2*/YLR020c encodes a novel steryl ester hydrolase of the yeast *Saccharomyces cerevisiae*. J. Biol. Chem., *280*, 13321–13328.

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

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

Ploier B., M. Scharwey, B. Koch, C. Schmidt, J. Schatte, G. Rechberger, M. Kollroser, A. Hermetter, G. Daum (2013). Screening for hydrolytic enzymes reveals Ayr1p as a novel triacylglycerol lipase in *Saccharomyces cerevisiae*. J. Biol. Chem., *288*, 36061–36072.

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

Rajakumari S., Rajasekharan R., Daum G. (2010) Triacylglycerol lipolysis is linked to sphingolipid and phospholipid metabolism of the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta, *1801*, 1314–1322.

Reue K. (2011). A thematic review series: lipid droplet storage and metabolism: from yeast to man. J. Lipid Res., *52*, 1865–1868.

Sandager L., Dahlqvist A., Banaś A., Ståhl U., Lenman M., Gustavsson M., Stymne S. (2000). An acyl-CoA:cholesterol acyltransferase (ACAT)-related gene is involved in the accumulation of triacylglycerols in *Saccharomyces cerevisiae*. Biochem. Soc. Trans., *28*, 700–702.

Sandager L., Gustavsson M.H., Ståhl U., Dahlqvist A., Wiberg E., Banas A., Lenman M., Ronne H., Stymne S. (2002). Storage lipid synthesis is non-essential in yeast. J. Biol. Chem., *277*, 6478–6482.

Sorger D., 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.

Sorger D., Athenstaedt K., Hrastnik C., Daum G. (2004). A yeast strain lacking lipid particles bears a defect in ergosterol formation. J. Biol. Chem., *279*, 31190–31196.

Thoms S., Debelyy M.O., Connerth M., Daum G., Erdmann R. (2011). The putative *Saccharomyces cerevisiae* hydrolase Ldh1p is localized to lipid droplets. Eukaryot. Cell, *10*, 770–775.

Tinkelenberg A.H., Liu Y., Alcantara F., Khan S., Guo Z., Bard M., Sturley S.L. (2000). Mutations in yeast *ARV1* alter intracellular sterol distribution and are complemented by human *ARV1*. J. Biol. Chem., *275*, 40667–40670.

Tiwari R., Köffel R., Schneiter R. (2007). An acetylation/deacetylation cycle controls the export of sterols and steroids from *S. cerevisiae*. EMBO J., *26*, 5109–5119.

Valachovic M., Klobucníková V., Griac P., 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.

Wagner A., Grillitsch K., Leitner E., Daum G. (2009). Mobilization of steryl esters from lipid particles of the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta, *1791*, 118–124.

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

Yu C., N.J. Kennedy, C.C. Chang, J.A. Rothblatt (1996). Molecular cloning and characterization of two isoforms of *Saccharomyces cerevisiae* acyl-CoA:sterol acyltransferase. J. Biol. Chem., *271*, 24157–24163.

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

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

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

6

THESIS SUMMARY AND DISCUSSION

Saccharomyces cerevisiae is an important model organism in life sciences. It was the first eukaryote whose genome was completely sequenced (Goffeau et al., 1996). In many respects yeast still is the major proving ground for functional genomics, proteomics or systems biology as reviewed by Suter *et al*. (2006). The *Saccharomyces* Genome Database (SGD) provides yeast researchers with the reference genomic chromosomal sequence and provides encyclopedic information about the yeast genes, proteins and other encoded features (Cherry et al., 2012). Much of the current knowledge of how eukaryotic cells work has come from studies with yeast, and this is especially true for research on lipid metabolism. Over the last few decades outstanding advancements have been made to identify the major enzymes involved in the pathways of lipid metabolism. The main cellular routes for synthesis, storage and degradation of lipid compounds are now known. However, some gaps still remained. One issue under discussion is the regulation of lipid metabolism. It occurs at many different levels, and the cellular lipid composition is not only extremely dependent on growth conditions such as nutrient availability, growth phase and pH, but also on many transcriptional control mechanisms that have been reported. Another intriguing open question is why yeast seems to have a high redundancy of enzymatic activities involved in lipid metabolism and under which conditions minor activities come to the fore. Especially interesting regarding regulatory mechanisms is the existence of multifunctional enzymes which also show dual localizations.

This Thesis was aimed at elucidating some selected aspects of the complex network of lipid metabolism in the yeast *S. cerevisiae*. Special emphasis was set on deciphering molecular mechanisms of regulatory aspects of non-polar lipid metabolism and the identification of novel enzymes involved in the mobilization of the main storage lipid molecules, the triacylglycerols. In the following, findings of each Thesis chapter will be summarized, discussed in the light of our recent knowledge from the literature, and results will be

connected to the overall Thesis topic “Molecular Enzymology of hydrolytic/lipolytic enzymes in the yeast *Saccharomyces cerevisiae*”. The contribution of this work to this field of lipid research will be described.

IDENTIFICATION OF NOVEL HYDROLYTIC/LIPOLYTIC ENZYMES

Previous studies from our lab identified Tgl3p, Tgl4p and Tgl5p as major triacylglycerol lipases of the yeast *S. cerevisiae* being localized to LD (Athenstaedt and Daum, 2003, 2005). In a triple deletion strain lacking these major lipolytic enzymes, the mobilization of triacylglycerols (TG) was strongly impaired but not completely abolished when cells were grown on glucose. In chapter 3 we show that a yeast strain lacking the three TG lipases and the sterol acyltransferases Are1p and Are2p grown on oleic acid is not only able to hydrolyze TG *in vivo*, but also to mobilize TG close to wild type level (see Figure 4.1). This result tempted us to speculate about the presence of further hydrolytic enzymes. Our experimental strategy to identify further enzymes possibly involved in the mobilization of TG was to design a functional proteome approach making use of specific fluorescent inhibitors of esterases and lipases as probes. The list of proteins detected in this screening (see Chapter 4, supplementary data) was narrowed to a subset of proteins with putative hydrolase or lipase function (see Table 4-4). TG content analysis (Figure 4.5), *in vivo* mobilization of TG (Figure 4.6 and Table 4-7) as well as *in vitro* enzyme assays (Figure 4.7) revealed Ayr1p as novel TG lipase and confirmed the lipolytic potential of Lpx1p *in vivo* that has been previously shown *in vitro* by Thoms *et al.* (2011). One open question of this study was why yeast cells already suffering from an excess of free fatty acids by supply from the medium still mobilize TG. Currently, we can only speculate that yeast resorts to different pools of lipids upon different requirements. One interpretation may be that fatty acids mobilized from TG may serve primarily for phospholipid synthesis or β-oxidation. However, proof for this model is still missing.

Another interesting aspect of this study (Ploier et al., 2013) was that we found two gene products as hits in the functional proteomic approach with currently unknown functions. The genome of *S. cerevisiae* is composed of about 6500 genes organized on 16 chromosomes. Only 5800 genes have been characterized so far meaning that nearly 1000 genes are still listed as uncharacterized on the *Saccharomyces* Genome Database (SGD), even twenty years

after sequencing the genome (Peña-Castillo and Hughes, 2007). This fact was the motivation for some further investigations on gene products of YBR056w and YKL050c. According to SGD, YBR056w encodes a putative glycoside hydrolase of the mitochondrial intermembrane space alias 17-beta-hydroxysteroid dehydrogenase-like protein (Huh et al., 2003; Vögtle et al., 2012); and Ykl050cp is seen as protein of unknown function that has a paralog, *EIS1*, a component of the eisosome required for proper eisosome assembly that arose from the whole genome duplication (Byrne and Wolfe, 2005). The protein is a target of the SCFCdc4 ubiquitin ligase complex and its transcription is regulated by Azf1p (Slattery et al., 2006; Tang et al., 2005). A minor hydrolytic potential of YBR056wp was proven in this study using artificial esterase substrates, although a role of either of these two proteins in the metabolism of non-polar lipids was not found. To elucidate the possible function of these proteins in the cell, phenotypic and lipid analyses as well as localization and gene expression studies were performed. A Myc-tagged protein variant of YBR056w could not be found as described in the literature in the mitochondrial fraction (Vögtle et al., 2012), but was predominantly detected in the cytosol and microsomal fractions (Barbara Koch, personal communication). This observation was in line with purification attempts where Ybr056wp could be easily purified directly from the homogenate without prior solubilization. Ykl050cp, on the other side, could not be purified from any subcellular fraction although some positive clones for the tagged construct were obtained. We could never detect the fusion protein in any fraction, not even in isolated plasma membrane, which was tried following the hint of SGD that Ykl050cp is a paralog of the eisosomal protein *EIS1*. The phenotypic analysis of deletion and overexpression-strains of these two candidates did not result in any effect. Moreover, analyses of non-polar lipids, sterols and fatty acids did not reveal significant alterations compared to wild type. However, phospholipid analyses showed changes in the amounts of total phospholipids as well as differences in the phospholipid composition. The amount of total phospholipids was slightly decreased in the two deletion strains, and increased levels of phosphatidylethanolamine were found at the expense of phosphatidic acid. The opposite effect could be observed in analyses with strains overexpressing YBR056w and YKL050c, respectively. These results led to the idea that a link to the Kennedy pathway (for a detailed description of this pathway see Chapter 1) might exist. Indeed, we observed that *EPT1* and *ECT1* were significantly down-regulated in strains lacking YBR056w and YKL050c, respectively. These preliminary data might serve as an indication that YBR056wp

and YKL050cp might play a role in the phospholipid metabolism of *S. cerevisiae*. Further analyses with these proteins will be necessary for a functional characterization of the two genes YBR056w and YKL050c. Their potential roles could further be investigated by *in vitro* phospholipase assays and combinations of deletions of genes involved in phospholipid metabolism.

In summary, results of this study showed that TG turnover is not only catalyzed by the main TG lipases Tgl3p, Tgl4p and Tgl5p, but may also be performed by a subset of lipases with minor lipolytic activities. One intriguing open question is why yeast cells rely on several lipases and what the contributions of the single lipases to TG mobilization are. An especially interesting aspect to be taken into account when discussing enzymes involved in the turnover of non-polar lipids is their subcellular localization. These enzymes can be found on various organelles including the endoplasmic reticulum, lipid droplets, peroxisomes and, as shown for the steryl ester hydrolase Yeh2p, even at the plasma membrane (Köffel et al., 2005). Studies on membrane association between organelles are a hot topic of current lipid research and will probably be able to shed more light on these questions. Our data suggest that the action of these enzymes is strongly growth-dependent and that they may come to the fore when the major enzymes are inactive or lacking.

REGULATORY MECHANISMS OF NON-POLAR LIPID METABOLISM

Besides the identification of Ayr1p as a novel triacylglycerol lipase, investigations on regulatory mechanisms of the steryl ester (SE) metabolism were another main topic of this Thesis. Especially interesting for us was the question how the two SE synthesizing enzymes, Are1p and Are2p, behave in a strain lacking the three SE degrading enzymes, Yeh1p, Yeh2p and Tgl1p (TM). Our results show that deletions of *TGL1*, *YEH1* and *YEH2* cause multiple effects of feedback regulation. This study revealed that the regulation of sterol acyltransferase activity in the TM did not occur on the gene expression levels or protein levels of Are1p and Are2p (Figure 5.4). Nevertheless, lipid formation analyzed by *in vivo* labeling experiments showed that a block of SE hydrolysis led to reduced SE formation whereas TG formation was enhanced. *In vitro* enzyme assays revealed that the activity of Are1p and Are2p was markedly reduced in the TM (see Figure 5.2 and Figure 5.5). These results indicated that SE synthases were subject to regulation on the enzyme level. Moreover, decreased amounts of sterols and fatty acids formed in the TM may contribute to

the reduced synthesis of SE (see Table 5-4), providing evidence of a metabolic and regulatory link between SE metabolism and the biosynthesis of sterols and fatty acids. Despite the findings described in this study, many questions regarding a regulatory mechanism of SE metabolism remain open. There are several aspects which may be considered for future investigations, including the systematic study of proteins interacting with enzymes involved in non-polar lipid metabolism. Partner-proteins regulating the enzymatic activities of SE synthesizing enzymes, and also the possibilities of lipids, i.e. the membrane environment of proteins with transmembrane domains, as modulators of protein functions may play a role. To get an idea how the lack of SE hydrolases changes the cellular setup, microsomal fractions of wild type and TM could be purified and their proteome and lipidome could be compared addressing modifications of metabolic routes in the lipid network. Moreover, regulatory posttranslational modifications including phosphorylation, ubiquitination or acetylations of non-polar lipid synthesizing enzymes could play a role in balancing storage and degradation of lipid molecules.

GENERAL CONCLUSION

Over the last few decades outstanding advancements have been made to identify the major enzymes involved in the main cellular routes for synthesis, storage and degradation of triacylglycerols and steryl esters. However, still some gaps remained. This Thesis aimed at elucidating novel aspects of the complex metabolic network of non-polar lipids in the yeast *S. cerevisiae*. Data presented in this study contribute to our understanding of non-polar lipid metabolism by the finding that Ayr1p was identified as novel TG lipase, and that SE hydrolases in the yeast cause a feedback control to upstream enzymatic steps. These results appear to be important for a balanced non-lipid metabolism under different environmental conditions and may have a broader impact on the interactive role of lipids as storage and membrane forming components. These data contribute to a more detailed knowledge of non-polar lipid turnover and set the stage to investigate regulatory aspects of non-polar lipid metabolism in more detail.

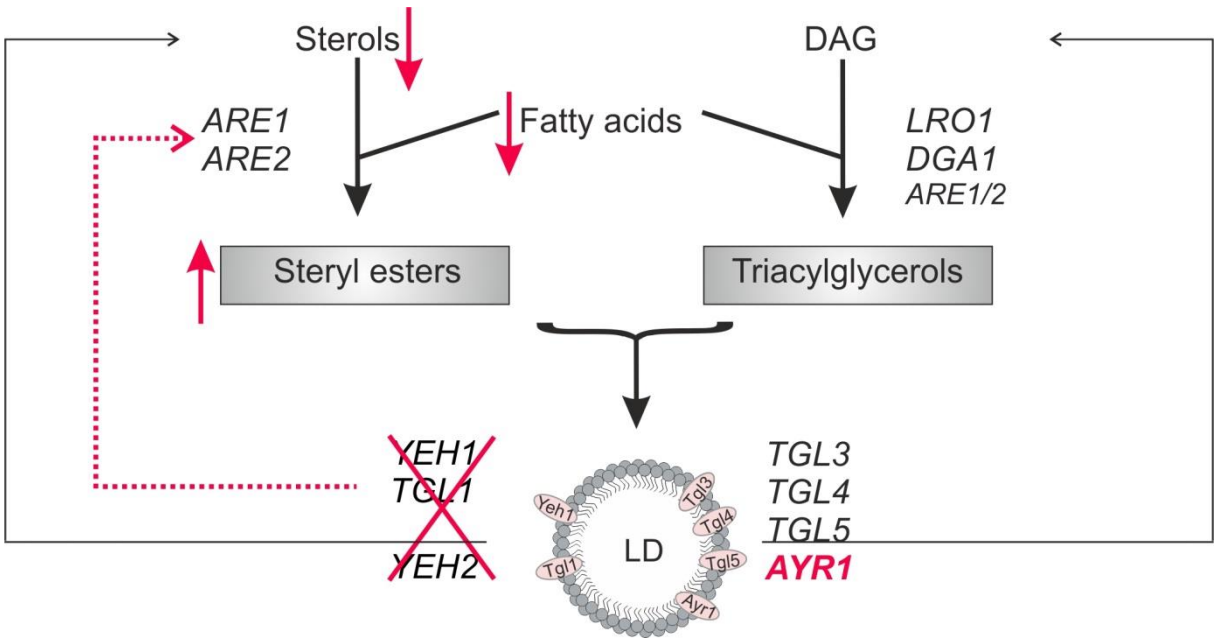

Figure 6.1: Graphical summary of results obtained in this PhD Thesis. Overview of non-polar lipid metabolism in *S. cerevisiae*, novel findings are highlighted in red: The major storage lipids of the yeast *S. cerevisiae* are steryl esters and triacylglycerols. Both are stored in organelle-like structures called lipid droplets (LD). Steryl esters are synthesized by Are1p and Are2p and are mobilized upon requirement by the three steryl ester hydrolases Yeh1p, Yeh2p and Tgl1p. A yeast strain lacking these hydrolases shows feedback regulation of steryl ester synthesis accompanied by reduced amounts of sterols and fatty acids. Triacylglycerols are synthesized by Lro1p and Dga1p, and with minor efficiency also by Are1p and Are2p. Triacylglycerol mobilization is catalyzed by the main lipases Tgl3p, Tgl4p and Tgl5p and by the newly identified triacylglycerol lipase Ayr1p.

REFERENCES

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.

Byrne, K.P., and Wolfe, K.H. (2005). The Yeast Gene Order Browser: combining curated homology and syntenic context reveals gene fate in polyploid species. Genome Res. *15*, 1456–1461.

Cherry, J.M., Hong, E.L., Amundsen, C., Balakrishnan, R., Binkley, G., Chan, E.T., Christie, K.R., Costanzo, M.C., Dwight, S.S., Engel, S.R., et al. (2012). *Saccharomyces* Genome Database: the genomics resource of budding yeast. Nucleic Acids Res. *40*, D700–705.

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., et al. (1996). Life with 6000 genes. Science *274*, 546, 563–567.

Huh, W.-K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. (2003). Global analysis of protein localization in budding yeast. Nature *425*, 686–691.

Köffel, 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.

Peña-Castillo, L., and Hughes, T.R. (2007). Why are there still over 1000 uncharacterized yeast genes? Genetics *176*, 7–14.

Ploier, B., Scharwey, M., Koch, B., Schmidt, C., Schatte, J., Rechberger, G., Kollroser, M., Hermetter, A., and Daum, G. (2013). Screening for hydrolytic enzymes reveals Ayr1p as a novel triacylglycerol lipase in *Saccharomyces cerevisiae*. J. Biol. Chem. *288*, 36061–36072.

Slattery, M.G., Liko, D., and Heideman, W. (2006). The function and properties of the Azf1 transcriptional regulator change with growth conditions in *Saccharomyces cerevisiae*. Eukaryot. Cell *5*, 313–320.

Suter, B., Auerbach, D., and Stagljar, I. (2006). Yeast-based functional genomics and proteomics technologies: the first 15 years and beyond. BioTechniques *40*, 625–644.

Tang, X., Orlicky, S., Liu, Q., Willems, A., Sicheri, F., and Tyers, M. (2005). Genome-wide surveys for phosphorylation-dependent substrates of SCF ubiquitin ligases. Methods Enzymol. *399*, 433–458.

Thoms, S., Debelyy, M.O., Connerth, M., Daum, G., and Erdmann, R. (2011). The putative *Saccharomyces cerevisiae* hydrolase Ldh1p is localized to lipid droplets. Eukaryot. Cell *10*, 770–775.

Vögtle, F.-N., Burkhart, J.M., Rao, S., Gerbeth, C., Hinrichs, J., Martinou, J.-C., Chacinska, A., Sickmann, A., Zahedi, R.P., and Meisinger, C. (2012). Intermembrane space proteome of yeast mitochondria. Mol. Cell. Proteomics MCP *11*, 1840–1852.

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10/2008-10/2010	**Graz University of Technology (Austria)** Master's degree Biotechnology, passed with excellence Title of Master Thesis: "Engineering of Sterol Synthesis in Yeast"
02/2009- 07/2009	**University of Sheffield (United Kingdom)** ERASMUS Programme
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08/2009-09/2009 and **12/2009-01/2010**	**Graz University of Technology** Project Student Assistant, Institute of Molecular Biotechnology
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2004-2009	Various internships (**Gynaecological Ward of the LKH; pharmacy**: Apotheke Eisteichgasse; Trainee at **AVL UK Ltd. Peterborough (United Kingdom)**; Various jobs as a tutor (mathematics, English, chemistry) and nanny for 2 children as a student job

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Languages	**German** (mother tongue), **English** (fluent, contractual level), **French** (advanced: Diploma DELF A1 and A2), **Italian** (basic), **Spanish** (basic)
Soft Skills	**01/2013- 12/2013** **"Karriereprogramm für Wissenschafterinnen 2013"** Seminars in time management, career planning, communication, rhetoric's and presentation skills, moderation, conflict management, self-management, negotiation strategies, group leading, motivation strategies and networking Seminars of the **"Bildungspass Forum"**: Team Development; conflict management Seminars of **„Rhetorics and Discussion"**of the "Steiermärkische Landesregierung"
Computer skills	Extensive knowledge of Windows system, Office, CorelDraw, Various bioinformatic tools (Databases, VNTI, Serialcloner, Lasergene programmes, BLAST, etc.)

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Distinctions

09/2013	**ÖGMBT poster award** at the 5th ÖGMBT meeting
08/2012	**GSA poster award** at the Yeast Genetics and Molecular Biology meeting, Princeton University, New Jersey, USA
02/2012	**Organizer of the 7th NAWI Graz Doctoral School-DocDay**
2012	**Master's degree with honour's**
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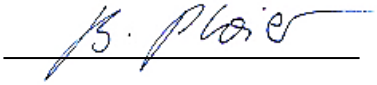

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PUBLICATIONS

Ploier B., Scharwey M., Koch B., Schmidt C., Schatte J., Rechberger G., Kollroser M., Hermetter A., and Daum, G.: Screening for hydrolytic enzymes revealed Ayr1p as a novel triacylglycerol lipase in *Saccharomyces cerevisiae*. *J Biol Chem* (2013), 288 (50), 36061-72.

Ploier B., Daum G., and Petrovič U.: Lipid metabolism and Lipidomics. *Molecular Mechanisms in Yeast Carbon Metabolism.* Springer (2014), Ed. Jure Piskur and Concetta Compagno (In press)

Schmidt C.*, **Ploier B.***, Koch B., and Daum G.: Analysis of yeast lipid droplet proteome and lipidome. *Methods in Cell Biology* (2013), 16, 15-37. doi: 10.1016/B978-0-12-408051-5.00002-4.
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Gsell M., Mascher G., Schuiki I., **Ploier B.**, Hrastnik C., Daum G.: Transcriptional Response to Deletion of the Phosphatidylserine Decarboxylase Psd1p in the Yeast *Saccharomyces cerevisiae*. *PLoS ONE* (2013) 8(10): e77380. doi:10.1371/journal.pone.0077380

Schmidt C., Athenstaedt K., Koch B., **Ploier B**., Daum G.: Regulation of the yeast triacylglycerol lipase Tgl3p by formation of non polar lipids. *Journal of Biological Chemistry* (2013), 288 (27), 19939-19948.

Souza C. M., Schwabe TM, Pichler H, **Ploier B**., Leitner E, Guan XL, Wenk MR, Riezman I, Riezman H: A stable yeast strain efficiently producing cholesterol instead of ergosterol is functional for tryptophan uptake, but not weak organic acid resistance. *Metabolic Engineering* (2011), 13(5), 555-569.

Bartholomae M., **Ploier B.**, Cheung, H., Ouellette, W, Zubieta, J.: Coordination polymers of Cu(II) and Cd(II) with bifunctional chelates of the type dipicolylamino-alkylcarboxylate, $(NC_5H_4CH_2)_2N(CH_2)nCO_2H$ (n = 1, 2, 3 and 4). *Inorganica Chimica Acta*(2010), 363 (8), 1659-1665.

Bartholomae MD, Vortherms AR, Hillier S., **Ploier B.,** Joyal J., Babich J., Doyle R.P, Zubieta J., Synthesis, Cytotoxicity, and Insight into the Mode of Action of $Re(CO)_3$ Thymidine Complexes. *ChemMedChem* (2010), 5(9), 1513-1529.

Koch B., Schmidt C., **Ploier B**., and Daum G.: Minimal changes in the C-terminus affect functionality and stability of yeast triacylglycerol lipase Tgl3p. *J Biol Chem* (2014, under revision)

Ploier B.*, Korber M*., Koch B., Schmidt C., and Daum G.: Regulatory link between steryl ester formation and hydrolysis in the yeast *Saccharomyces cerevisiae*.
* equally contributed (submitted)

Schmidt C., Athenstaedt K., Koch B., **Ploier B**., Zellnig G., and Daum G.: Defects in triacylglycerol lipolysis affect synthesis of triacylglycerols and steryl esters in the yeast.
(BBA 2014, under revision)

ORAL AND POSTER PRESENTATIONS

05/2014	**3rd European Symposium on Microbial Lipids** (Hamburg, Germany), talk
09/2013	**5th ÖGMBT Annual Meeting** (Innsbruck, Austria), talk and poster
06/2013	**30th International Specialised Symposium on Yeast** (Stará Lesná, Slovakia), talk
04/2013	**Graduate Seminar DK Molecular Enzymology** (Graz, Austria), talk
12/2012	**"Enzymes and Signals" Joint Research Summit** (Graz, Austria), poster presentation
08/2012	**GSA Yeast Genetics and Molecular Biology meeting** (Princeton University, USA), poster presentation
10/2012	**NAWI Graz day** (Graz, Austria), poster presentation
04/2010	**ACIB Science days** (Fürstenfeld, Austria), poster presentation