DISSERTATION

Topology of Tgl3p, the main Triacylglycerol Lipase of the Yeast

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

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Summary

Tgl3p, Tgl4p and Tgl5p are the major triacylglycerol (TG) lipases of the yeast *Saccharomyces cerevisiae*, a highly suitable and well established model organism for studies of lipid biology and biochemistry. These hydrolytic enzymes were demonstrated to be located to lipid droplets (LD) where they are responsible for degradation and turnover of TG. Different experiments from our laboratory revealed a dual function of these enzymes. Tgl3p, Tgl4p and Tgl5p are not only required for the hydrolosis of TG but also contribute to phospholipid metabolism as lysophospholipid acyltransferases. It has been shown that both enzyme activities work completely independently from each other due to distinct and independent active centres for lipolysis and acyltransferase reaction. The present work focuses on the topology of Tgl3p, the main TG lipase of the yeast *S. cerevisiae*. With the strategy of limited proteolysis by Proteinase K we were able to show that the N-terminus of Tgl3p faces the cytosolic site of the organelle whereas the C-terminus of this lipase plays a critical role in protein stability and is buried within the LD. Furthermore, our investigations revealed two aspartate residues within the C-terminus which are crucial for the lipase activity of the enzyme. Interestingly, Tgl3p shows a dual localization within yeast cells. It is found on LD and in the endoplasmic reticulum (ER). This is a challenging situation for proteins, as the surface of LD consists of phospholipid monolayer whereas the ER is built from a phospholipid bilayer. Results obtained in this work suggest that the C-terminus of Tgl3p is also facing the cytosolic site when the protein is present in the ER. This fact might explain the reduced half-life and inactivation of the enzyme in the ER. We hypothesize that Tgl3p has to undergo structural rearrangements to become fully functional in the phospholipid monolayer of LD.

Zusammenfassung

Tgl3p, Tgl4p und Tgl5p sind die wichtigsten Triacylglyceridlipasen der Hefe *Saccharomyces cerevisiae*. Diese hydrolytischen Enzyme befinden sich auf Lipid droplets (LD), wo sie für den Abbau und den Umsatz von Triglyceriden (TG) verantwortlich sind. Die vorliegende Arbeit konzentriert sich auf die Topologie von Tgl3p, der wichtigsten TG-Lipase der Hefe *S. cerevisiae*, einem sehr gut geeigneten und seit langem etablierten Modellorganismus für Untersuchungen zum Lipidstoffwechsel. Verschiedene Experimente aus unserem Labor zeigten eine doppelte Funktion dieser Enzyme. Tgl3p, Tgl4p und Tgl5p sind nicht nur für die Hydrolyse von TG erforderlich, sondern tragen auch zum Phospholipidmetabolismus als Lysophospholipidacyltransferasen bei. Diese beiden Enzymaktivitäten arbeiten durch unterschiedliche aktive Zentren völlig unabhängig voneinander. Die vorliegende Arbeit beschäftigt sich mit der Aufklärung der Topologie von Tgl3p auf LD der Hefe. Mithilfe limitierter Proteolyse durch Proteinase K konnten wir zeigen, dass der N-Terminus auf der cytosolischen Seite der LD zu finden ist, während der C-Terminus dieser Lipase eine entscheidende Rolle bei der Proteinstabilität spielt und sich auf der dem Innern des LD zugewandten Seite befindet. Ferner konnten wir mit unseren Untersuchungen zwei Aspartatreste im C-Terminus von Tgl3p identifizieren, die entscheidend für die Lipase-Aktivität des Enzyms sind. Interessanterweise zeigt Tgl3p eine duale Lokalisierung in Hefezellen. Der Großteil des Proteins befindet sich auf LD und ein kleinerer Anteil im Endoplasmatischen Retikulum (ER). Dies ist eine herausfordernde Situation für Proteine, da die Oberfläche der LD aus einer Phospholipid-Einzelschicht besteht, während das ER durch eine Phospholipid-Doppelschicht aufgebaut wird. Ergebnisse dieser Arbeit weisen darauf hin, dass der C-Terminus von Tgl3p auf der zytosolischen Seite des ER zu finden ist. Dies könnte eine Erklärung für die reduzierte Halbwertszeit und Inaktivierung der beiden aktiven Zentren des Enzyms sein, wenn es sich im ER befindet. Wir vermuten, dass Tgl3p strukturelle Umlagerungen durchläuft, wenn es während der LD Biogenese von der Phospholipid-Doppelschicht des ER in die Phospholipid-Monolayermembran der LD wechselt um voll funktionstüchtig zu werden.

Aim and Hypothesis

All types of eukaryotic cells contain intracellular lipid droplets (LD) (1). These organelles are specialized to store non-polar lipids such as triacylglycerols (TG) and steryl esters (SE). TG and SE are low-volume and high-energy reserve materials serving as an internal depot for sterols and fatty acids, which are important building blocks for membrane biogenesis and a source of cellular energy. LD of the yeast *Saccharomyces cerevisiae* are small spherical organelles with an approximate diameter of ~400 nm and consist of 95% non-polar lipids with approximately equal amounts of TG and SE, each (2). TG and SE seem to be present in an ordered arrangement instead of being randomly distributed with TG forming the inner core of the LD which is surrounded by several shells of SE most likely with some TG intercalated. In contrast to other organelles, the surface of LD is covered by a phospholipid monolayer (3).
The major TG lipases of the yeast, Tgl3p, Tgl4p and Tgl5p were identified in the proteome of LD proteins (4, 5). All three lipases share the conserved GXSXG lipase motif and a patatin domain instead of a typical α/β hydrolase fold (8). Tgl3p shows the highest lipolytic activity of the three major TG lipases in the yeast, and a deletion of *TGL3* causes accumulation of TG up to 200% compared to wild type cells (5). Interestingly, Tgl3p also harbors an H-XXXX-D acyltransferase motif and also fulfills biosynthetic functions as lysophosphatidylethanolamine acyltransferase (6). Multiple enzymatic activities were also shown for Tgl4p and Tgl5p. Tgl5p acts also as a lysophospholipid acyltransferase; and Tgl4p, the functional orthologue of the mammalian adipocyte triacylglycerol lipase (ATGL), is a multifunctional enzyme exhibiting SE hydrolase, phospholipase A2 and acyl-CoA dependent lysophospholipid acyltransferase activities *in vitro* (7, 8). Interestingly, several LD proteins including Tgl3p show a dual localization in LD and in the ER (9, 10). This is an intriguing observation taking into account that these proteins have to adapt to two different membrane environments.
The aim of this PhD thesis was to elucidate topological aspects of Tgl3p in more detail at a molecular level. For this purpose the specific roles of N- and C-terminal stretches of this lipase regarding stability and functionality were investigated. Furthermore, we started to investigate the topology of LD surface proteins in general in a new approach by applying traditional methods such as proteinase K treatment of isolated LD in combination with mass spectrometry analysis. Results obtained in this PhD thesis are a step forward to understand the interaction of Tgl3p with LD and identified new protein regions important for lipase activity.

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CHAPTER I

Storage lipids of yeasts:

A survey of non-polar lipid metabolism in *Saccharomyces cerevisiae*, *Pichia pastoris* and *Yarrowia lipolytica*

Barbara Koch[1], Claudia Schmidt[1], and Günther Daum[#]

Institute of Biochemistry, Graz University of Technology, Austria

[1]Contributed equally to this manuscript and should be considered as co-first authors

[#]Corresponding author: Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria; Tel.: +433168736462; Fax: +433168736952; E-mail: guenther.daum@tugraz.at

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Abstract

Biosynthesis and storage of non-polar lipids such as triacylglycerols and steryl esters have gained much interest during the last decades because defects in these processes are related to severe human diseases. The baker's yeast *Saccharomyces cerevisiae* has become a valuable tool to study eukaryotic lipid metabolism because this single cell microorganism harbors many enzymes and pathways with counterparts in mammalian cells. In this article we will review aspects of triacylglycerol and steryl ester metabolism and turnover in the yeast which have been known for a long time, and combine them with new perceptions of non-polar lipid research. We will provide a detailed insight into the mechanisms of non-polar lipid synthesis, storage, mobilization and degradation in the yeast *S. cerevisiae*. The central role of lipid droplets in these processes will be addressed with emphasis on the prevailing view that this compartment is more than only a depot for triacylglycerols and steryl esters. Dynamic and interactive aspects of lipid droplets with other organelles will be discussed. Results obtained with *S. cerevisiae* will be complemented by recent investigations of non-polar lipid research with *Yarrowia lipolytica* and *Pichia pastoris*. Altogether, this review article provides a comprehensive view of non-polar lipid research in yeast.

Introduction

The yeast *Saccharomyces cerevisiae* is a well-established and reliable model organism to study lipid biology and biochemistry, because lipid biosynthetic routes in this microorganism are similar to higher eukaryotes, e. g. plants and mammals. Similar to other eukaryotic cells, the yeast needs to balance non-polar lipid synthesis and turnover to maintain lipid homeostasis. To avoid possible toxic and membrane disturbing effects of free fatty acids (FA) and sterols, they are stored in the biologically inert form of non-polar lipids. The two major non-polar lipids of the yeast *S. cerevisiae* are triacylglycerols (TG) and steryl esters (SE). The main function of these non-polar lipid classes is to serve as a reservoir of energy and building blocks for membrane lipids. Since non-polar lipids lack charged groups and therefore do not integrate into the phospholipid membrane bilayer of organelles, they are stored in specific subcellular compartments named lipid droplets (LD) (Zweytick *et al.*, 2000a).

LD, also known as lipid particles, oil bodies or oleosomes, show a distinct and unique structure which is conserved in most eukaryotes. In *S. cerevisiae*, TG are randomly packed in the center of these droplets and surrounded by several shells of SE (Czabany *et al.*, 2008). This compact and highly hydrophobic core is covered by a phospholipid monolayer with a small but specific set of proteins embedded. During the last few years the protein equipment of LD has been studied intensively and LD proteins were identified and characterized in some detail (Fei *et al.*, 2011; Grillitsch *et al.*, 2011). Most of these LD proteins play a role in lipid metabolism and homeostasis. However, LD were also discussed to contribute to other cellular functions such as storage and degradation of protein aggregates (Fujimoto *et al.*, 2008).

Under certain conditions, e.g. in the absence of sufficient nutrients, TG lipases located to the surface of LD hydrolyze TG to diacylglycerols (DG) and free FA (Athenstaedt & Daum, 2003; 2005). In the yeast, FA can then be further used either for energy production by breakdown during β-oxidation or for the formation of biological membranes by reincorporation into newly formed complex lipids. Moreover, DG can serve as second messengers in signal transduction (Becker & Hannun, 2005) and as a substrate for phospholipid synthesis via the so-called Kennedy pathway (for reviews see Carman & Han, 2011; Henry *et al.*, 2012). Sterols set free from SE by SE hydrolases can also be incorporated into membrane bilayers to regulate membrane stability, fluidity and permeability (Sharma, 2006). In higher eukaryotes, sterols can serve as precursors for the formation of steroid hormones (Bisgaier *et al.*, 1985; Hu *et al.*, 2010).

In this review article, we will focus on non-polar lipid synthesis, storage and turnover in the yeast *S. cerevisiae*. Anabolic as well as catabolic pathways and the enzymes involved in these processes will be described in detail. Additionally, we will report on lipid/protein composition and formation of the unique structure of LD, the storage compartment for non-polar lipids. We will highlight LD as dynamic structures and focus on recently identified interactions and interplays with other organelles, e.g. the endoplasmic reticulum (ER), mitochondria or peroxisomes. Finally, we will describe recent investigations of non-polar lipid research with *Pichia pastoris* and *Yarrowia lipolytica*. The reader is also referred to other recent review articles addressing non-polar lipid biology (Czabany *et al.*, 2007; Rajakumari *et al.*, 2008; Brasaemle & Wolins, 2012; Kohlwein *et al.*, 2013).

Non-polar lipid synthesis in *Saccharomyces cerevisiae*

Fatty acid biosynthesis

TG and SE stored in LD are the major non-polar lipid classes not only in *S. cerevisiae*, but also in other yeasts (Leber *et al.,* 1994; Ivashov *et al.,* 2012; Athenstaedt *et al.,* 2006). The final step for the synthesis of TG and SE is the acylation of DG and sterols, respectively. Therefore, FA are major building blocks of non-polar lipids. FA can be provided by either *de novo* synthesis, by uptake from the media, or by endogenous lipid turnover. In this review article which is focused on the final steps of non-polar lipid formation we will only briefly address the complex network of FA biosynthesis. For a more detailed description of FA synthesis and metabolism the reader is referred to Black and DiRusso (2007); Tehlivets *et al.* (2007); and Schneiter and Kohlwein (1997).

The first step in FA biosynthesis is catalyzed by acetyl-CoA carboxylase encoded by *ACC1/FAS3* (Roggenkamp *et al.,* 1980; Hasslacher *et al.,* 1993). This enzyme is essential for *de novo* synthesis of long chain FA in the cytosol and catalyzes the carboxylation of acetyl-CoA to malonyl-CoA by incorporation of CO_2. Acc1p contains biotin as a covalently bound cofactor. In *S. cerevisiae*, a second enzyme of this kind is encoded by *HFA1* which is responsible for the mitochondrial acetyl-CoA carboxylase activity (Hoja *et al.,* 2004). Despite overall sequence similarity between Acc1p and Hfa1p, the mitochondrial acetyl-CoA carboxylase is not able to complement for the loss of activity in *acc1*Δ strains. Malonyl-CoA is then further metabolized by FA synthases (FAS) in a cyclic series of elongation steps to form FA with typically 14-18 carbon atoms. Cytosolic FAS contains two subunits, namely Fas1p (β subunit) and Fas2p (α subunit). Six copies of each subunit form a hexameric enzyme complex (α6β6) consisting of multiple catalytic domains (Kuziora *et al*., 1983; Lynen 1980; Schweizer *et al*., 1986; Wieland *et al*., 1979; Maier *et al*., 2010; Grininger 2014). *S. cerevisiae* contains a second FAS complex (type II FAS system), which catalyzes mitochondrial FA biosynthesis completely independently from the cytosolic FAS apparatus (Hiltunen *et al.,* 2009). Except octanoic acid, the direct precursor for the synthesis of lipoic acid, the range of FA produced by the mitochondrial FAS pathway has not yet been determined (Brody *et al.,* 1997).

Very long chain fatty acid (VLCFA) synthesis up to C-26 takes place in the ER. Formation of C-20 to C-26 from C-14 to C-18 FA requires sequential elongation steps which are similar to FA *de novo* synthesis. *ELO1*, *ELO2* and *ELO3* genes encode components of the membrane-

bound FA elongation system (Oh *et al.,* 1997; Schneiter *et al.,* 2000; Toke & Martin, 1996). While Elo1p shows a high specificity for carboxy-terminal elongation of C-14 to C-16 FA (Schneiter *et al.,* 2000), Elo2p is involved in the elongation of FA up to 24 carbons (Oh *et al.,* 1997). Elo3p shows broader substrate specificity and is essential for the conversion of C-24 to C-26 FA species. Paul *et al.* (2006) demonstrated that very long chain FA are essential for cellular functions, because deletion of both *ELO2* and *ELO3* resulted in synthetic lethality.

A large portion of phospholipids and TG in *S. cerevisiae* contains C16:1 and C18:1 FA (Grillitsch *et al.,* 2011). In contrast to other fungi, *S. cerevisiae* harbors only monounsaturated FA. Unsaturation of FA takes place in the ER and is catalyzed by the Δ9-fatty acid desaturase Ole1p (for review see Martin *et al.,* 2007). Ole1p which was identified as FA desaturase by Stukey *et al.* (1989) forms a double bond in saturated acyl-CoA substrates through an oxygen and NADH dependent mechanism. Strains deleted of *OLE1* lack unsaturated FA synthesis and require supplementation with oleic acid for growth (Resnick & Mortimer, 1966). The rat liver stearoyl-CoA desaturase can efficiently replace Ole1p in *S. cerevisiae* (Stukey *et al.,* 1990). *OLE1* gene expression is highly regulated and depends on growth conditions (Martin *et al.,* 2007). Different studies showed that *OLE1* transcription is regulated by FA levels (McDonough *et al.,* 1992) as well as temperature and oxygen availability (Kwast *et al.,* 1999; Nakagawa *et al.,* 2002). Additionally, *OLE1* transcription is induced by the two homologous transcription factors Spt23p and Mga2p (Hoppe *et al.,* 2000; Rape *et al.,* 2001; Chellappa *et al.,* 2001; Zhang *et al.,* 1999). Both transcription factors are anchored to the ER as precursors and become activated in an ubiquitin/proteasome-dependent processing. The processing of Spt23p and Mga2p depends on the fatty acid pools.

Alternatively to FA biosynthesis, exogenous FA from the medium can be taken up for incorporation into several lipid classes. FA need to be converted to activated intermediates by reacting with coenzymeA (CoA) through thioesterification in an ATP dependent manner. The yeast possesses several long chain acyl-CoA synthetases encoded by *FAA1, FAA2, FAA3, FAA4* and a very long chain acyl-CoA synthetase encoded by *FAT1* (Johnson *et al.,* 1994; Knoll *et al.,* 1994; Black & DiRusso, 2007). The major acyl-CoA synthetase Faa1p is responsible for most of the long chain acyl-CoA synthetase activity and is also required for FA transport (Faergeman *et al.,* 1997). Faa1p and Faa4p activate exogenous FA taken up from the medium, but also endogenous FA from the degradation of phospholipids, TG and SE (Black & DiRusso, 2007). In contrast, acyl-CoA synthetase Faa2p is assumed to be required for providing activated FA for β-oxidation and has been localized to peroxisomes.

The function of another acyl-CoA synthetase encoded by *FAT2* is still unclear (Blobel & Erdmann, 1996; van Roermund *et al.*, 2000). Activated FA in the form of acyl-CoAs are important substrates for TG and SE biosynthesis. Furthermore FA production for the synthesis of phospholipids is crucial to maintain the physical properties of biomembranes (for review see de Kroon *et al.*, 2013).

Synthesis of phosphatidic acid and diacylglycerols

DG contain a glycerol moiety esterified with two FA and serve as the direct precursors for the formation of TG. The main route for the generation of DG is by dephosphorylation of phosphatidic acid (PA). In *S. cerevisiae*, two alternative routes, namely the glycerol-3-phosphate (G-3-P) and the dihydroxyacetone phosphate (DHAP) pathway lead to the formation of PA via several acylation steps. In the G-3-P pathway, the first acylation step in the *de novo* synthesis of PA is carried out by two different enzymes, Gat1p/Gpt2p and Gat2p/Sct1p, leading to the formation of 1-acyl-G-3-P also known as lysoPA (Zheng & Zou, 2001). As shown in Figure 1, these two enzymes with overlapping function catalyze the same reaction by transferring an activated FA (acyl-CoA) to the *sn*-1 position of G-3-P. Yeast Gat1p and Gat2p differentially contribute to TG formation (Marr *et al.*, 2012). Both enzymes are located to the ER, but Gat1p has also been localized to LD where the highest specific G-3-P acyltransferase activity was found (Athenstaedt *et al.*, 1999a). The two enzymes prefer different FA as substrates. While Gat1p efficiently uses a broad range of FA as acyl donors, Gat2p prefers FA with 16 carbon atoms (Zheng & Zou, 2001). Both enzymes seem to utilize unsaturated FA preferentially. Single deletions of *GAT1* and *GAT2* exhibit decreased G-3-P acyltransferase activity, a markedly lower PA pool than wild type, but also an increased phosphatidylserine (PS) to phosphatidylinositol (PI) ratio. Zheng and Zou (2001) demonstrated synthetic lethality of *GAT1* and *GAT2* deletions.

Gat1p and Gat2p do not only acylate G-3-P, but also utilize DHAP as substrate (Athenstaedt *et al.*, 1999a; Zheng & Zou, 2001), thus exhibiting DHAP acyltransferase activity as well. Similar to G-3-P acyltransferase activity, DHAP acyltransferase activity was found both in the ER and LD with the latter subcellular fraction harboring the highest specific activity. Noteworthy, G-3-P/DHAP acyltransferase activity was also found in mitochondria, which seem to be the only compartment with a preference for DHAP over G-3-P as substrate of this reaction (Athenstaedt *et al.*, 1999a). As shown in Figure 1, acylation of DHAP by Gat1p and Gat2p results in the formation of 1-acyl-DHAP. This intermediate needs to be reduced to

lysoPA by the 1-acyl-DHAP reductase Ayr1p in an NADPH dependent manner (Racenis *et al.,* 1992; Athenstaedt & Daum, 2000). Similar to other enzymes involved in this pathway, Ayr1p shows dual localization to the ER and LD. This dual localization of different enzymes involved in lipid metabolism supported the view of a close relationship of these two organelles which will be discussed later in this review. Interestingly, Athenstaedt and Daum (2000) showed that Ayr1p is the only 1-acyl-DHAP reductase in LD, because deletion of this gene resulted in complete loss of activity in this fraction. In contrast, the ER fractions from *ayr1*Δ deletion strains still showed low 1-acyl-DHAP reductase activity. Therefore, Ayr1p which is not essential in yeast may not be the only enzyme of this kind in the ER. Recently, Ploier *et al.* (2013) identified Ayr1p also as a novel TG lipase based on results of a functional proteome screening. This result extends and supports the view that some enzymes of the lipid metabolism network are multifunctional as will be discussed later in this review.

LysoPA is further acylated to diacylglycerol-3-phosphate, also known as PA. As shown in Figure 1, this reaction is catalyzed by two enzymes encoded by *SLC1* and *SLC4/ALE1/LPT1* which use activated FA as substrate. Slc1p was originally characterized as a suppressor gene involved in sphingolipid metabolism and as a fatty acyltransferase (Nagiec *et al.,* 1993). The gene product was able to complement the growth defect of an *Escherichia coli* strain mutated in the 1-acyl-*sn*-G-3-P acyltransferase *plsC*. The gene product of *SLC1*, one of the first eukaryotic *sn*-2-acylglycerol fatty acyltransferase genes cloned, transfers an acyl-group from an activated FA to the *sn*-2 position of lysoPA to form PA (Figure 1). Slc1p of yeast uses preferentially C18:1, C22:1 and C24:0 acyl-CoAs as substrates (Athenstaedt & Daum, 1999; Zou *et al.,* 1997). Localization studies revealed that similar to other enzymes involved in lipid metabolism Slc1p was dually located to the ER and LD (Athenstaedt & Daum, 1997; 1999).

Using synthetic genetic array analysis (Tong *et al.,* 2001) with an *slc1*Δ strain, a second gene named *SLC4/ALE1/LPT1* was identified which encodes an acyltransferase using a variety of lysophospholipid species including lysoPA as substrates (Jain *et al.,* 2007; Riekhof *et al.,* 2007; Benghezal *et al.,* 2007). Deletion of this gene showed synthetic lethality with *SLC1*. As Ale1p utilizes lysophosphatidylcholine (lysoPC), lysoPI, lysoPS, lysophosphatidylethanolamine (lysoPE) and lysoPA as substrates, and different activated FA including palmitoyl-CoA, palmitoleoyl-CoA, stearoyl-CoA or oleoyl-CoA as co-substrates, the enzyme was regarded as a general lysophospholipid acyltransferase (Jain *et al.,* 2007). Ale1p shows a preference for unsaturated acyl-CoA substrates and was demonstrated to be the only lysoPC acyltransferase in *S. cerevisiae*. Furthermore, Ale1p catalyzes the major

lysoPE acyltransferase activity (Riekhof *et al.*, 2007). Localization studies revealed that most of the Ale1p activity was present in the ER and mitochondria. Moreover, Ale1p activity was found to be highly enriched in the so called MAM, the mitochondria associated membrane fraction. Slc1p and Ale1p are members of the membrane-bound *O*-acyltransferase family. They also play a role in phospholipid acyl-chain remodeling which includes deacylation of a phospholipid at the *sn*-2 position by phospholipase A_2 and reacylation by lysophospholipid acyltransferases (Lands, 1960; Chen *et al.*, 2007).

Ayciriex *et al.* (2012) recently identified a novel lysoPA acyltransferase encoded by *LOA1* (lysophosphatidic acid:oleoyl-CoA acyltransferase 1) which is involved in TG homeostasis. Loa1p is dually located to LD and the ER and displays lysoPA acyltransferase activity *in vitro* using oleoyl-CoA and lysoPA as substrates. No other acyltransferase activities were observed when either G-3-P or other lysophospholipids were used as substrate. Deletion of *LOA1* resulted in a decreased amount of TG and in changed LD morphology. Additionally, the number of LD was significantly decreased in the absence of Loa1p.

It is worth mentioning that the major TG lipases of the yeast, which are responsible for most of TG degradation from LD (see Section *Mobilization and degradation of triacylglycerols*) also act as lysophospholipid acyltransferases (Rajakumari & Daum, 2010a, 2010b). The TG lipases Tgl3p, Tgl4p and Tgl5p use a variety of lysophospholipids for acylation, although with different efficiency. Tgl4p and Tgl5p preferentially catalyze the formation of PA by acylation of lysoPA, whereas the major TG lipase Tgl3p uses rather lysoPE as a substrate than lysoPA. The contribution of these enzymes to phospholipid synthesis was emphasized in experiments using single and triple mutants. Single deletions as well as the *tgl3*Δ*tgl4*Δ*tgl5*Δ strain showed decreased levels of phospholipids. In these strains, the level of PA was markedly reduced to ~40% of wild type (Rajakumari *et al.*, 2010). Interestingly, the effect observed with the triple mutant was similar to single mutants.

Besides *de novo* synthesis of PA as described above, PA can also be generated by removing the head group of a phospholipid by the action of phospholipase D (Pettitt *et al.*, 2001). It has to be noted that in addition to its important role in TG biosynthesis, PA is also used as a substrate for the synthesis of membrane phospholipids through the intermediate CDP-DG (for review see Carman & Henry, 1999).

Dephosphorylation of PA yielding DG and inorganic phosphate occurs in a step catalyzed by phosphatidate phosphatases (PAP, 3-*sn*-phosphatidate phosphohydrolase) (Carman & Han, 2006; Smith *et al.*, 1957) (Fig. 2). This reaction is regarded as the rate-limiting step of TG

synthesis. In eukaryotic cells, PAP activity plays a central role in TG and phospholipid synthesis through generation of DG and lipid-signaling molecules (Wang *et al.,* 2006). Additionally, PAP is involved in the transcriptional regulation of phospholipid synthesis. Mg^{2+} dependent and Mg^{2+} independent forms of PAP have been identified in *S. cerevisiae* (Carman, 1997; Oshiro *et al.,* 2003). The Mg^{2+} dependent forms of the enzyme (PAP1 type) require Mg^{2+} ions for catalytic activity (Lin & Carman, 1989; Han *et al.,* 2006). Pah1p (phosphatidic acid phosphatase) is responsible for most of the PAP1 enzyme activity in the yeast and is located to the cytosol and the membrane fraction of the cell (Huh *et al.,* 2003; Lin & Carman, 1989). The importance of this enzyme is underlined by different effects observed with a *pah1*Δ deletion strain. Strains deleted of *PAH1* accumulate PA and show reduced amounts of DG (Han *et al.,* 2006). In the stationary growth phase, the TG content of *pah1*Δ is reduced to 50% of wild type (Fakas *et al.,* 2011). Moreover, deletion of *PAH1* results in a marked change of the phospholipid composition in the exponential growth phase and in a temperature sensitive growth phenotype.

In contrast to PAP1 enzymes, PAP2 type enzymes do not need Mg^{2+} ions as cofactors and utilize beside PA a variety of other lipid phosphates as substrates (Faulkner *et al.,* 1999; Toke *et al.,* 1998a; 1998b). The major yeast PAP2 enzymes encoded by *DPP1* and *LPP1* are located to the vacuole (Han *et al.,* 2004) and Golgi (Huh *et al.,* 2003), respectively. Dpp1p prefers diacylglycerol pyrophosphate (DGPP) as a substrate (Wu *et al.,* 1996), whereas Lpp1p shows similar substrate specificity for PA and DGPP (Furneisen & Carman, 2000). Single deletions of these genes cause a marked decrease in Mg^{2+} independent hydrolysis of several isoprenoid phosphates. Deletion of both genes resulted in a decreased hydrolysis of dolichyl phosphate, dolichyl pyrophosphate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate (Faulkner *et al.,* 1999). However, individual enzymes are neither essential under standard growth condition nor do mutants defective in these genes exhibit any morphological abnormalities.

As remaining Mg^{2+} dependent PAP activity in a *pah1*Δ*dpp1*Δ*lpp1*Δ strain indicated the presence of other gene(s) encoding for enzyme(s) of this kind, Chae *et al.* (2012) screened for novel PAPs enzymes. Liquid chromatography/tandem mass spectrometry (MS) analysis of PAP activity-enriched samples from *pah1*Δ*dpp1*Δ*lpp1*Δ revealed multiple putative phosphatases. These experiments identified *APP1* encoding an enzyme which confers ~30% of PAP activity in wild type yeast cells. A *pah1*Δ*dpp1*Δ*lpp1*Δ*app1*Δ quadruple mutant did not show any residual PAP activity, indicating that these four proteins are the only PAP

enzymes in *S. cerevisiae*. Chae *et al.* (2012) also demonstrated that Pah1p is the only PAP affecting TG synthesis.

It has to be mentioned that DG cannot only be formed by dephosphorylation of PA. Degradation of glycerophospholipids catalyzed by phospholipase C and turnover of TG may also provide an additional portion of DG. Finally, DG can also be formed in the course of sphingolipid synthesis. The inositol-phosphoceramid synthase Aur1p which catalyzes the formation of inositol-phosphoceramide from ceramide and PI releases DG as a byproduct (Nagiec *et al.*, 1997).

Triacylglycerol biosynthesis

DG formed from PA can be further converted to TG by acyl-CoA dependent or independent acylation (Figure 2). The most prominent acyl-CoA dependent diacylglycerol acyltransferase (DGAT) of the yeast is encoded by *DGA1* (orthologue of mammalian DGAT), a member of the DGAT2 gene family (Oelkers *et al.*, 2002; Sorger & Daum, 2002). Dga1p catalyzes acylation of DG utilizing different acyl-CoAs as substrates, e. g. oleoyl-CoA, palmitoyl-CoA and myristoyl-CoA. Dga1p requires the presence of Mg^{2+} and K^{+} for efficient catalytic action. Localization studies performed by Sorger and Daum (2002) showed that acyl-CoA dependent DGAT activity was highly enriched in LD, but also in microsomal fractions. Whereas Dga1p was the only acyl-CoA dependent DGAT in LD, deletion of *DGA1* alone did not result in an absolute loss of DGAT activity in microsomes, indicating the presence of another enzyme of this kind in this fraction. *DGA1* is not essential in yeast and a deletion strain does not show any growth defect. Nevertheless, Dga1p was postulated to be the major TG synthase of *S. cerevisiae* since the overall amount of TG is more than 40% reduced in a *dga1*Δ deletion strain compared to wild type (Sandager *et al.*, 2002). Oelkers *et al.* (2002) showed by incorporation of [^{3}H]oleate into TG that the contribution of Dga1p to TG synthesis was growth phase dependent. Cells deleted of *DGA1* grown to the stationary growth phase unveiled a 50% reduction of TG synthesis, whereas in the logarithmic growth phase a deletion of *DGA1* resulted only in a ~20% reduction of TG synthesis.

Two other proteins, the two SE synthases Are1p and Are2p, also catalyze acyl-CoA dependent TG formation, although with minor efficiency (Sandager *et al.*, 2000). The relative contribution of these enzymes to TG formation *in vivo* is still a matter of dispute.

The acyl-CoA independent TG synthesis pathway requires a glycerophospholipid as an acyl donor. Lro1p was identified as a phospholipid:diacylglycerol acyltransferase (PDAT) by

screening the yeast genome for lecithin:cholesterol acyltransferase homologues (Dahlqvist *et al.,* 2000). Lro1p (lecithin:cholesterol acyltransferase-related open reading frame) shares 27% overall sequence identity with the human lecithin:cholesterol acyltransferase found in blood which transfers a FA from PC to cholesterol to form cholesteryl esters (Glomset, 1968). In contrast to its homologue, the yeast Lro1p mediates esterification of DG by transferring an acyl-group to DG (see Figure 2) preferentially utilizing the *sn*-2 acyl chain of PE or PC (Dahlqvist *et al.,* 2000). PDAT activity was measured in yeast microsomes, but in contrast to Dga1p not in LD (Sorger & Daum, 2003). A yeast strain deleted of *LRO1* showed no growth defect on different carbon sources, but PDAT activity was completely missing (Dahlqvist *et al.,* 2000). Noteworthy, in contrast to human lecithin:cholesterol acyltransferase, Lro1p did not form steryl esters. Oelkers *et al.* (2002) demonstrated that the contribution of Lro1p to TG synthesis is growth dependent. A strain deleted of *LRO1* grown to the exponential growth phase showed less than 30% incorporation of [^{3}H]oleate into TG compared to wild type. In contrast, the overall TG content of *lro1*Δ strains was only moderately changed in the stationary growth phase (Sandager *et al.,* 2002). Therefore, Lro1p seems to be the major TG synthase during the exponential growth.

Interestingly, Horvath *et al.* (2011) demonstrated a metabolic link between yeast TG synthesis and phosphatidylethanolamine metabolism by Lro1p. The activity of Lro1p was markedly reduced in *cki1*Δ*dpl1*Δ*eki1*Δ*,* a mutant which cannot form phosphatidylethanolamine through the cytidyldiphosphate ethanolamine (CDP-Etn) branch of the so-called Kennedy pathway. It was assumed that insufficient supply of phosphatidylethanolamine as a substrate for the phospholipid:diacylglycerol acyltransferase reaction of Lro1p was the reason for the strongly reduced Lro1p activity and the resulting decreased TG level.

Independent of the growth phase, a *dga1*Δ*lro1*Δ double deletion mutant shows only marginal TG synthesis, which can be attributed to the residual TG synthase activity of Are1p and Are2p. Similar to *dga1*Δ and *lro1*Δ single deletion strains, a double mutant exhibits only minor growth defects and is still able to form LD (Oelkers *et al.,* 2002). However, in cells lacking both TG synthases LD appear to be strongly reduced in size and number (Czabany *et al.,* 2008).

Steryl ester biosynthesis

Formation of sterols

Sterols, the direct precursors of SE, are essential components of the plasma membrane where they affect fluidity/rigidity and permeability. However, an excess of sterols or their appearance in the "wrong" membrane may cause toxic effects in the cell (Henneberry & Sturley, 2005). The most abundant sterol in *S. cerevisiae* is ergosterol, which differs slightly from cholesterol, the predominant sterol in mammalian cells, by unsaturation at C-7,8 in the ring structure, unsaturation at C-22 in the side chain and a methyl group at C-28. The major sterols in plants, sitosterol and stigmasterol, are similar to cholesterol but have a slightly modified side chain (for review see Daum *et al.,* 1998). Ergosterol can either be taken up from the growth medium or produced endogenously by oxygen dependent biosynthesis. In this review article, sterol biosynthesis will only be briefly addressed. For a more detailed description of sterol formation, homeostasis and transport the reader is referred to Henneberry and Sturley (2005); Pichler (2005); Jacquier and Schneiter (2012); and Wriessnegger and Pichler (2013).

Under aerobic conditions the major portion of intracellular ergosterol is formed by synthesis in the ER, although localization of some enzymes catalyzing late steps of biosynthesis was also reported in LD or secretory vesicles (Zinser *et al.,* 1993). Enzymes involved in ergosterol formation and the respective enzymatic reactions have been studied and characterized in detail using mutants defective in ergosterol biosynthesis, the so-called "erg" -mutants (Parks *et al.,* 1999). As examples, mutations in *ERG9* encoding the squalene synthase led to obligatory sterol auxotrophy (Grabowska *et al.,* 1998); and deletion of *ERG2* encoding the C8 sterol isomerase, which converts fecosterol to episterol, led to ergosterol auxotrophy, accumulation of aberrant sterols and compromised growth (Palermo *et al.,* 1997; Silve *et al.,* 1996).

Formation of ergosterol from its first precursor acetyl-CoA involves more than 20 enzymes and catalytic reactions (for reviews see Daum *et al.,* 1998; Pichler, 2005). The initiate part of ergosterol synthesis is the formation of farnesyl pyrophosphate by the branched-chain isoprenoid pathway, which provides molecules required not only for ergosterol synthesis but also for protein modification, protein glycosylation and electron transport (Casey *et al.,* 1992; van der Rest *et al.,* 1995). Since products of this catalytic sequence are essential for many cell biological reactions, mutations in this part of the pathway are lethal. The second part of

ergosterol biosynthesis is the conversion of farnesyl diphosphate to the end-product, ergosterol, involving several catalytic steps. Farnesyl diphosphate is converted to squalene in an NAD(P)H dependent manner catalyzed by the squalene synthase Erg9p (Jennings *et al.,* 1991) which was localized to the ER. Squalene is further converted to squalene epoxide by Erg1p in the presence of oxygen (Jahnke & Klein, 1983; Jandrositz *et al.,* 1991). Erg1p shows dual localization to LD and the ER (Leber *et al.,* 1998), although *in vitro* the enzyme exhibits activity only in the ER, indicating that an additional component in this compartment may be needed for activity. Erg7p, the lanosterol synthase, catalyzes the next step in the sterol biosynthetic cascade producing the first sterol, lanosterol, which contains already the characteristic sterol ring structure (Corey *et al.,* 1994; Shi *et al.,* 1994). Finally, a set of different Erg proteins forms the sterol intermediates zymosterol, fecosterol and episterol and finally the end product ergosterol (for reviews see Parks *et al.,* 1999; Pichler, 2005).

Interestingly, squalene which serves as precursor for the synthesis of sterols, steroids and ubiquinons, was also identified as a storage lipid accumulating in yeast LD. Squalene accumulates in yeast cells overexpressing *HMG1* encoding the HMG-CoA reductase (Polakowski *et al.,* 1998) or deleted of *ERG1* and *ERG7* (Jahnke & Klein, 1983). Spanova *et al.* (2010) showed that a mutant deleted of *HEM1* accumulated large amounts of squalene almost exclusively in LD. Surprisingly, in a yeast strain lacking TG and SE synthesizing enzymes enhanced squalene synthesis did not stimulate LD formation. Under these conditions, squalene was found in cellular membranes, especially in microsomes. Ta *et al.* (2012) demonstrated an influence of squalene on LD growth and distribution. Accumulation of squalene was associated with the clustering of LD as shown by fluorescence and electron microscopy. An *erg1*Δ strain, which is defective in sterol biosynthesis and accumulates squalene at large amounts, showed an abnormal LD morphology and LD clustering. Reducing the high amount of squalene restored normal LD formation.

To identify interactions between lipid biosynthetic pathways, Shin *et al.* (2012) analyzed yeast strains with deregulated sterol and FA biosynthesis. It became evident that a yeast strain accumulated sterols and squalene when acetyl-CoA carboxylase *ACC1* was overexpressed. The linkage between the two pathways was due to transcriptional co-regulation of *ACC1* and *HMG1*. In addition to *HMG1* other genes involved in ergosterol biosynthesis such as *HMG2, ERG13, ERG11, ERG5* and *ERG28* were upregulated upon overexpression of *ACC1*. However, the two SE synthases *ARE1* and *ARE2* were expressed at the same level as in wild type.

Formation of steryl esters

In eukaryotic cells, an excess of sterols can be converted to hydrophobic SE and stored in the core of cytosolic LD. A constant rate of SE formation is maintained during exponential growth of the yeast, but a strong increase occurs upon the entry of the culture into the stationary growth phase (Bailey & Parks,1975). Homology searches based on sequence alignment with the human acyl-CoA:cholesterol acyltransferase (ACAT) led to identification of two yeast acyl-CoA:sterol acyltransferases (ASAT) encoded by *ARE1* and *ARE2* (ACAT-related enzymes) which synthesize SE. These two enzymes share 49% sequence identity and approximately 24% identity with the mammalian ACAT. Are1p and Are2p are, like Slc1p and Ale1p, members of the membrane-bound *O*-acyltransferase family (Pagac *et al.,* 2011) and located to the ER (Zinser *et al.,* 1993; Zweytick *et al.,* 2000b). Are1p and Are2p form SE by transferring an activated FA to the hydroxyl group at the C3-position of a sterol molecule (Figure 3). Although Are proteins do not show any preference for different FA, they utilize different sterols for esterification *in vivo* (Zweytick *et al.,* 2000b). Whereas Are2p prefers ergosterol as a substrate, Are1p esterifies ergosterol as well as ergosterol precursors, especially lanosterol. *In vitro* the major ASAT activity was attributed to Are2p accounting for 65-75% of total ASAT activity (Yu *et al.,* 1996). In an *are2*Δ single deletion strain, the overall SE content was decreased to ~30% of wild type. In contrast, deletion of *ARE1* did not affect the overall amount of SE at all under standard growth condition (Zweytick *et al.,* 2000b). Noteworthy, Are1p was demonstrated to contribute to SE synthesis under anaerobic conditions when the oxygen dependent ergosterol biosynthesis was disturbed (Hronská *et al.,* 2004; Valachovic *et al.,* 2001). Single deletions of *ARE1* or *ARE2,* respectively, did not result in any growth defect (Yang *et al.,* 1996). A mutant deleted of both *ARE1* and *ARE2* completely lacks SE. Hence, the two proteins seem to be the only ASATs of the yeast. Interestingly, also the *are1*Δ*are2*Δ double deletion strain does not exhibit any growth phenotype, although the lack of SE leads to accumulation of free ergosterol in the cell (Zweytick *et al.,* 2000b). However, double mutants exhibit a significant decrease in sterol biosynthesis indicating a regulatory role of Are proteins in sterol formation (Yang *et al.,* 1996). Arthington-Skaggs *et al.* (1996) showed that in the absence of Are proteins, the sterol C-5 desaturase Erg3p which converts episterol to ergosta-5,7,24(28)-trienol was down regulated. Sorger *et al.* (2004) demonstrated that cellular level and stability of Erg1p were dramatically reduced in the absence of SE synthases.

Tinkelenberg *et al.* (2000) screened for yeast mutants that were not viable in the absence of sterol esterification. A deletion of *ARV1* encoding for a protein which mediates sterol transport from the ER to the plasma membrane was shown to cause lethality in the *are1*Δ*are2*Δ background. Cells lacking Arv1p bear a defect in sterol uptake and display an altered intracellular sterol distribution. Shechtman *et al.* (2011) recently extended the study with *arv1*Δ strains and demonstrated by using fluorescence and electron microscopy that in addition to sterol accumulation and subcellular membrane expansion, also LD formation and vacuolar fragmentation were affected under so-called sterol-loading conditions. The authors demonstrated that on media supplemented with cholesterol an *arv1*Δ strain with an additional mutation in *UPC2* showed increased LD proliferation. These LD were formed mostly from SE. The paralogous transcription factors Upc2p and Ecm22p were identified as sterol regulatory element binding proteins affecting the transcription of several sterol biosynthetic genes, e.g. *ERG2* and *ERG3* (Vik & Rine, 2001). Additionally, Upc2p was reported to activate sterol uptake (Wilcox *et al.*, 2002).

Dysfunction in non-polar lipid synthesis

Although storage lipids play an important role in lipid homeostasis, formation of non-polar lipids is not essential in *S. cerevisiae*. A yeast strain lacking all four non-polar lipid synthesizing enzymes, the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant, is completely devoid of non-polar lipids and hence lacks LD formation (Sandager *et al.*, 2002). This strain is still viable and shows only a minor growth defect under standard growth conditions but was reported to be sensitive to exogenous unsaturated FA (Garbarino *et al.*, 2009). Similar to the *are1*Δ*are2*Δ double deletion strain the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant showed a significant defect in sterol biosynthesis (Sorger *et al.*, 2004).

In contrast to the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ strain which is completely devoid of LD, some mutations and variation of growth conditions lead to an increased number and size of LD. Fei *et al.* (2011) identified ten yeast mutants producing so-called supersized LD. These yeast strains showed an increased amount of total TG and SE leading to LD that were up to 50 times larger than in wild type. Among the affected genes the authors identified *FLD1*, the homologue of mammalian seipin, and *CDS1*, *INO2*, *INO4*, *CHO2* and *OPI3* which encode enzymes involved in phospholipid synthesis and regulation of phospholipid metabolism. A common feature of these mutants was the increased level of cellular PA. Fei *et al.* (2011) hypothesized that PA may play an important role in fusion of LD leading to supersized

organelles. Furthermore, Connerth *et al.* (2010) demonstrated that yeast cells grown on oleate supplemented media showed strongly increased TG synthesis and enhanced proliferation of LD. Under these culture conditions, SE synthesis was strongly inhibited due to a negative effect of free FA on Are proteins.

Non-polar lipid synthesis in *Yarrowia lipolytica* and *Pichia pastoris*

Y. lipolytica has been isolated from various lipid-rich media like sewage or oil-polluted media (Nicaud, 2012). This yeast is able to use hydrophobic substrates as the sole carbon source (Fickers *et al.*, 2005). *Y. lipolytica* can accumulate lipids up to 40% of cell dry weight and linoleic acid to more than 50% of total FA (Ratledge & Wynn, 2002). Because of its ability to accumulate substantial amounts of lipids, *Y. lipolytica* was frequently used to study mechanisms of fat uptake, storage and mobilization.

The enzymes involved in non-polar lipid synthesis of *Y. lipolytica* are similar to those of *S. cerevisiae* (Beopoulos *et al.*, 2009). One exception, however, is the existence of ATP-citrate lyases encoded by *ACL1* and *ACL2*, which catalyze production of acetyl-CoA by cleavage of citrate. These enzymes may be responsible for the increased formation of FA precursors and thus for oleaginocity. No such *ACL* genes were identified for the non-oleaginous yeast *S. cerevisiae*. Similar to *S. cerevisiae* TG synthesis in *Y. lipolytica* requires activated FA (acyl-CoA) and G-3-P. The three enzymatic reactions leading to DG formation involve enzymes closely related and named after the *S. cerevisiae* genes. Acylation of G-3-P yielding lysoPA is performed by Sct1p and acylation of lysoPA to produce PA is catalyzed by Slc1p. Furthermore, a *Y. lipolytica* gene encoding PAP (YALI0D27016g) showing 39% sequence identity to *PAH1* from *S. cerevisiae* forms DG from its precursor PA (Beopoulos *et al.*, 2009).

The last step of TG synthesis in *Y. lipolytica* is catalyzed by *DGA1* and *LRO1* (YALI0E32769g and YALI0E16797g). The major TG synthases in this yeast share ~50% identities to their counterparts in *S. cerevisiae* (Athenstaedt, 2011). Single deletions of *DGA1* or *LRO1* result in a decrease of total TG, but a double deletion strain still contains a minor amount of TG which may be synthesized by other unidentified TG synthases. *Y. lipolytica* produces only small amounts of SE, normally less than five percent of all storage lipids. Are1p (YALI0F06578g) is the major SE synthase in this yeast and shares 30% sequence

identity to its counterpart in *S. cerevisiae*. Deletion of *ARE1* in *Y. lipolytica* resulted in a complete loss of SE (Beopoulos *et al.*, 2012) indicating that this enzyme is essential for SE synthesis. Are2p from *Y. lipolytica* (YALI0D07986g) has only minor homology (17%) to Are1p but is more closely related to the DGAT from the plant *Perilla frutescens.* Actually, Are2p was shown to be a major contributor to TG synthesis but not to SE synthesis. Therefore, Beopoulos *et al.* (2012) suggested that the gene product of *ARE2* from *Y. lipolytica* should be reclassified as Dga2p, an acyl-CoA dependent DG acyltransferase of the DGAT1 family. Such enzymes have been identified in mammals and plants but not in *S. cerevisiae*.

Lipid accumulation, in particular accumulation of TG, is not well understood in *Y. lipolytica*. However, several attempts were made to identify enzymes involved in TG synthesis. Mlícková *et al.* (2004) demonstrated a link between β-oxidation and lipid accumulation. These authors analyzed morphological changes of *Y. lipolytica* grown on oleic acid medium and the effect of *POX* deletions on lipid accumulation. *POX1* to *POX5* encode acyl-CoA oxidases which catalyze the rate-limiting step of peroxisomal β-oxidation. Using electron microscopy it was shown that the size of LD and their composition depended on *POX* genes. A *pox2*Δ*pox3*Δ*pox5* triple mutant harbored only very few and small intracellular LD. In contrast, overexpression of *POX2* led to the formation of very large LD. It was concluded that the gene product of *POX2*, the Aox2p, regulates the size of the cellular TG pool.

Dulermo and Nicaud (2011) described the role of the so-called G-3-P shuttle and β-oxidation pathway in lipid accumulation. Gut2p, the G-3-P dehydrogenase, oxidizes G-3-P to DHAP, whereas Gpd1p, another G-3-P dehydrogenase, is involved in the reduction of DHAP to G-3-P. Recently, it was demonstrated that deletion of *GUT2*, overexpression of *GPD1* or both mutations together result in a strongly increased level of G-3-P and TG accumulation (Beopoulos *et al.*, 2008; Dulermo & Nicaud, 2011). Additionally, the level of glycerol was decreased in such strains. The authors hypothesized that G-3-P phosphatases are missing in *Y. lipolytica* which seems to contain a unique glycerol metabolism mainly devoted to G-3-P formation for subsequent TG synthesis. Additionally, Beopoulos *et al.* (2008) demonstrated that a combination of *GUT2* and *POX* deletions led to a significant alteration of the lipid composition and LD morphology. It was observed that a *gut2*Δ*pox1-6*Δ strain grown on oleic acid medium contained one to three large LD, whereas *gut2*Δ contained a large numbers of small LD. Transcriptional analyses of different TG accumulating strains revealed that lipid accumulation was caused by over-expression of genes encoding TG synthesizing enzymes

(Dulermo & Nicaud, 2011). As an example, over-expression of *GPD1* in *gut2*Δ*pox1-6*Δ or *pox1-6*Δ led to increased expression of *DGA1*. In contrast, expression of TG lipases *TGL3* and *TGL4* was negatively affected by deletion of *GUT2* or overexpression of *GPD1*. These results indicate that expression of genes involved in TG homeostasis is regulated by G-3-P shuttle and β-oxidation.

Similar to *S. cerevisiae*, LD proliferation in *Y. lipolytica* can be enhanced by growing cells on oleate instead of glucose as a carbon source (Athenstaedt *et al.,* 2006). Under such conditions, the size of LD was increased 3.8-fold compared to wild type, and LD exhibited a markedly lower ratio of TG to SE. The reason for the low non-polar lipid level in cells grown on glucose might be that ATP-citrate lyase becomes inactivated by an excess of glucose in the media (Papanikolaou *et al.,* 2002). Wang *et al.* (2013) demonstrated that deletion of *MIG1,* which is a key component of the glucose repression pathway (Gancedo, 1998), resulted in increased lipid biosynthesis and thus in increased LD formation. In cells lacking *MIG1* an up-regulation of *GPD1, GUT1* and *GUT2,* (DG biosynthesis); *KGD1*, the isocitrate dehydrogenase *ID*, (citrate formation); and *ME1, ACL1* and *ACL2* (FA biosynthesis) was observed (Wang *et al.,* 2013). Interestingly, biosynthesis of oleic acid was induced in the mutant.

P. pastoris is a methylotrophic yeast and widely used for recombinant protein expression (Daly & Hearn, 2005). For a better understanding of lipid homeostasis in this industrial yeast, Ivashov *et al.* (2013) investigated non-polar lipid synthesis in *P. pastoris*. Homology searches revealed two DGATs, Dga1p and Lro1p and one ASAT, Are2p, which were identified as enzymes catalyzing the final steps of TG and SE formation, respectively. Dga1p from *P. pastoris* shares 40% sequence identity with the orthologue from *S. cerevisiae,* and 33% with the orthologue from *Y. lipolytica*. The PDAT Lro1p and ASAT Are2p from *P. pastoris* share 49% and 40% sequence identity with their respective counterparts from *S. cerevisiae,* and 52% and 42.5% identity with those from *Y. lipolytica*. In contrast to *S. cerevisiae,* where Dga1p is the major contributor to TG synthesis, Lro1p seems to be the more potent TG synthase in *P. pastoris*. Deletion of *LRO1* led to a marked decrease of TG, whereas deletion of *DGA1* hardly affected the total TG content in this yeast. Are2p seems to be the only SE synthase in *P. pastoris,* since deletion of this gene resulted in an almost complete loss of SE. Noteworthy, similar to *Y. lipolytica*, *P. pastoris* produces only small amounts of SE. Are2p alone in the absence of TG synthesizing enzymes is not sufficient to initiate LD formation. Similar to *S. cerevisiae* and *Y. lipolytica,* LD formation can be enhanced when *P. pastoris* is

grown on oleate as the only carbon source. Under these conditions, cells accumulate a large amount of TG.

Non-polar lipid storage in yeast

Lipid droplets of *Saccharomyces cerevisiae*

The products of non-polar lipid synthesis, TG and SE, are stored in specialized organelles termed LD. All eukaryotic cells and even some prokaryotes of the gram-positive genera such as *Rhodococcus* or *Streptomyces* species contain such intracellular LD (Zweytick *et al.*, 2000a).

Experiments employing small-angle X-ray scattering and differential scanning calorimetry provided a first insight into the internal structure of LD from *S. cerevisiae.* These analyses showed that TG form the inner core of a LD surrounded by several shells of SE, most likely with some TG intercalated (Czabany *et al.*, 2008). TG and SE are the major components of LD from *S. cerevisiae* contributing 45% to 50%, each, to the total mass of LD (Leber *et al.*, 1994). Squalene and sterols (0.5% and < 0.3%, respectively) are only minor components of LD when cells are grown under standard growth conditions. The highly hydrophobic core of LD is covered by a phospholipid monolayer which contains 57% PC, 21% PI and 16% PE. PA and PS contribute with about 2%, each, to the phospholipid composition of LD. Cardiolipin and dimethyl-PE were not detectable in the LD (Tauchi-Sato *et al.*, 2002; Grillitsch *et al.*, 2011). The high abundance of PI is characteristic for the LD surface phospholipid monolayer. Proteins are present in LD only at an amount of 2.6% of the total mass.

It is assumed, that the ER is the origin of the surface monolayer of LD, although the process of LD biogenesis is still a matter of dispute. Several models of LD biogenesis have been discussed in the literature, such as the “lensing model”, “bicell formation” and “vesicle formation” (Guo *et al.*, 2009). The lensing model and the bicell formation model share the idea of TG accumulation between the two membrane leaflets of the ER. After reaching a critical size, LD bud off the ER membrane (Figure 4). The phospholipid monolayer of LD may develop from the outer leaflet of the ER bilayer membrane (lensing model) or from both leaflets (bicell formation model). In a theoretical approach to understand LD formation, biophysical models were applied to mimic LD biogenesis. These models suggest, that

phospholipid demixing is the driving force for the budding of LD from the ER membrane when LD reach a critical size (Zanghellini *et al.,* 2010). The large amounts of PC and PI present in LD may indicate that specific phospholipids are important for structure and function of LD (Grillitsch *et al.,* 2011). The least favored model of LD biogenesis is based on the "secretory vesicle" hypothesis. This model suggests that at the beginning of LD formation secretory vesicles filled with TG and covered by a phospholipid bilayer are built from the ER membrane. During a maturation process, the bilayer membrane may be rearranged to form the surface phospholipid monolayer membrane of mature LD (Guo *et al.,* 2009; Murphy & Vance, 1999). An intimate relationship between LD and ER is common to all three LD biogenesis models. Indeed, LD seem to be tightly associated with the ER membrane, which was recently underlined by functional assays (Robenek *et al.,* 2006; Szymanski *et al.,* 2007; Perktold *et al.,* 2007; Jacquier *et al.,* 2011). When expression of Dga1p or Lro1p, which are both localized to the ER, was induced in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ mutant background, LD formation was observed at the ER membrane. Nascent LD became decorated with specific membrane proteins which were in the beginning of the process randomly distributed over the ER membrane. The transport of ER membrane proteins to the LD was found to be fast, energy and temperature as well as COPI and COPII independent and occurred in both directions. These findings supported the idea of transient or permanent interactions of ER membranes and LD (Jacquier *et al.,* 2011; Zehmer *et al.,* 2009). Fld1p, the yeast homologue of seipin, seems to play a very important role in LD formation, size and inheritance. An *fld1*Δ strain harbors irregular populations of LD in terms of quantity, size and shape. A portion of 30% of *fld1*Δ cells comprise LD with a size of 0.5 – 1.5 µm, whereas LD of a wild type strain reach a size between 0.2 and 0.4 µm (Fei *et al.,* 2008). Fld1p is a homooligomer of about 9 subunits and assumed to facilitate trafficking of lipids and proteins between the ER and LD (Binns *et al.,* 2010). This idea was supported by the work of Wolinski *et al.* (2011). These authors showed that in a wild type strain Tgl3p, the main TG lipase of *S. cerevisiae*, predominantly localized to distinct spots on the LD surface. After stimulation of lipolysis, Tgl3p homogenously distributed over the surface of LD. In an *fld1*Δ strain, however, Tgl3p was already spread over the LD surface without induction of lipolysis. Thus, the increased TG content in *fld1*Δ strains might be result of an impaired access of TG lipases to their TG substrate.

Recently, it was shown that Pah1p, the major PA phosphatase of the yeast, also plays a role in LD assembly (Adeyo *et al.,* 2011). DG synthesized by Pah1p appears to be important for LD

formation independent of its role as TG precursor. It was hypothesized that DG causes bending of the ER membrane and promotes bud formation as a direct result of membrane instability. Alternatively, DG may play an indirect role in LD biogenesis by recruiting proteins which are important for LD formation (Adeyo *et al.,* 2011). Further insight to the role of Pah1p in LD biogenesis was recently provided by Karanasios *et al.* (2013). These authors showed that the C-terminal acidic tail of Pah1p was crucial for the regulation of LD biogenesis and the translocation of Pah1p from the cytosol to the ER membrane. The latter process requires dephosphorylation of Pah1p by the Nem1p-Spo7p transmembrane phosphatase complex although the mechanism is poorly understood. Thus, DG synthesized by Pah1p localized to the ER may build a platform for LD biogenesis (Karanasios *et al.,* 2013; Adeyo *et al.,* 2011).

In higher eukaryotes, LD are covered by scaffolding proteins such as oleosins in plants and perilipins in animal cells. *S. cerevisiae* does not have homologues of such scaffolding proteins. However, Jacquier *et al.* (2013) found that expression of oleosins and perlipins in yeast promote formation of LD from the ER. Both oleosins and perlipins were properly targeted to LD in *S. cerevisiae,* and both polypeptides were able to induce LD formation in mutants containing elevated levels of non-polar lipids in the ER phospholipid bilayer. Such a scenario can be created by deletion of *PAH1* which causes a decreased number of LD and enrichment of non-polar lipids in the ER (Adeyo *et al.,* 2011). Results presented by Jacquier *et al.* (2013) may suggest that LD scaffolding proteins promote sequestration of non-polar lipids from the ER bilayer and thereby induce LD formation. A possible reason why *S. cerevisiae* does not require homologues of oleosins or perlipins for LD formation might be the rather small size of yeast LD compared to LD from plants and animal cells. Thus, the need of scaffolding proteins may be limited or the function of scaffolding proteins may be taken over by other proteins found in LD.

Proteome of lipid droplets from *Saccharomyces cerevisiae*

A small but specific set of proteins is embedded in the surface phospholipid monolayer of yeast LD. MS was used as a sensitive method to analyze the proteome of purified LD from *S. cerevisiae*. The protein equipment of LD varies under different growth condition. Grillitsch *et al.* (2011) identified 49 proteins as constituents of LD from cells grown on glucose, whereas 54 proteins were found on LD from cells grown on media containing oleate as a carbon source. Only 25 proteins were detected in both LD variants. These proteins appear to be a

"basic protein equipment" of LD (Table 1). Most of these 25 proteins are involved in lipid metabolism such as proteins catalyzing ergosterol biosynthesis (Erg1p, Erg6p, Erg7p, Erg27p), TG and SE degradation (Tgl3p, Tgl4p, Tgl5p, Tgl1p), and long chain FA synthesis or FA transport (Faa1p, Pdr16p). As mentioned above, homologues of perilipins or oleosins, scaffolding proteins affecting structural properties of LD in mammalian and plant cells, have not been found (Brasaemle, 2007).

Tight interaction of LD with other cell compartments (ER, peroxisomes, mitochondria) and limitations in LD purification procedures causing contamination of LD with other organelles have to be considered as possible problems leading to false data of MS analysis (Pu *et al.*, 2011; Jacquier *et al.,* 2011). To overcome these problems a new method was recently introduced, namely high-resolution proteomics based on the correlation of profiles of organelle samples with known organelle markers (Krahmer *et al.,* 2013). With this method it was possible to identify 111 proteins in LD fractions from *Drosophila* S2. It was possible to identify 111 proteins in LD fractions based on MS and protein correlation profiles. Among these proteins were well-characterized proteins such as *Brummer*, the drosophila TG lipase homologue of ATGL, as well as proteins previously unknown as LD components. The latter group contained proteins functionally related to LD such as a lysoPA acyltransferase or a glycosyltransferase. However, LD from yeast have not yet been analyzed with this new method.

Despite various experimental efforts it is not yet known how LD proteins reach their destination. Primary amino acid targeting consensus sequences on LD proteins have not been identified. In mammalian cells, however, it was shown that the N-terminal amphipathic helices of viperin and NS5A, two proteins of the hepatitis C virus, were crucial for targeting these proteins to LD in virus host cells (Hinson & Cresswell, 2009; Brass *et al.*, 2002). Also hydrophobic stretches of the N- or C-terminus of proteins were shown to affect their targeting to LD. Twenty-eight amino acids of the hydrophobic N- terminus of the putative methyltransferase AAM-B were shown to be necessary and sufficient to target the protein to LD of HeLa cells, COS7 cells and yeast (Zehmer *et al.*, 2008). When the hydrophobic C-termini of Erg1p, Erg6p and Erg7p from S. *cerevisiae* were removed, targeting of these proteins to LD was disturbed and polypeptides accumulated in the ER (Müllner *et al.,* 2004). However, C-terminal stretches of Erg1p, Erg6p or Erg7p were not sufficient to direct a GFP-hybrid to LD. Oleosins from plants harbor probably the best elucidated targeting motifs. They are comprised of a tripartite structure with a central hydrophobic core of about 70 amino

acids (Abell *et al.,* 1997). This core contains a proline knot motif consisting of three prolines within a 12 residue sequence flanked by hydrophilic stretches on each site. However, no such motifs were found in LD proteins from the yeast.

Many LD proteins display a dual localization and are also found in the ER. The proteins studied best with this respect are Erg1p, Erg6 and Tgl3p from *S. cerevisiae* (Zinser *et al.,* 1993; Leber *et al.,* 1998; Schmidt *et al.,* 2013). This dual localization raises the question how proteins can cope with two different membrane environments, namely a phospholipid monolayer in LD and a phospholipid bilayer in the ER. Structural rearrangement of the proteins might be a possibility to adapt to the two types of membrane environments, but experimental data for such a process are still missing.

Lipid droplets from *Yarrowia lipolytica* and *Pichia pastoris*

The yeast *Y. lipolytica* can be cultivated on hydrophobic carbon sources such as alkanes or FA as sole carbon sources. LD from *Y. lipolytica* grown on glucose have a diameter of ~ 650 nm and are thus markedly larger than LD from *S. cerevisiae.* However, when *Y. lipolytica* was cultivated on oleate containing media LD reached a size of 2.5 µm in the stationary growth phase of the cultures (Athenstaedt *et al.,* 2006). TG is the major non-polar lipid of *Y. lipolytica* and only small amounts of SE and free ergosterol were detectable which resulted in a TG to SE ratio of about 11 (Athenstaedt *et al.,* 2006). The variation of the carbon source, however, did not only affect the size of LD but also the protein equipment. MS analysis of LD from *Y. lipolytica* grown on glucose or oleate as carbon source, respectively, showed adaption of the protein pattern. Twenty-one proteins were identified on LD from cells grown on glucose, whereas 30 proteins were identified on LD from cells grown on oleate (Athenstaedt *et al.,* 2006). According to sequence homology with *S. cerevisiae* proteins, the identified LD proteins from *Y. lipolytica* contribute to FA activation (Faa1p), phospholipid synthesis (Sct1p, Slc1p), and TG and SE metabolism. Recently, the first TG lipases of *Y. lipolytica* identified as LD components by MS analysis were functionally characterized. The *Y. lipolytica* genes YALI0D17534g and YALI0F10010g are closely related to *S. cerevisiae* Tgl3p and Tgl4p and were therefore named YlTgl3p and YlTgl4p (Dulermo *et al.,* 2013). *Y. lipolytica yltgl3*Δ and *yltgl4*Δ deletion mutants showed a 2-fold TG accumulation compared to wild type. Contrary to *S. cerevisiae,* YlTgl4p seems to be the major TG lipase of *Y. lipolytica*.

LD of the methylotrophic yeast *P. pastoris* have been studied in some detail. These investigations revealed that more than 90% of non-polar lipids contained in LD are TG and only small amounts of SE were found. Thus, the TG to SE ratio in *P. pastoris* is ~15 which is remarkably higher than in *S. cerevisiae* and *Y. lipolytica* (Ivashov *et al.,* 2012) (Table 2). The small portion of SE seems to be result of the low amount of sterols formed in *P. pastoris.* A limited number of proteins was identified by MS analysis on the surface of LD from *P. pastoris* compared to the proteome of LD from *S. cerevisiae* (Ivashov *et al.,* 2012). The function of most proteins identified in *P. pastoris* LD is still hypothetical and only based on blast and motif searches. Some proteins are orthologues of *S. cerevisiae* LD proteins, such as ergosterol, phospholipid and sphingolipid biosynthesis enzymes (Erg1p, Erg6p, Erg7p, Erg27p, Slc1p and Tsc10p); enzymes involved non-polar lipid degradation (Tgl1p); and proteins involved in FA metabolism (Faa1p, Fat1p and Eht1p).

Lipid droplet interaction with other organelles

A number of studies addressed the question of LD interaction with other organelles. Respective experiments were carried out with cellular systems from yeast to mammalian cells (for reviews see Murphy *et al.,* 2009; Dugail, 2013). Apart from the intimate relationship of LD and ER during LD formation, numerous studies mainly with mammalian cells suggested further interactions between these two organelles. In several LD proteome analyses proteins belonging to the Rab-family were identified with Rab18p being the best studied example (Murphy *et al.,* 2009). Ozeki *et al.* (2005) showed that the Rab18p GTP/GDP status regulated the interaction of LD and ER. Only Rab18p with GTP bound was localized to LD. Furthermore, Rab18p recruitment to LD was observed in response to lipolytic stimulation and Rab18p-positive LD were found in close proximity to ER membranes (Martin *et al.,* 2005). Thus, Rab18p appears to be responsible for the interaction of ER and LD which might be prerequisite for the transport of lipids mobilized from LD in mammalian cells (Ozeki *et al.,* 2005).

Moreover, LD can often be found in close proximity to mitochondria, peroxisomes or lysosomes (Turró *et al.,* 2006; Liu *et al.,* 2007; Sturmey *et al.,* 2006; Schrader, 2001). LD are a source of FA which may serve as substrates for β-oxidation. As β-oxidation of *S. cerevisiae* occurs exclusively in peroxisomes, contact between LD and peroxisomes was considered to be important for direct transfer of FA between these organelles. Indeed, tight interaction of peroxisomes and LD was observed when *S. cerevisiae* was grown on oleate containing media

which is required for the induction of peroxisome proliferation (Binns *et al.,* 2006). When cells were starved and forced to use FA from TG stored in LD, protrusions of peroxisomes into LD were seen and termed pexopodia. In an interactomic study with a bimolecular fluorescent complementation assay the LD proteins Erg6p and Pet10p showed protein-protein interactions with several peroxisomal proteins, many of them being involved in transport processes (Pu *et al.,* 2011). This finding might be a further indication that the tight interaction between these organelles serves metabolite transport. However, peroxisome proliferation does not rely on FA as substrates for β-oxidation derived from TG of LD. Work from our laboratory (Connerth *et al.,* unpublished data) showed that a *tgl3*Δ*tgl4*Δ*tgl5*Δ mutant which cannot mobilize FA from LD depots can still form peroxisomes. Thus, direct supply of FA from external sources appears to be sufficient for supplying substrate to the site of β-oxidation.

Interaction of LD and mitochondria, which are the site for β-oxidation in mammalian cells, was observed in skeletal muscle cells. Interestingly, some LD appear to share portions of their surface membranes as organelle boundaries as shown by transmission electron microscopy (Shaw *et al.,* 2008; Pu *et al.,* 2011). Interaction of LD proteins with mitochondrial resident proteins was also found in *S. cerevisiae*. According to Pu *et al.* (2011) Erg6p interacts with the mitochondrial protein Mcr1p, which is involved in ergosterol biosynthesis. Thus, interaction of LD with other organelles may be a mechanism for the supply of lipids to other cellular compartments.

Non-polar lipid mobilization and turnover in yeast

Mobilization and degradation of triacylglycerols

TG stored in LD of yeast are degraded via a cascade of hydrolysis reactions to DG, monoacylglycerols (MG) and FA (Figure 5). The major TG lipases of the yeast *S. cerevisiae* identified so far are Tgl3p, Tgl4p and Tgl5p (Athenstaedt & Daum, 2003; 2005). All three lipases share the highly conserved GXSXG lipase motif within a patatin domain instead of a typical α/β hydrolase fold present in hydrolytic enzymes (Ollis *et al.,* 1992). The α/β hydrolase fold consists of a mostly parallel eight stranded β sheet surrounded on both sides by α helices, and the active site contains a catalytic serine-histidine-aspartate triade (Brady *et al.,* 1990). In contrary, the patatin domain has a different topology and shows an α/β/α-

architecture. The active center consists of a catalytic serine-aspartate dyad (Rydel *et al.,* 2003).

Tgl3p from *S. cerevisiae* was first discovered by a proteome analysis as a major component of LD and was the first fully characterized TG lipase from the yeast (Athenstaedt *et al.,* 1999b; Athenstaedt & Daum, 2003). The finding that TG mobilization was not completely stopped in a *tgl3*Δ strain led to the identification of two further TG lipases, Tgl4p and Tgl5p (Athenstaedt & Daum, 2005; Kurat *et al.,* 2006). Tgl3p, the major TG lipase of the *S. cerevisiae,* shows the highest lipolytic activity *in vivo* and *in vitro* of all yeast TG lipases known so far, and a deletion of *TGL3* results in the accumulation of TG to ~200% compared to wild type (Athenstaedt & Daum, 2005). Tgl3p preferentially utilizes TG containing C14:0, C16:0 and C26:0, and deletion of *TGL3* results in the accumulation of TG containing these three FA species. Moreover, Tgl3p is also a DG hydrolase although with minor efficiency (Kurat *et al.,* 2006). The DG lipase activity was shown by overexpression of *TGL3* in a *tgl3*Δ*tgl4*Δ background. This manipulation led to an accumulation of DG up to 6% of total lipids, whereas overexpression of *TGL4* in the same strain background caused an accumulation of DG up to 12% of total lipids. These data suggested that Tgl3p not only accepts TG as substrate but also contributes to DG hydrolysis *in vivo*.

Lack of Tgl3p affects sporulation of *S. cerevisiae* (Athenstaedt & Daum, 2005). A homozygous diploid strain deleted of *TGL3* was not able to form spores after 21 days, whereas such a defect was not observed with *tgl4*Δ*/tgl4*Δ and *tgl5*Δ*/tgl5*Δ mutants. These strains showed similar sporulation behavior as wild type and formed spores after 3 to 4 days. Interestingly, a *tgl4*Δ*tgl5*Δ double deletion also resulted in a negative effect on sporulation, as the homozygous double deletion strain *tgl4*Δ*tgl5*Δ*/tgl4*Δ*tgl5*Δ revealed only poor spore formation after 16 days (Athenstaedt & Daum, 2005). In contrast to *tgl4*Δ and *tgl5*Δ single deletion strains the *tgl4*Δ*tgl5*Δ double mutant accumulated substantial amounts of TG. However, the cellular TG level does not appear to be crucial for sporulation (see below).

In addition to lipase activity, Tgl3p and Tgl5p harbor acyltransferase activity with broad substrate specificity (Rajakumari & Daum, 2010a). The active centers for anabolic and catabolic enzyme activities of both enzymes work independently of each other. Experiments employing site directed mutagenesis revealed that the function of Tgl3p as PE forming acyltransferase was required for proper spore formation. Tgl4p, the functional orthologue of the mammalian adipocyte triglyceride lipase (ATGL), is also active as SE hydrolase, *sn2*-specific PLA_2 and acyl-CoA dependent acyltransferase *in vitro* (Kurat *et al.,* 2006;

Rajakumari & Daum, 2010b). The finding that Tgl3p, Tgl4p and Tgl5p are multifunctional enzymes suggested that TG degradation is linked to other lipid metabolic pathways. Indeed, it was shown, that deletion of each of the three TG lipases resulted in a reduced amount of all three major types of yeast sphingolipids, namely inositolphosphoceramide, mannosyl-inositolphosphoceramide and mannosyl-diinositolphosphoceramide. Furthermore, lipase deficient mutants were also compromised in PI synthesis (Rajakumari *et al.*, 2010; Gaspar *et al.*, 2011). These results indicated that non-polar lipid degradation and synthesis of membrane-forming lipids, especially PI and sphingolipids, are linked processes to maintain lipid homeostasis in growing cells (Gaspar *et al.*, 2011). A further involvement of non-polar lipid metabolism in phospholipid synthesis and homeostasis was provided by Mora *et al.* (2012). These authors showed that enzymes of non-polar lipid synthesis and mobilization play an important role in FA trafficking and consequently in phospholipid remodeling.

The initial breakdown of TG to DG performed by Tgl3p, Tgl4p, and Tgl5p is a relatively well studied process. In contrast, the subsequent steps of non-polar lipid degradation need to be elucidated in more detail. Specific yeast DG lipases have not been identified so far, although Tgl3p and Tgl4p were shown to accept DG as substrates (Kurat *et al.*, 2006). The first identified MG lipase of *S. cerevisiae* was Yju3p, the functional orthologue of the human MG lipase (Heier *et al.*, 2010). This lipase shows a 1000-fold higher specific activity than Tgl3p and Tgl4p. However, deletion of *YJU3* resulted only in low accumulation of MG and no specific phenotype, probably due to rapid turnover of MG.

Evidence about regulation of TG lipases from *S. cerevisiae* is limited. It was demonstrated that lipolysis is most active during the logarithmic growth phase of yeast cells (Kurat *et al.*, 2006). Moreover, it was shown that Tgl4p is a direct target of the cyclin-dependent kinase Cdk1p. Tgl4p can be phosphorylated by Cdk1p/Cdc28p at threonine 675 and, to a lesser extent, at serine 890 during G1/S transition of the cell cycle (Ubersax *et al.*, 2003; Kurat *et al.*, 2009). These phosphorylation events are necessary to stimulate the lipolytic activity of Tgl4p. A further indication for a tight link of the cell cycle to lipid metabolism was the finding that Tgl3p appeared as a target of the cyclin dependent kinase Pho85p, a non-essential kinase, in a global analysis of protein phosphorylation in yeast (Ptacek *et al.*, 2005). Pho85p is involved in regulating the cellular response to nutrient levels, environmental conditions and progression through the cell cycle (Huang *et al.*, 2007). Thus, the activity of TG lipases seems to be linked to the cell cycle and to environmental conditions which

regulate the release of FA and other lipid components upon cellular requirements of membrane lipid biosynthesis (Kurat *et al.,* 2009).

Protein stability and localization of Tgl3p are affected by TG substrate limitation and the presence of LD in yeast. Schmidt *et al.* (2013) showed that in wild type cells Tgl3p is not exclusively localized to LD but also found in the ER. The presence or absence of the TG substrate in LD affects the distribution of Tgl3p between LD and ER. Lack of LD in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ mutant or depletion of TG in a *dga1*Δ*lro1*Δ strain caused complete or partial re-localization of Tgl3p to the ER resulting in protein instability. Interestingly, the gene expression of *TGL3* was only slightly affected in strains lacking TG

Mobilization and degradation of steryl esters

SE contribute with ~45% of the total mass to the non-polar lipid pool of LD from *S. cerevisiae* and play an important role in cellular sterol homeostasis (Leber *et al.,* 1994; Zinser *et al.,* 1993). A crucial step in understanding sterol homeostasis in the yeast was the discovery of three hydrolases with a predicted α/β-hydrolase fold, Tgl1p, Yeh1p and Yeh2p, which are most likely the only SE hydrolases in *S. cerevisiae* (Köffel *et al.,* 2005; Jandrositz *et al.,* 2005; Müllner *et al.,* 2005; Wagner *et al.,* 2009). Sequence analysis revealed that these enzymes are paralogues of the human acid lipase family (Köffel *et al.,* 2005). Tgl1p, Yeh1p and Yeh2p contain the conserved GXSXG lipase motif, although the first glycine of this motif is replaced by a serine in Yeh1p and an alanine in Yeh2p. Yeh1p and Tgl1p localize to LD whereas Yeh2p is enriched in the plasma membrane (Köffel *et al.,* 2005; Jandrositz *et al.,* 2005; Müllner *et al.,* 2005). The three SE hydrolases show a certain substrate specificity. Yeh2p and Tgl1p exhibit a slight preference for zymosterol esters. Tgl1p also hydrolyses TG *in vitro* but does not significantly contribute to TG turnover *in vivo* (Müllner *et al.,* 2005; Wagner *et al.,* 2009; Jandrositz *et al.,* 2005). Yeh1p hydrolyses fecosteryl esters more efficiently than ergosteryl and zymosteryl esters (Wagner *et al.,* 2009). Interestingly, aerobic/anaerobic growth conditions of yeast cells affect the activity of Yeh1p. As *S. cerevisiae* is a facultative anaerobic organism it can grow in the absence of oxygen when supplemented with sterols and oleic acid. Anaerobic conditions or deletion of *HEM1* turned out to be useful for studying sterol dependency of the yeast and were widely used to investigate sterol biosynthesis, transport and degradation. It was shown that Yeh1p was equally utilizing cholesteryl, lanosteryl and ergosteryl esters under heme-deficient conditions, while Yeh2p and Tgl1p were inactive (Köffel & Schneiter, 2006). These results indicated that

the activity of the three steryl ester hydrolases Yeh1p, Yeh2p and Tgl1p is differentially regulated by the presence or absence of oxygen.

A novel regulatory mechanism of sterol homeostasis was discovered by Tiwari *et al.* (2007). These authors showed that a cycle of acetylation and deacetylation controlled the sterol export from *S. cerevisiae*. Acetylation of sterols requires catalysis of the acetyltransferase Atf2p whereas deacetylation of sterol acetates is accomplished by Say1p. Deletion of *SAY1* resulted in secretion of acetylated sterols into the culture media indicating that the acetylation status of sterols was crucial for their secretion. The acetylation/deacetylation cycle might function as a lipid proofreading mechanism, as sterol precursors as well as non-natural sterols such as pregnenolone were acetylated by Atf2p, whereas the respective acetates were neither deacetylated by Say1p nor exported from the cell. Thus, the sterol acetylation cycle might be regarded as detoxification mechanism to protect the yeast cell from potentially toxic steroid-like compounds. This view was supported by the finding that yeast cells expressing *ATF2* and *SAY1* are able to grow in the presence of the toxic plant-derived allylbenzene eugenol. A similar mechanism may exist in mammalian cells as two homologues of Say1p were found in the human genome, which were able to take over the function of Say1p in yeast cells. As acetylation of sterols renders derivatives even more hydrophobic the question arose how these acetylated sterols are secreted. Choudhary and Schneiter (2012) identified a family of conserved proteins, the pathogen-related yeast (PRY) proteins, as a class of sterol-binding proteins in *S. cerevisiae*. These authors showed that Pry1p and Pry2p were able to bind sterol and sterol acetate *in vitro*. Deletion of *PRY1* and *PRY2* in *S. cerevisiae* completely blocked the secretion of acetylated sterols. Taken together, sterol homeostasis is not only affected by biosynthesis, storage and degradation, but also by complex mechanisms of lipid quality control, detoxification and secretion.

Conclusions and perspectives

Many key players involved in non-polar lipid synthesis, storage and mobilization of the yeast have been studied and characterized extensively within the last few years. Particularly, the protein equipment of LD from *S. cerevisiae*, *Y. lipolytica* and *P. pastoris* has gained much attention, and LD proteins were identified and characterized in some detail (Fei *et al.*, 2011; Grillitsch *et al.*, 2011; Athenstaedt *et al.*, 2006; Ivashov *et al.*, 2012). Despite these efforts, many functions of these enzymes still remained elusive. Several enzymes of the non-polar

lipid metabolic network do not only have one function but exhibit two or more enzymatic activities. Consequently, these enzymes may contribute to anabolic as well as catabolic processes. Tgl3p is a prominent example for such an enzyme exhibiting dual functionality in TG degradation and phospholipid synthesis, respectively. The occurrence of overlapping functions of non-polar lipid metabolizing enzymes makes non-polar lipid homeostasis an even more complex system which we just begin to understand.

Several questions related to yeast non-polar lipid biology remained open so far. As an example, it is not known how non-polar lipid hydrolytic enzymes locate to LD get access to their substrates. LD models predict that proteins are embedded in the surface phospholipid monolayer of the organelle. However, the substrates for TG lipases and SE hydrolases form the hydrophobic core of LD, and the access of enzymes to the substrates appears to be a problem. To address this question a more detailed knowledge of the topology of LD proteins is required. Also, little is known about targeting and insertion of LD proteins into the phospholipid monolayer of LD. Moreover, several LD proteins are dually localized to the ER and LD (Schmidt *et al.,* 2013; Sorger *et al.,* 2004). Consequently, they are able to integrate into both phospholipid monolayer and bilayer membranes. How the different membrane environments affect the stability and function of these enzymes needs to be investigated. More detailed studies of structural properties of LD proteins will help to answer this question. Another not yet understood aspect of non-polar lipid metabolism is the regulation of the enzymes involved. As described in this review article we are only at the beginning to understand how the different branches of the general lipid metabolic network are linked to non-polar lipid metabolism. Enzymes which catalyze reactions at some branching points of lipid metabolism such as Pah1p have only recently been characterized. Regulation by enzyme modification, i. e. by phosphorylation, is another possibility to modulate enzymatic activities. Examples of this kind are the multifunctional enzymes Tgl3p and Tgl4p which were shown to be targets of kinases. However, the molecular mechanism balancing catabolic and anabolic activities of these enzymes needs to be elucidated.

In summary studies of non-polar lipid synthesis, storage and mobilization as well as LD biology with the yeast contributed significantly to our understanding of these processes in a general way. A combination of biochemical, molecular biological and cell biological methods as well as lipidome and proteome analyses have set the stage for future studies addressing the open questions mentioned above.

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ASAT, acyl-CoA:sterol acyltransferase; ATGL, adipocyte triglyceride lipase; CoA, coenzymeA; DG, diacylglycerols; DGAT, diacylglycerol acyltransferase; DGPP, diacylglycerol pyrophosphate; DHAP, dihydroxyacetone phosphate; ER, endoplasmic reticulum; FA, fatty acids; FAS, fatty acid synthase; G-3-P, glycerol-3-phosphate; LD, lipid droplets; MG, monoacylglycerols; MS, mass spectrometry; PA, phosphatidic acid; PAP, phosphatidate phosphatase; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SE, steryl esters; TG, triacylglycerols; VLCFA, very long chain fatty acids;

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Tables

Table 1 Lipid droplet proteins from *S. cerevisiae* identified by proteome analysis. LD from *S. cerevisiae* grown either on glucose or oleic acid supplemented media were analyzed by MS. Proteins detected in both LD variants are listed (Grillitsch *et al.*, 2011). LD, lipid droplets; ER, endoplasmic reticulum; M, mitochondria; C, cytosol; PM; plasma membrane; G, Golgi; End, endosomes; nEnv, nuclear envelope; Mic, microsomes; mem, integral to membrane; V, vacuole.

Gene	Systematic name	Localization	Function
AYR1	YIL124W	LD/ER/M/C	NADPH-dependent 1-acyl-dihydroxyacetone phosphate reductase
CPR5	YDR304C	C/ER	Peptidyl-prolyl cis-trans isomerase
EHT1	YBR177C	LD/M	Acyl-coenzymeA:ethanol O-acyltransferase
ERG1	YGR175C	ER/LD	Squalene epoxidase
ERG6	YML008C	ER/LD/M	Delta(24)-sterol C-methyltransferase
ERG7	YHR072W	ER/LD/PM	Lanosterol synthase
ERG 27	YLR100W	ER/M	3-Keto sterol reductase
FAA1	YOR317W	LD/PM/M	Long chain fatty acyl-CoA synthetase
GTT	YIR038C	C/G	BAR domain-containing protein
HFD1	YMR110C	M/LD/End	Putative fatty aldehyde dehydrogenase
NUS1	YDL193W	ER/LD/nEnv	Putative prenyltransferase
OSH4	YPL145C	C/G/ext	Oxysterol binding protein
PDI1	YCL043C	ER	Disulfide isomerase
PDR16	YNL231C	LD/Mic/PM/C	Phosphatidylinositol transfer protein
PET10	YKR046C	LD	Unknown
RRT8	YOL048C	LD	Unknown
SLC1	YDL052C	LD	1-Acyl-sn-glycerol-3-phosphate acyltransferase
TGL1	YKL140W	LD/mem	Steryl ester hydrolase
TGL3	YMR313C	LD	Triacylglycerol lipase
TGL4	YKR089C	LD	Triacylglycerol lipase
TGL5	YOR081C	LD	Triacylglycerol lipase
UBX2	YML013W	ER/M	Protein involved in ER-associated protein

			degradation
VPS66	YPR139C	C	Cytoplasmic protein of unknown function involved in vacuolar protein sorting
YJU1	YKL094W	LD/C/M/PM	Monoglyceride lipase
YPT7	YML001W	V/M	GTPase

Table 2 Composition of lipid droplets from *S. cerevisiae, P. pastoris* and *Y. lipolytica.* Components of LD were analyzed from yeast strains grown to the stationary growth phase on glucose media. TG, triacylglycerols; SE, steryl esters (Leber *et al.*, 1994; Ivashov *et al.*, 2012; Athenstaedt *et al.*, 2006).

	S. cerevisiae (µg/µg protein)	*P. pastoris* (µg/µg protein)	*Y. lipolytica* (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

FIGURE 1

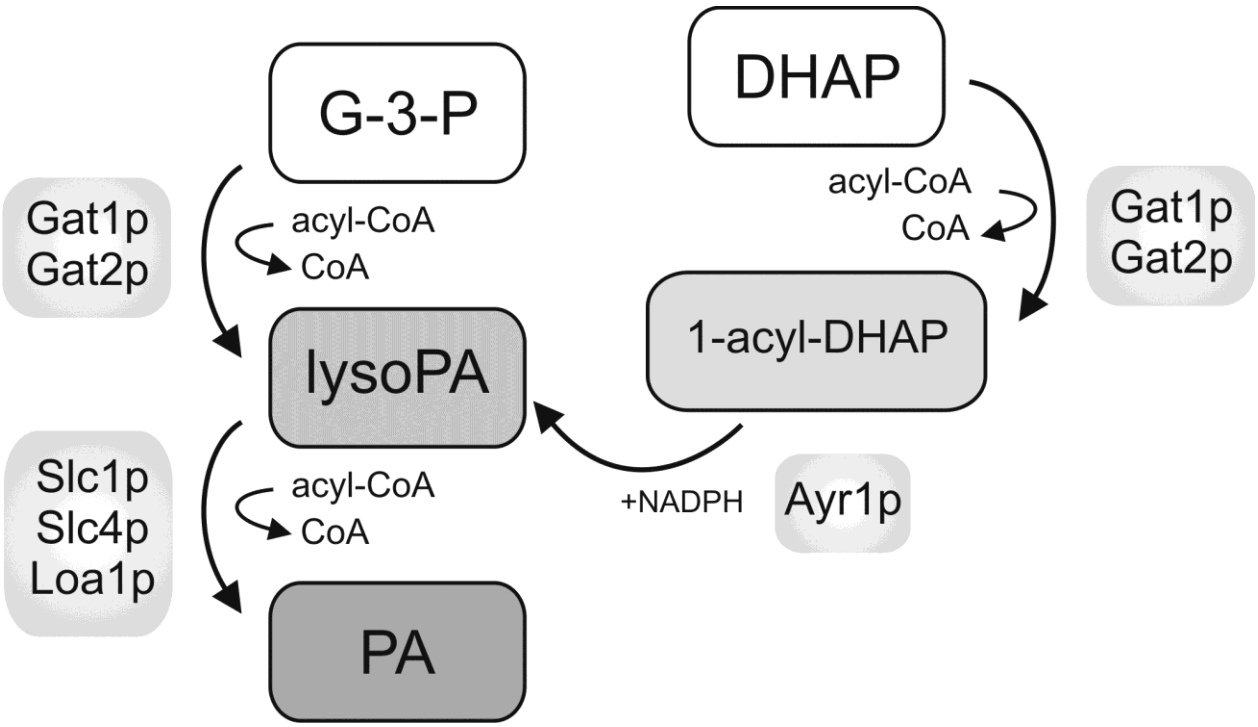

Fig. 1 Formation of phosphatidic acid in the yeast *S. cerevisiae.* LysoPA can be either formed through the G-3-P or the DHAP pathway. Both reactions require acylation of the respective precursors by Gat1p or Gat2p, respectively. 1-Acyl-DHAP needs to be reduced to lysoPA by Ayr1p in an NADPH dependent manner. LysoPA is further acylated by Slc1p, Slc4p or Loa1p to form PA. DHAP, dihydroxyacetone phosphate; G-3-P, glycerol-3-phosphate; PA, phosphatidic acid.

FIGURE 2

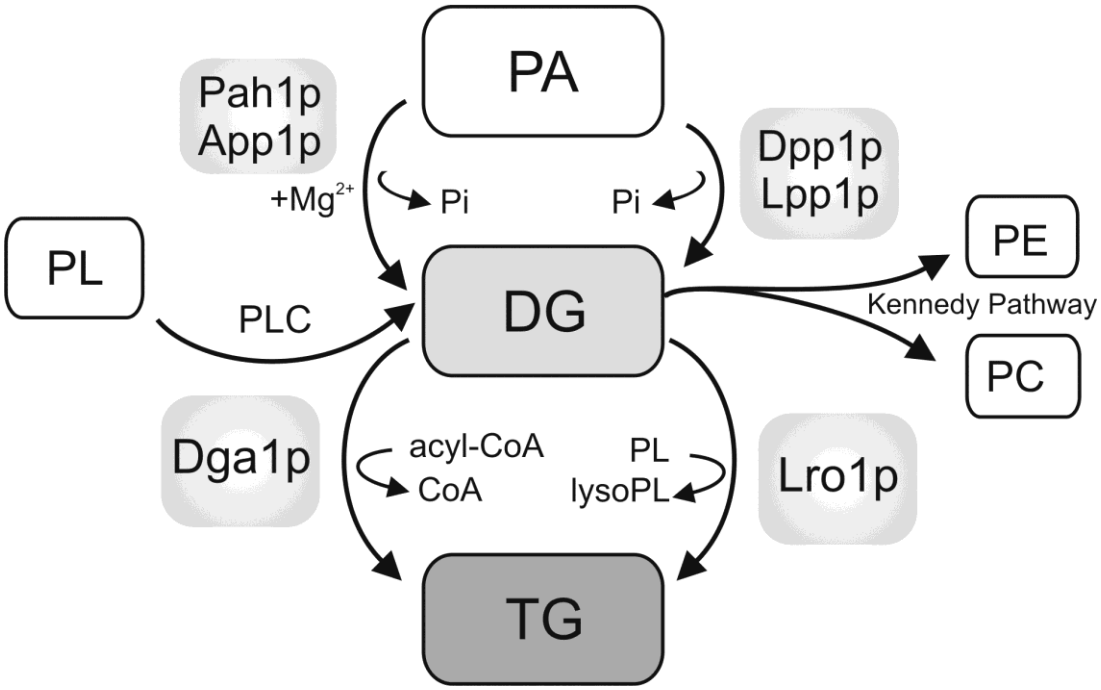

Fig. 2 Formation of diacylglycerol and triacylglycerol in the yeast *S. cerevisiae.* Formation of DG by dephosphorylation of PA is catalyzed by Pah1p and App1p in an Mg^{2+} dependent, or by Dpp1p and Lpp1p in an Mg^{2+} independent reaction. DG is then acylated to form TG by either an acyl-CoA dependent reaction catalyzed by Dga1p or an acyl-CoA independent step catalyzed by Lro1p. DG can also be formed by degradation of glycerophospholipids catalyzed by phospholipase C. DG can also be used as precursor for PC and PE synthesis via the Kennedy pathway. DG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; PLC, phospholipase C; TG, triacylglycerol.

FIGURE 3

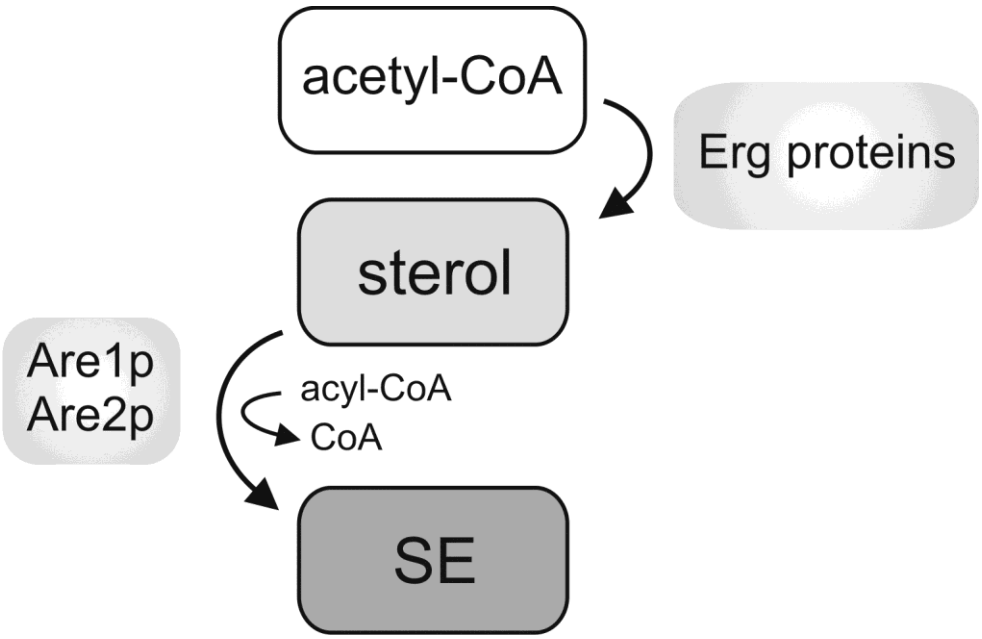

Fig- 3 Formation of sterols and steryl esters in the yeast *S. cerevisiae*. Sterols are formed from their very first precursors acetyl-CoA through several catalytic steps catalyzed by Erg proteins. Sterols are then further acylated in an acyl-CoA dependent reaction performed by Are1p or Are2p, respectively. SE, steryl esters.

FIGURE 4

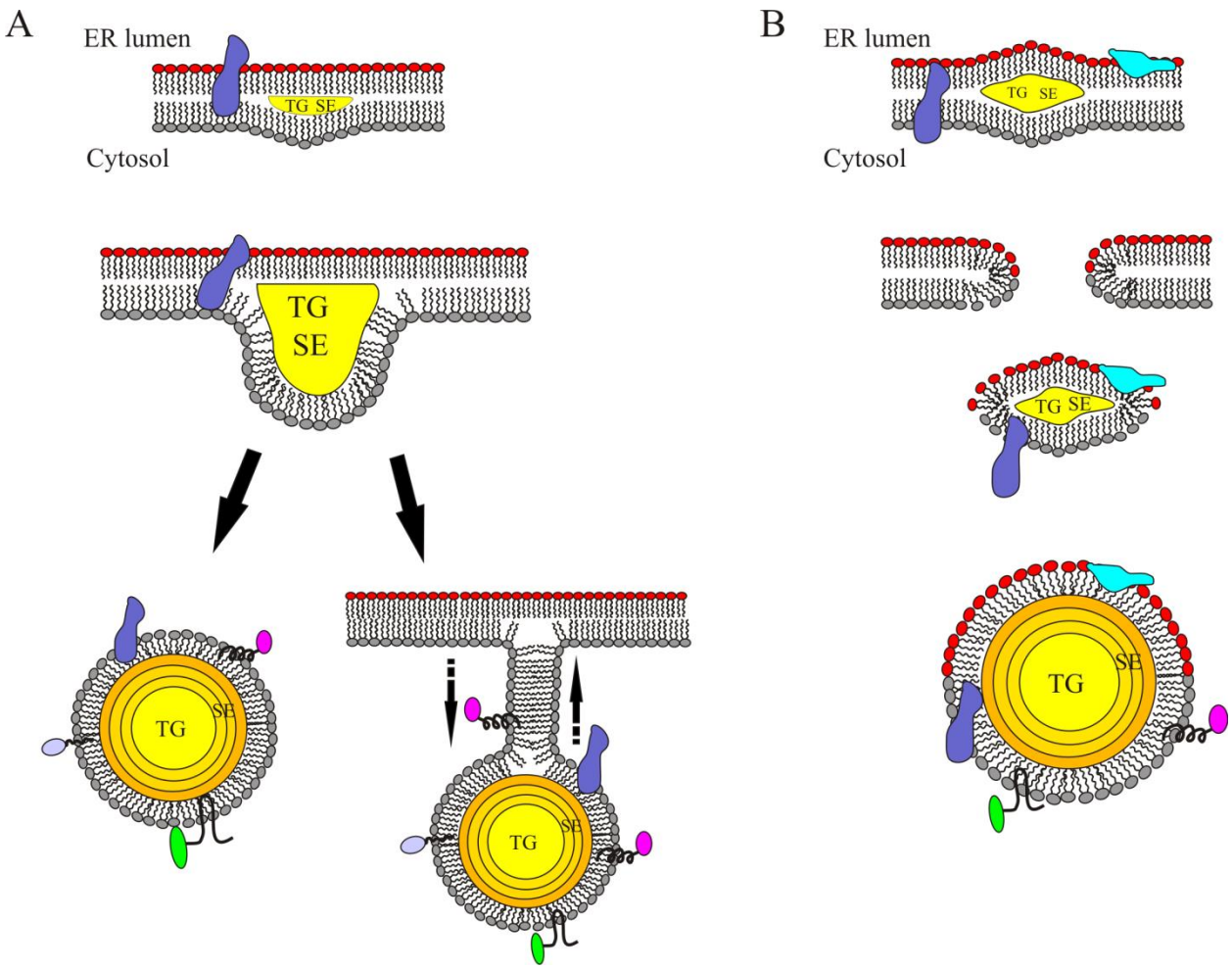

Fig. 4 Models of lipid droplet biogenesis. A: TG accumulates between the two leaflets of the ER membrane. After reaching a critical size, the growing LD buds off the ER membrane. Continuities between the ER membrane and the LD might deliver LD proteins from the ER to their destination. According to this model, the phospholipid monolayer of the LD is derived from the outer leaflet of the ER membrane. B: The bicell formation model also suggests TG accumulation between the two leaflets of the ER, but the phospholipid monolayer of the LD contains components of the inner and outer leaflet of the ER membrane. SE, steryl esters; TG, triacylglycerol. Adapted from Guo *et al.* (2009), Murphy and Vance (1999), and Czabany *et al.* (2008).

FIGURE 5

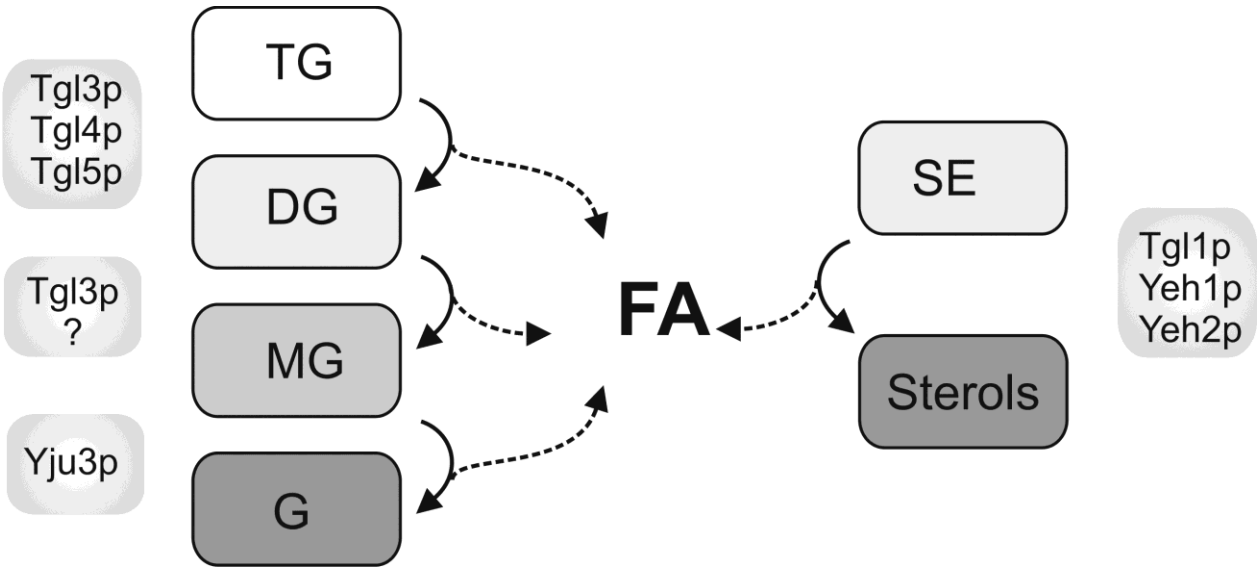

Fig. 5 Mobilization of triacylglycerols and steryl esters in the yeast *S. cerevisiae.* TG is degraded in a cascade of hydrolytic reactions. Tgl3p, Tgl4p and Tgl5p catalyze the hydrolysis of TG to DG and FA. Tgl3p is also a DG lipase. Other DG lipases of the yeast have not yet been identified. Yju3p catalyzes hydrolysis of MG to glycerol and FA. Tgl1p, Yeh1p and Yeh2p are SE hydrolases of *S. cerevisiae* catalyzing the hydrolysis of SE to sterols and FA. DG, diacylglycerol; FA, fatty acids; G, glycerol; MG, monoacylglycerol; SE, steryl esters; TG, triacylglycerol

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CHAPTER II

Analysis of Yeast Lipid Droplet Proteome and Lipidome

Claudia Schmidt[1], Birgit Ploier[1], Barbara Koch and Günther Daum*

Institute of Biochemistry, Graz University of Technology, Austria

[1]Contributed equally to this manuscript and should be considered as co-first authors

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

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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 & Daum, 2006; Zweytick, Athenstaedt, & Daum, 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, Tgl4 and Tgl5, respectively (Athenstaedt & Daum, 2003, 2005; Zimmermann et al., 2004), enzymes of sterol biosynthesis (Leber et al., 1998; Milla et al., 2002; Zinser, Paltauf, & Daum, 1993; Caldas & Herman, 2003; Ohashi, Mizushima, Kabeya, & Yoshimori, 2003; van Meer, 2001), or more specifically seipin in mammalian cells and Fld1p in yeast (Fei et al., 2008; Fei, Du, & Yang, 2011; Wolinski, Kolb, Hermann, Koning, & Kohlwein, 2011). Also in plant LD certain enzymes of lipid metabolism were detected (for review see Baud & 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, Zweytick, Jandrositz, Kohlwein, & Daum, 1999; Czabany, Athenstaedt, & Daum, 2007; Leber, Zinser, Zellnig, Paltauf, & Daum, 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 & 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 & 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, Wang, Fu, Bielby, & Yang, 2009; Fujimoto, Ohsaki, Cheng, Suzuki, & Shinohara, 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, Connerth, Zellnig, & Daum, 2009). This effect is only one example for 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 for 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^{TM} (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, Rosebrough, Farr, and Randall (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^{TM}, 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^{TM} 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^{TM} 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^{TM} 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^{TM}LC/MALDI Insert metal target (ABSciex). Mass spectra are acquired on a 4800 TOF/TOF^{TM} 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 be almost 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, Lees, and Sloane Stanley (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 is 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 for various organelles. A Western Blot analysis using a typical set of antibodies directed against marker proteins from *S. cerevisiae* is shown in Fig. 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.

However, due to the tight interaction and contacts of LD to other organelles like the ER, mitochondria and peroxisomes (Kohlwein et al. 2013) on 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 (Grillitsch et al., 2011; Fei et al., 2011; Ivashov et al., 2012; Athenstaedt et al., 2006). 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. (2011) 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, Kimmel, Londos, & Hurley, 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, Zweytick, Leber, Turnowsky, & Daum, 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. Fig. 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 Fig. 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 Fig. 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 Fig. 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 Fig. 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.

Phospholipids of LD are usually separated by two-dimensional TLC and analyzed as described in the Methods section. Fig. 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.

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* (Fig. 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.

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 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 & Aggelis, 2002).

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 is 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.

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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.

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Tables

Table 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 µg/µg protein	*P. pastoris* µg/µg protein	*Y. lipolytica* 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

FIGURE 1

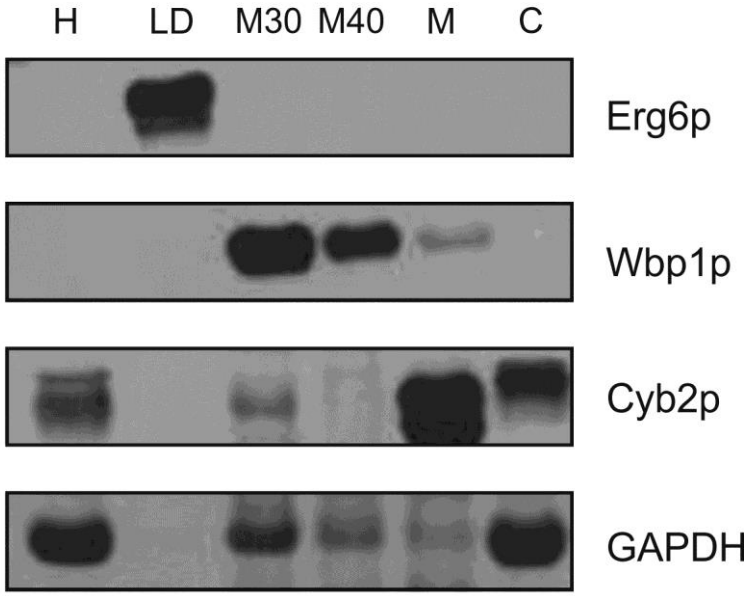

Fig. 1 Quality control of lipid droplets isolated from *S. cerevisiae.* Cells were grown to the stationary phase on glucose medium. Western blot analysis of 10 µg protein from each fraction was performed as described in the Methods section. Homogenate (H), LD (LD), 30,000xg microsomes (M30), 40,000xg 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 b_2; L-lactate cytochrome-c oxidoreductase; mitochondrial marker), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; cytosolic marker).

FIGURE 2

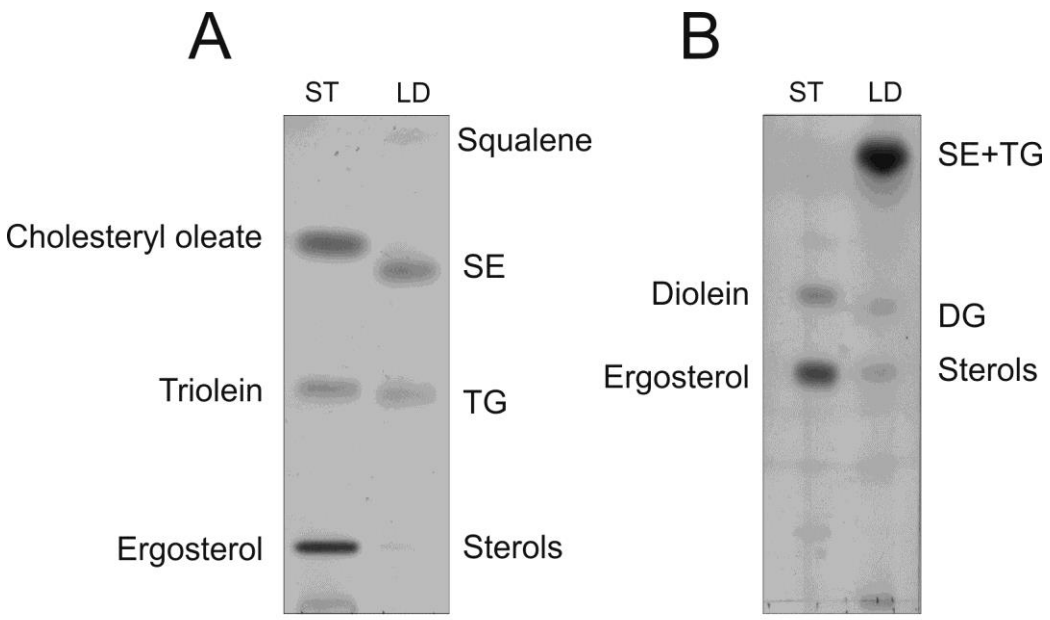

Fig. 2 Non-polar lipid analysis of lipid droplets from *S. cerevisiae.* For the TLC analysis of non-polar lipids different solvent systems were used (see Methods section). Cells were grown to the stationary phase on glucose medium. Lipids were extracted and separated using light petroleum/diethyl ether/acetic 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.

FIGURE 3

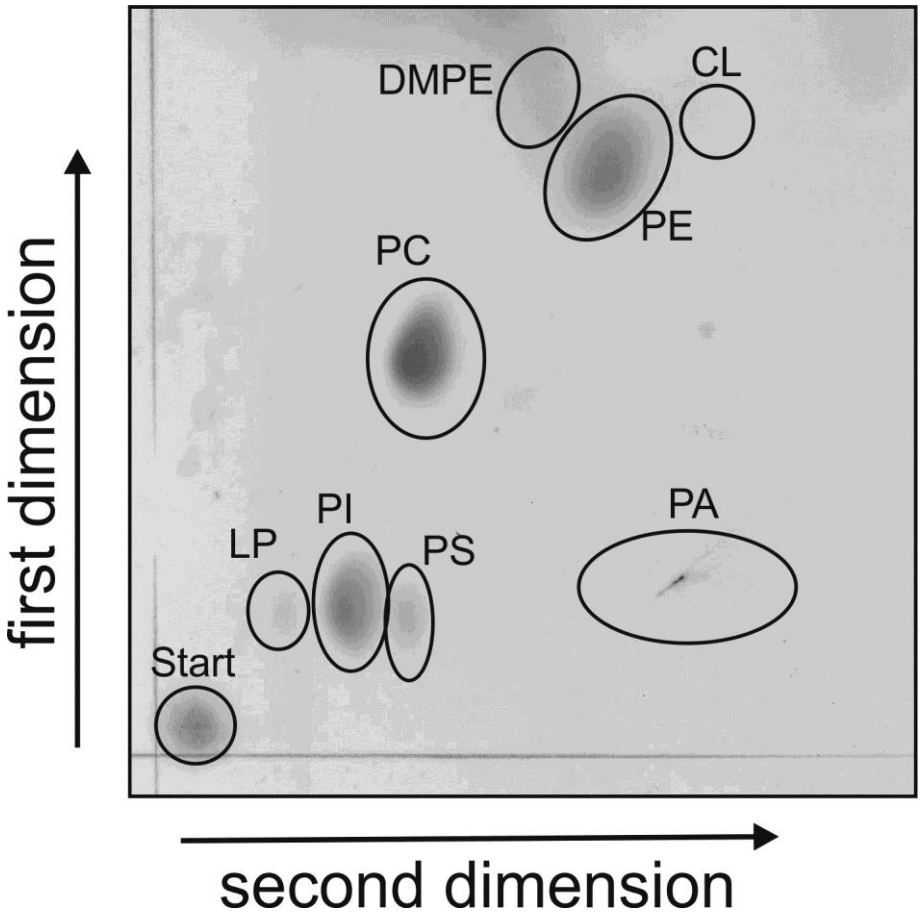

Fig. 3 Phospholipid analysis of lipid droplets from *S. cerevisiae.* Two dimensional TLC of individual phospholipids from *S. cerevisiae* LD was performed as described in the Methods section. Cells were grown to the stationary phase on glucose medium. Lipids were extracted and separated using chloroform/methanol/25% 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.

FIGURE 4

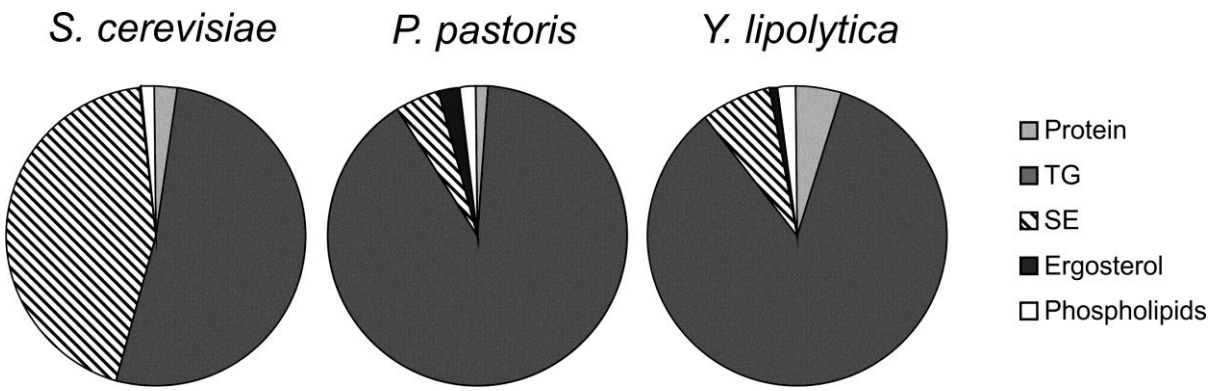

Fig. 4 Major components of lipid droplets from *S. cerevisiae, P. pastoris* and *Y. lipolytica.* Data were obtained from Athenstaedt et al. (2006); Ivashov et al. (2012) and Leber et al., (1994). Relative amounts of TG (triacylglycerols), SE (steryl esters), ergosterol, phospholipids and protein were calculated according to analytical data.

CHAPTER III

Modification of the C-terminus Affect Functionality and Stability of Yeast Triacylglycerol Lipase Tgl3p

Barbara Koch, Claudia Schmidt, Birgit Ploier and Günther Daum

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

[#]To whom correspondence should be addressed: Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria; Tel.: +433168736462; Fax: +433168736952; E-mail: guenther.daum@tugraz.at

Key words: Lipid droplets, triacylglycerol, lipase, topology, *S. cerevisiae*

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Abstract

Lipid droplets are specific organelles for the storage of triacylglycerols and steryl esters. They are surrounded by a phospholipid monolayer with a small but specific set of proteins embedded. Assembly and insertion of proteins into this surface membrane is an intriguing question of lipid droplet biology. To address this question we studied the topology of Tgl3p, the major triacylglycerol lipase of the yeast Saccharomyces cerevisiae, on lipid droplets. Employing the method of limited proteolysis of lipid droplet surface proteins we found that the C-terminus of Tgl3p faces the inside of the organelle, whereas the N-terminus is exposed at the cytosolic side of lipid droplets. Detailed analysis of the C-terminus revealed a stretch of seven amino acids which are critical for protein stability and functionality. The negative charge of two aspartate residues within this stretch is crucial for lipase activity of Tgl3p. A portion of Tgl3p which is located to the endoplasmic reticulum exhibits a different topology. In the phospholipid bilayer of the endoplasmic reticulum the C-terminus faces the cytosol which results in instability of the protein. Thus, the topology of Tgl3p is important for its function and strongly dependent on the membrane environment.

Introduction

All types of eukaryotic cells such as plant, mammalian, and non-mammalian cells and even some prokaryotes belonging to the Gram positive genera such as Rhodococcus or Streptomyces contain intracellular lipid droplets (LD[1]) (1). These organelles are specialized to store non-polar lipids such as triacylglycerols (TG) and steryl esters (SE). TG and SE are low-volume and high-energy reserve materials serving as an internal depot for sterols and fatty acids, which are important building blocks for membrane biogenesis and a source of cellular energy. Although LD show significant variation in size, shape and composition in various cell types, they appear to have a general and very special structure. LD of the yeast Saccharomyces cerevisiae are small spherical organelles with an approximate diameter of ~400 nm (2). They consist of 95% non-polar lipids with approximately equal amounts of TG and SE, each. TG and SE seem to be ordered instead of randomly distributed with TG forming the inner core of the LD which is surrounded by several shells of SE most likely with some TG intercalated. In contrast to other organelles, the surface of LD is covered by a phospholipid monolayer (3).

Several proteome analyzes from *S. cerevisiae* identified a small set of about 60 proteins on the surface of LD which can adjust to different growth conditions (4, 5). Among the prominent LD proteins, the major TG lipases of the yeast, Tgl3p, Tgl4p and Tgl5p were identified (6, 7). All three lipases share the conserved GXSXG lipase motif and a patatin domain instead of a typical α/β hydrolase fold (8). The patatin domain not only differs in the secondary structure from a canonical α/β hydrolase fold, but also in the catalytic site of the enzyme (9). The active center of patatin domain containing lipases consists of a serine-aspartate dyad, whereas the active site of a typical lipase with an α/β hydrolase fold comprises a catalytic serine-aspartate-histidine triad.

Tgl3p shows the highest lipolytic activity of the three major TG lipases in the yeast, and a deletion of *TGL3* causes accumulation of TG up to 200% compared to wild type cells (7). Interestingly, previous work from our group showed that Tgl3p also harbors an H-XXXX-D acyltransferase motif and also functions as lysophosphatidylethanolamine acyltransferase (8). A *tgl3*Δ strain exhibits a reduced amount of total phospholipids which may be regarded as proof for the role of Tgl3p as acyltransferase in vivo. Multiple enzymatic activities were also shown for Tgl4p and Tgl5p. Tgl5p acts also as a lysophospholipid acyltransferase; and Tgl4p, the functional orthologue of the mammalian adipocyte triacylglycerol lipase (ATGL), is a

[1] Abreviations used are: LD, lipid droplets; ER, endoplasmic reticulum; PK, proteinase K; QM, quadruple mutant; SE, steryl esters; TG, triacylglycerols

multifunctional enzyme exhibiting SE hydrolase, sn2-specific PLA2 and acyl-CoA dependent lysophospholipid acyltransferase activities in vitro (10, 11). Most recently, it was demonstrated that Ayr1p, previously identified as an NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase, also acts as TG lipase (12, 13). These findings clearly illustrate the complex and not yet sufficiently elucidated dynamic network of lipolytic enzymes in the yeast.

During the last decade, our fundamental knowledge about LD constantly increased. However, several important questions remained open, especially those concerning the biogenesis and the assembly of LD. It has been hypothesized that N- or C-terminal hydrophobic stretches of LD proteins are responsible for targeting and anchoring these polypeptides to LD. Removal of hydrophobic C-terminal stretches of the LD proteins Erg1p, Erg6p and Erg7p disturbed the targeting of these proteins to the LD and led to their accumulation in the ER (14). However, C-terminal stretches of the respective proteins were not sufficient to direct a GFP-hybrid to LD. Thus, our knowledge of targeting and anchoring of proteins to LD is still limited. Interestingly, several LD proteins including Tgl3p show a dual localization in LD and in the ER (15–17). This is surprising on one hand, because these proteins have to assemble in two different membrane environments, namely a phospholipid monolayer in LD and a phospholipid bilayer in the ER. How proteins adapt to these two different membrane environments is not known yet. On the other hand, the dual localization of these proteins in LD and ER can be explained by the close relationship of these two compartments (18).

In the present work, topological aspects of Tgl3p, a typical representative of LD proteins, were studied in some detail at the molecular level. The specific roles of N- and C-termini of the protein were addressed with special emphasis on the stability and functionality of Tgl3p. We show that the C-terminus contains small stretches of amino acids whose presence or absence decides about the fate of the protein. The molecular link between topology and functionality of Tgl3p is discussed.

Experimental procedures

Strains and culture conditions

All yeast strains used in this study are listed in Table 1. Cells were grown aerobically to either logarithmic or stationary growth phase at 30°C in YPD media containing 1% yeast extract, 2% glucose and 2% peptone. Plasmid containing yeast strains were cultured in minimal media containing 0.67% yeast nitrogen base (U.S. Biochemical Corp.), 2% glucose and the respective amino acid supplements. Growth was monitored by measuring OD_{600}.

Genetic Techniques

All primers and plasmids used in this study are listed in Tables 2, 3 and 4. 500 bp of the promoter region and 300 bp of the terminator region of *TGL3* were cloned into a pRS315 plasmid. Insertion cassettes were obtained by PCR using genomic DNA from *S. cerevisiae*. The promotor region of *TGL3* was inserted by cleavage with NotI and BamHI, the terminator region by cleavage of PstI and HindIII. *TGL3* and all tagged and truncated variants of *TGL3* were obtained by PCR and inserted into the pRS315 plasmid containing the promoter and terminator region with BamHI and PstI. All constructs were confirmed by sequencing and then transformed into *S. cerevisiae* BY4741 *tgl3*Δ employing the high efficiency lithium acetate transformation protocol (19). After transformation, cells were plated on minimal media lacking the respective amino acids for selection and incubated for 2-3 days at 30°C.

Isolation and characterization of subcellular fractions

Yeast LD and microsomal fractions were obtained at high purity from cells grown to the stationary phase according to published procedures (20, 21). The protein concentration of the isolated fractions was analyzed by the method of Lowry et al. (22) using bovine serum albumin as a standard. Prior to protein quantification, samples of LD fractions were delipidated with 2-3 volumes of diethyl ether. The organic phase was removed and samples were dried under a stream of nitrogen. Then, proteins were precipitated with trichloroacetic acid at a final concentration of 10% and solubilized in 0.1% SDS, 0.1% NaOH.

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (23) using 10%, 12.5% or 15% separation gels. Western blot analysis was performed as described by Haid and Suissa (24) using the following antibodies: Anti-HA-Peroxidase conjugated, High Affinity (3F10) (Roche) (1:1,000); Anti-c-Myc antibody monoclonal (9E10) Sigma-Aldrich produced in mouse (1:1,000); Anti-Ayr1 (produced in rabbit in house) (1:5,000); Anti-Wbp1 (1:5,000) (produced in rabbit and kindly provided by H. Pichler); secondary anti-rabbit

horseradish peroxidase-conjugated antibody (Sigma-Aldrich) (1:15,000); secondary anti-mouse horseradish peroxidase-conjugated antibody (Sigma-Aldrich) (1:15,000). Proteins were visualized using an enhanced chemiluminescence detection substrate (Thermo Scientific) following the manufacturer's instructions.

Preparation of total cell extracts for lipid analysis and thin layer chromatography (TLC)

Yeast cells were grown to the stationary growth phase and an equivalent of 30 OD_{600} units was harvested. Cells were disintegrated by vigorous shaking (Vortex) in the presence of glass beads for 10 min at 4°C, and lipids were extracted as described by Folch et al. (25) using chloroform/methanol (2:1; v/v). For quantification of TG, lipid extracts were applied to Silica Gel 60 plates. Chromatograms were developed in an ascending manner by a two-step developing system. First, light petroleum/diethyl ether/acetic acid (35:15:1, per vol.) was used as mobile phase, and plates were developed to 2/3-length of the plate. Then, plates were dried briefly and further developed to the top of the plate using the second mobile phase consisting of light petroleum/diethyl ether (49:1, v/v). Separated bands were visualized by irreversible staining. For this purpose the TLC plates were dipped into a charring solution consisting of 0.63 g MnCl2*4 H_2O, 120 mL water, 120 mL methanol, and 8 mL concentrated sulfuric acid, and incubated in a heating chamber at 105°C for at least 30 min. Bands were quantified by densitometric scanning at 400 nm using a CAMAG TLC Scanner 3.

Proteinase K protection assay

LD were isolated as described above and samples corresponding to 20 µg LD protein were used for Proteinase K treatment. Proteinase K (Roche) was added to a ratio of 1:100 and the incubation was carried out on ice. As control experiments, LD were solubilized in the presence of 1% Triton-X100. The reaction was stopped by adding trichloroacetic acid at a final concentration of 15% and vigorous vortexing. Proteins were precipitated for 1 h and centrifuged in a table top centrifuge at 4°C for 30 min. The protein pellet was washed with ice-cold water and centrifuged again. Aliquots were used for Western Blot analysis as described above.

Results

A Proteinase K protection assay reveals the orientation of the C- and N-terminus of Tgl3p.

LD are the only organelles of the cell which are covered by a phospholipid monolayer. This fact raises the intriguing question how proteins are inserted into and/or anchored to the surface membrane of this compartment. To address this question we designed an assay for studying the topology of one prominent LD protein, the TG lipase Tgl3p. This assay is based on limited proteolysis of surface components with proteinase K (PK) which allowed us to test for the orientation of N-terminus and C-terminus of Tgl3p in the phospholipid monolayer membrane of LD. For this purpose we constructed a double tagged variant of Tgl3p carrying an N-terminal Myc-tag and an HA-tag at the C-terminus (Figure 1A). This construct was cloned into a low copy vector and transformed into a *tgl3*Δ deletion strain. The functionality of the constructed Myc-Tgl3p-HA hybrid was verified by comparing the TG content of a *tgl3*Δ strain carrying an untagged version of *TGL3* to a *tgl3*Δ mutant and the strain harboring the double tagged variant of Tgl3p. As Tgl3p is the major TG lipase in *S. cerevisiae*, deletion of *TGL3* resulted in a marked TG accumulation compared to wild type (Figure 1B). The strain carrying the Myc-Tgl3p-HA protein did not accumulate TG and thus behaved like a wild type strain. Conclusively, the tags did not affect the functionality of Tgl3p, and PK protection assays were carried out with this construct. As an additional control, the functionality of Tgl3p variants tagged only at the N-terminus or C-terminus were tested. As can be seen from Figure 1B both variants were fully functional and exhibited TG levels similar to wild type.

The effect of PK treatment on LD surface proteins from isolated LD was monitored at several time points after stopping proteolysis by addition of TCA. Samples were then analyzed by Western Blots using antibodies directed against the N- and C-terminal tags of Tgl3p. Figure 1C shows a characteristic experiment of this type. As can be clearly seen, the N-terminus of Tgl3p was easily accessible to PK. After 2.5 min hardly any signal of the N-terminus of Tgl3p was detectable. This result indicated that the N-terminus of Tgl3p faces the cytosolic side of LD protruding into the cytosolic environment.

Western Blot analysis with Myc-tagged Tgl3p as described above also detected a truncated version of the protein (see Figure 1C). Whether this additional band was an unspecific fragment arising during LD purification or result of specific proteolytic cleavage is currently not known. Interestingly, similar results were obtained with N-terminally HA-tagged Tgl2p, a mitochondrial resident lipase (26). As full length and truncated Myc-Tgl3p were degraded by PK with similar efficiency (see Figure 1), and data of this experiments served to demonstrate the instability of the N-terminus of Tgl3p, results were not biased.

In contrast to the N-terminus, the C-terminus of Tgl3p was protected from digestion by PK. During incubation with the proteinase, several smaller fragments of Tgl3p appeared gradually. After 20 min two stable protein bands were still visible, indicating that PK did not have access to the 20 kD fragment of the C-terminus (Figure 1D). Thus, we conclude that the C-terminus of Tgl3p is protected from degradation by membranes and orientated towards the inside of the organelle. Control experiments with LD samples solubilized with Triton X-100 revealed that N-terminally as well as C-terminally tagged variants of Tgl3p were completely digested by PK in the presence of the detergent. This observation indicated that the folding of the two termini per se did not affect the obtained results.

As a control for incubation conditions, we chose to test the effect of PK treatment on Erg6p, a Δ(24)-sterol C-methyltransferase (Figure 1E); and Ayr1p, an NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase which was recently identified as another TG lipase of *S. cerevisiae* (12, 13) (Figure 1F);. Both proteins had been identified before as typical LD proteins. A small part of Erg6p seemed to be accessible to PK, whereas Ayr1p was not affected at all after 20 min of PK treatment. Obviously, these two LD proteins are rather deeply embedded in the phospholipid monolayer of LD and therefore protected against the PK treatment.

C-terminal truncations of TGL3 result in protein instability

The finding, that the C-terminus of Tgl3p was inaccessible to proteolysis with PK led us to speculate that this part of the protein might be important for anchoring and/or targeting of the protein to LD. It had been shown before that C-terminal hydrophobic stretches of LD proteins were responsible for their association with the organelle (14). To test this hypothesis for Tgl3p, we constructed three different C-terminally variants of N-terminally HA-tagged Tgl3p which were truncated of 5 kD (43 amino acids), 2.4 kD (20 amino acids) and 1 kD (10 amino acids), respectively (Figure 2A). LD and microsomal fractions were isolated from the respective strains, and the subcellular distribution of the truncated Tgl3p variants was compared to the full length protein (Figure 2B). Full length Tgl3p and all Tgl3p variants were detected through their N-terminal HA-tag. The ER resident protein Wbp1p was used as an ER marker, and Ayr1p was analyzed as a typical LD protein. As already shown before (17), full length HA-Tgl3p showed a dual localization with the majority of the protein found in LD and a small amount in the 30,000 g and 40,000 g microsomal fractions. Surprisingly, all three C-terminally truncated variants of HA-Tgl3p could not be detected at all under standard Western Blot conditions. Only overloading samples 7-fold revealed minimal amounts of truncated

Tgl3p proteins present in LD (Figure 2C). Loss of stable Tgl3p also resulted in a massive loss of Tgl3p function as strains harboring the truncated variants of Tgl3p accumulated TG (Figure 2D).

As all truncated variants of Tgl3p were present in a low copy vector with the endogenous promoter and terminator region, we also tested whether the loss of protein was due to insufficient gene expression. Therefore, the respective mRNA levels were quantified by RT-PCR. As can be seen from Figure 2E, mRNA levels of truncated Tgl3p variants were rather increased than reduced. Thus, decreased transcription efficiency was not the reason for the low protein levels. To further test the possible influence of the lipid/membrane environment on the stability of the protein, we expressed the truncated variants of Tgl3p in the QM strain. In this strain, wild type Tgl3p associated only with the phospholipid bilayer of the ER (Figure 2F). As truncated variants of Tgl3p were equally unstable in the QM and in wild type we concluded that the protein degradation occurred immediately after the translation process. Taken together, our results suggested that C-terminal truncations of Tgl3p caused instability of the protein. Even minimal truncations, e.g. 1 kD, were obviously sufficient to compromise the protein severely. As minor amounts of Tgl3p were still found in the LD, we concluded that the C-terminus was rather involved in protein stability and anchoring than targeting of the protein to LD.

Effect of single and double amino acid exchanges in the C-terminus on stability and functionality of Tgl3p

Experiments described above demonstrated that 10 amino acids of the C-terminus are important and critical for the stability of Tgl3p. Therefore, we wished to go into further detail and dissect the role of this part of the protein. First, we removed the last six C-terminal amino acids of Tgl3p (RARRSR) and tested distribution of the protein between LD and microsomal fractions (Figure 3, A and B). These experiments showed that removing the last six amino acids of the protein led to reduction of the amount but did not change targeting of the protein to LD. Also the TG content of this mutant was similar to wild type with only minor accumulation of TG, which may be due to the reduced amount of Tgl3p (Figure 3C). As a second step to elucidate the role of the C-terminus we deleted the amino acids FKLDDII which overlap the 1 kD and 2.4 kD truncation points (Figures 2A and 3A). Interestingly, deletion of these seven amino acids resulted in a highly unstable protein and, not surprisingly, in TG accumulation (Figure 3, B and C).

As a last step of molecular investigations we wished to clarify the role of individual amino acids within the FKLDDII region. For this purpose, we constructed Tgl3p variants harboring the amino acid exchanges F630A, L632A, D633A/D634A, and I635A/I636A; and analyzed microsomal and LD fractions from these strains (Figure 4, A and B). Interestingly, the protein stability of all Tgl3p constructs bearing single and double amino acid exchanges was the same as full length HA-Tgl3p. Also the localization of the Tgl3p variants was not altered. Surprisingly, however, HA-Tgl3p D633A/D634A showed TG accumulation comparable to a *tgl3*Δ deletion strain, whereas all other amino acid exchanges had no effect on Tgl3p functionality (Figure 4C). Thus, HA-Tgl3p D633A/D634A was the only mutation which compromised activity of Tgl3p but not stability. To test whether both aspartates or only one were responsible for the loss of lipase activity, we also constructed Tgl3p bearing D633A or D634A single amino acid exchanges and compared the TG content with a *tgl3*Δ strain. Interestingly, lipase activity of Tgl3p D633A and Tgl3p D634A was the same as wild type (Figure 4D). Consequently, both aspartates in combination are important and essential for the lipase activity of Tgl3p.We further speculated whether the loss of the negative charge caused by the aspartate to alanine exchange was the reason for the loss of lipase activity. Therefore, we exchanged the two aspartates against two glutamates to conserve the negative charge. As can be seen from Figure 4E, the Tgl3p D633E/D634E variant displayed lipase activity, although at a slightly reduced level. Thus, it appears that the negative charge(s) in the C-terminus are crucial for TG lipase activity of Tgl3p.

The N-terminus of Tgl3p is less sensitive to truncations than the C-terminus

Experiments described above demonstrated that the C-terminus of Tgl3p, which is protected by the surface membrane of LD is critical for stability and activity of the protein. In contrast, the N-terminus of Tgl3p was easily digested by PK and thus faces the cytosol (see Figure 1C). To test possible effects of N-terminal truncations on targeting and stability of Tgl3p we constructed strains carrying 1 kD, 2 kD, and 5 kD N-terminal truncations of Tgl3p and performed localization analysis (Figure 5, A and B). All constructs, including the full length Tgl3p control carried C-terminal HA-tags. As can been seen from Figure 5B, targeting of the protein to LD was not affected by all N-terminal truncations. The stability of the 1 kD, 2 kD and 5 kD N-terminal truncated variants of Tgl3p was only slightly reduced. The TG levels of the strains carrying the 1 kD and 2 kD truncations were comparable to wild type, indicating that the lipase activity was not affected (Figure 5C). The 5 kD truncation of the N-terminus of Tgl3p, however, resulted in significant loss of activity (Figure 5C).

The C-terminus of Tgl3p is facing the cytosolic side when inserted into the endoplasmic reticulum

Schmidt et al. (17) showed that a small portion of Tgl3p is found in the ER of wild type cells. In the absence of LD, i.e. in an *lro1*Δ*dga1*Δ*are1*Δ*are2*Δ quadruple mutant (QM) strain (27), localization of Tgl3p was completely restricted to the ER although in an inactive form. Furthermore, it was shown that the stability of Tgl3p was strongly reduced in the ER of the QM, whereas Tgl3p in LD from a wild type strain was very stable. However, the reason for the short half-life of Tgl3p in the QM remained unclear, and we speculated that inefficient assembly of Tgl3p into the bilayer of the ER in contrast to embedment of the protein in the monolayer membrane of LD might cause this problem. To test this hypothesis we isolated microsomes from the QM and analyzed the topology of Tgl3p in this compartment by PK assays with emphasis on the role of the C-terminus of the enzyme. For these experiments we used C-terminally Myc-tagged Tgl3p (Figure 6). Wpb1p and Kar2p were used as ER markers to ensure the integrity of the microsomal vesicles. Kar2p, a prominent luminal ER protein, was only digested by PK in the presence of detergent. Similarly, Wbp1p, a type I membrane protein with a large luminal domain was only degraded when Triton X-100 was added during incubation with the protease (28). These control experiments confirmed the integrity of microsomal vesicles in a right-side-out orientation. As can be seen from the Western Blot, the C-terminus of Tgl3p was easily degraded by PK indicating its exposure to the cytosol. This orientation of the C-terminus of Tgl3p in the ER is in sharp contrast to LD. As the C-terminus of Tgl3p is crucial for its stability as demonstrated by the above mentioned experiments, we conclude that exposure of this part of the protein to the environment in ER fractions may be the reason for the short half-life of Tgl3p in this compartment.

Discussion

Tgl3p, the major TG lipase from *S. cerevisiae* plays a critical role in TG mobilization and also contributes to phospholipid metabolism through its lysophospholipid acyltransferase activity (8). Recently, Schmidt et al. (17) studying regulatory aspects of this lipase showed that the activity of Tgl3p was strongly influenced by its localization, and localization depended on substrate availability. Our work presented in this study extends these investigations to the topology of Tgl3p which led to the identification of protein domains that are critical for the stability and activity of Tgl3p.

Proteinase K (PK) protection assays using tagged variants of Tgl3p revealed that the N-terminus of the protein faces the cytosolic site of the LD, whereas a ~20 kD stretch of the C-terminus appears to be protected by the surface membrane of the organelle. Surprisingly, in silico analysis demonstrated that the C-terminus of Tgl3p is not explicitly hydrophobic and therefore not typical for insertion into membranes. The question remains how such a non-hydrophobic domain can be inserted into a membrane or even protrudes into the highly hydrophobic core of LD. Several mechanisms describing the assembly of proteins in the LD surface have been discussed, but all of them propose direct anchoring of the proteins via hydrophobic helices (29, 30). Thus, the possibility remains that Tgl3p might be associated with a partner protein which allows assembly in the form of a complex into the LD surface. Interestingly, Ayr1p has been identified among some other proteins as a possible partner protein of Tgl3p in a split GFP screening (31). Currently, however, there is no further experimental evidence supporting the view of an interaction of Tgl3p with Ayr1p, and formation of LD surface complexes containing Tgl3p remains pure speculation at present. Our results obtained with Ayr1p (see Figure 1F) suggest that this protein is deeply embedded within the LD. PK got only access to Ayr1p when LD were incubated at 37°C and vigorously vortexed during the treatment (data not shown). These results obtained with Ayr1p are in sharp contrast to Tgl3p. Also Erg6p, another typical LD protein seems to be largely inaccessible to PK treatment (Figure 1E). This result is in line with previous studies from our lab (14) which showed that removal of C-terminal hydrophobic domains of Erg6p abolished LD association of the protein. Conclusively, it appears that different types of LD proteins with different hydrophobicity exist, which may result in different topology of these proteins at the LD surface.

Results discussed above led us to speculate that the C-terminus of Tgl3p may play an important role in targeting of the protein to LD. To test this hypothesis we performed experiments with three different C-terminally truncated variants of Tgl3p (see Figure 2, A-C). These experiments, however, indicated that the C-terminus of Tgl3p was rather involved in protein stability than in the targeting of the protein to LD. All truncated variants were found to be rather unstable, but residual amounts of the protein were found in LD. We expressed the truncated variants of Tgl3p in the QM strain to explore a possible influence of the membrane/lipid environment on protein stability. In this strain, wild type Tgl3p accumulated with the phospholipid bilayer of the ER due to the lack of LD (Figure 2F). As truncated variants of Tgl3p were equally unstable in the QM and in wild type we concluded that the protein degradation occurred immediately after the translation process. Further and more

detailed molecular analysis of the C-terminus of Tgl3p revealed a region of seven amino acids (FKLDDII) which seems to be critical for protein stability (see Figure 3, A-C). Interesting, single or double amino acid exchanges within this sequence did not affect stability (see Figure 4A and B). Most surprisingly we found that a double exchange of D633A/D634A led to an inactive Tgl3p protein and to a strain which accumulated TG like a *tgl3*Δ strain. This situation is unique insofar as despite correct targeting the lipase activity of this Tgl3p variant was severely compromised. Comparable results were obtained previously in our lab by Schmidt et. al. (17) who showed that Tgl3p mutants inactivated by site directed mutagenesis in the GXSXG motif targeted normally to lipid droplets. Thus, it appears that activity of Tgl3p is not required for correct targeting.

Previous in silico analysis had revealed with, that Tgl3p harbors more likely a patatin domain than a canonical α/β hydrolase fold of lipases. The patatin domain differs significantly from a classical hydrolase fold not only in the secondary structure but also in the organization of the active site. Instead of a catalytic triad build from a histidine, serine and aspartate or glutamate, a patatin domain active site is formed by a serine and aspartate dyad. Thus, it is tempting to speculate, that one of the aspartates D633 or D634 may be part of the catalytic dyade. However, there are arguments which speak against this view. First, an amino acid exchange of only one aspartate, either D633 or D634, respectively, had no effect on the lipase activity as TG accumulation was not observed in these strains (Figure 4D). Secondly, D633 and D634 are not located within the predicted patatin domain where one would usually expect the catalytic active serine – aspartate dyad. Interestingly, however, a double amino exchange of D633 and D634 of Tgl3p to glutamate residues rescued the lipase activity (Figure 4E). Thus, the negative charge(s) in this position seems to be important for the functionality of Tgl3p. As the 3-dimensional structure of Tgl3p is not yet available, the possibility of the above mentioned interaction of amino acid residues remains hypothetical.

In a previous study from or lab Schmidt et al. (17) had shown that the half-life of Tgl3p is dramatically reduced when the protein was located to the ER. Experiments described in the present study enabled us to explain this effect at the molecular level. Given the fact that the C-terminus of Tgl3p is very important for its stability, exposure of this part of the protein at the cytosolic side of the ER may be the reason for the instability of Tgl3p in this compartment. The membrane bilayer environment of the ER may cause the unfortunate topology of Tgl3p in this organelle, whereas the phospholipid monolayer of the LD surface seems to provide the more appropriate surrounding for the enzyme.

In contrast to the C-terminus, the N-terminus of Tgl3p is obviously less important for subcellular localization and functionality of the protein. The N-terminus of Tgl3p appears to protrude from the LD surface into the cytosol which makes it susceptible to degradation. However, truncations up to 5 kD at the N-terminus of Tgl3p are not harmful for protein stability. As all N-terminally truncated variants of Tgl3p were localized to LD, we conclude that at least 5 kD of the N-terminus do not contain targeting sequences to LD.

In summary, our study provides a detailed molecular insight into the membrane topology of Tgl3p, the major TG lipase of the yeast, with emphasis on the specific role of the C-terminus of the protein. We were able to narrow critical amino acid stretches important for stability and functionality of the protein down to seven or even two amino acids. Thus, to be or not to be an active lipase in the appropriate subcellular location seems to be a matter of minor changes in the primary sequence of Tgl3p.

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Table 3: Yeast strains used in this study

Strain	Genotype	Source
Wild type	BY4741 *Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
Δ*tgl3*	BY4741; *tgl3Δ::kanMX4*	Euroscarf
QM Δ*tgl3*	BY4741; *dga1Δ:: kanMX4 lro1Δ:: kanMX4 are1Δ:: kanMX4 are2Δ:: kanMX4 tgl3Δ::hisMX6*	This study

Table 2: Primers used in this study

The abbreviations used are as follows: Fw, forward; Rev, reverse; underlined small letters, restriction sites; italic letters, tags, start or stop codons.

Primer	Sequence (5'- 3')
Promotor Fw	aaaagcggccgcGCTCCCTTGTTTAATAGCTT
Promotor Rev	aaaaggatccGCTTAACGGCAACTCAAAG
Terminator Fw	aaaactgcagTATCGTTTCCACTTTTTTCTG
Terminator Rev	aaaagcttGACCGGTTTTGCAAAGGACG
Tgl3 HA (N-term) Fw	aaaaggatccATG*tacccatacgatgttcctgactatgcg*AAGGAAACGGCGCAGG
Tgl3 HA (C-term) Rev	aaaactgcagCTA*cgcatagtcaggaacatcgtatgggta*CCTACTCCGTCTTGCTCTTA
Tgl3 MYC (N-term) Fw	aaaaggatccATG*gaacaaaagctaatctccgaggaagacttg*AAGGAAACGGCGCAGG
Tgl3 Fw	aaaaggatccATGAAGGAAACGGCGCAGG
Tgl3 Rev	aaaactgcagCTACCTACTCCGTCTTGCTCTTA
1 kDa truncation (C-term)	aaaactgcag*cta*TAATTTAAATTCGACTGCAC
2.4 kDa truncation (C-term)	aaaactgcag*cta*AATTAAAGCTAGTGCGGGCC
5 kDa truncation (C-term)	aaaactgcag*cta*ACCTTCAATAATCCTTGTTAATG
1 kDa truncation (N-term)	aaaaggatcc*atg*GTGTCTGCTGTAATACCGACC
2 kDa truncation (N-term)	aaaaggatcc*atg*AAAAACTGGATACTGCGTGTAG
5 kDa truncation (N-term)	aaaaggatcc*atg*ATCACCGATATTTATTTCTTC
Deletion of RARRSR Rev	ttttctgcag*cta*TATTATGTCGTCTAATTTAAA
Deletion of FKLDDII Fw	aagacaagatgtgcagtcgaaAGAGCAAGACGGAGTAG
Deletion of FKLDDII Rev	ctactccgtcttgctctTTCGACTGCACATCTTGTC
Exchange of F630A Fw	GATGTGCAGTCGAAgcTAAATTAGACGAC
Exchange of F630A Rev	GTCGTCTAATTTAgcTTCGACTGCACATC
Exchange of L632A Fw	GTGCAGTCGAATTTAAAgcAGACGACATAATAAGAGC
Exchange of L632A Rev	GCTCTTATTATGTCGTCTgcTTTAAATTCGACTGCAC
Exchange of D633A Fw	TGTGCAGTCGAATTTAAATTAGcCGACATAATAAGAGCAAGACGGAGT
Exchange of D633A Rev	ACTCCGTCTTGCTCTTATTATGTCGgCTAATTTAAATTCGACTGCACA
Exchange of D634A Fw	TGTGCAGTCGAATTTAAATTAGACGcCATAATAAGAGCAAGACGGAGT
Exchange of D634A Rev	ACTCCGTCTTGCTCTTATTATGgCGTCTAATTTAAATTCGACTGCACA
Exchange of DD633/634AA Fw	TGTGCAGTCGAATTTAAATTAGcCGcCATAATAAGAGCAAGACGGAGT
Exchange of DD633/634AA Fw	ACTCCGTCTTGCTCTTATTATGgCGgCTAATTTAAATTCGACTGCACA
Exchange of II635/636AAFw	GTCGAATTTAAATTAGACGACgcagcaAGAGCAAGACGGAGTAGGTAG
Exchange of II635/636AARev	CTACCTACTCCGTCTTGCTCTtgctgcGTCGTCTAATTTAAATTCGAC

Table 3: Primers used for RT-PCR

The abbreviations used are as follows: Fw, forward; Rev, reverse

Primer	Sequence (5' – 3')
RT Act1-Fw	CCAGCCTTCTACGTTTCCATCCAAG
RT Act1-Rev	GACGTGAGTAACACCATCACCGGA
RT Tgl3-FW	GCCAACAATCCGAGCATAACGGAG
RT Tgl3-Rev	TGGTGCCAAGTATGGTCTCGCCA

Table 4: Plasmids used in this study

Name	Relevant information	Source
pRS315	CEN, *LEU2*,	Sikorsky and Hieter, 1989
pRS315-MYC-TGL3-HA	CEN, *LEU2*,	This study
pRS315-HA-TGL3	CEN, *LEU2*	This study
pRS315-HA-tgl3-1kD	CEN, *LEU2*, *TGL3* is N-Term HA tagged and C-term truncated (last 9 aa missing)	This study
pRS315-HA-tgl3-2.4kD	CEN, *LEU2*, *TGL3* is N-Term HA tagged and C-term truncated (last 20 aa missing)	This study
pRS315-HA-tgl3-5kD	CEN, *LEU2*, *TGL3* is N-Term HA tagged and C-term truncated (last 43 aa missing)	This study
pRS315-TGL3-HA	CEN, *LEU2*	This study
pRS315-1kD-tgl3-HA	CEN, *LEU2 TGL3* is C-Term HA tagged and N-term truncated (first 9 aa missing)	This study
pRS315-2kD-tgl3-HA	CEN, *LEU2*, *TGL3* is C-Term HA tagged and N-term truncated (first 18 aa missing)	This study
pRS315-5kD-tgl3-HA	CEN, *LEU2*, *TGL3* is C-Term HA tagged and N-term truncated (first 43 aa missing)	This study
pRS315-HA-tgl3-RARRSR	CEN, *LEU2*, *TGL3* is N-Term HA tagged and the last 6 aa (RARRSR) are missing	This study
pSR315-HA-tgl3-FKLDDII	CEN, *LEU2*, *TGL3* is N-Term HA tagged and the aa 630-636 (FKLDDII) are missing	This study
pRS315-HA-tgl3-DDAA	CEN, LEU2, *TGL3* is N-Term HA tagged and aa D633 D634 are both replaced by A	This study
pRS315-HA-tgl3-DDEE	CEN, LEU2, *TGL3* is N-Term HA tagged and aa D633 D633 are both replaced by E	This study
pRS315-HA-tgl3-D633A	CEN, LEU2, *TGL3* is N-Term HA tagged and aa D633 is replaced by A	This study
pRS315-HA-tgl3-D634A	CEN, LEU2, *TGL3* is N-Term HA tagged and aa D634 is replaced by A	This study
pRS315-HA-tgl3-IIAA	CEN, LEU2, *TGL3* is N-Term HA tagged and aa I635 I636 are both replaced by A	This study
pRS315-HA-tgl3-F630A	CEN, LEU2, *TGL3* is N-Term HA tagged and aa F630 is replaced by A	This study
pRS315-HA-tgl3-L632A	CEN, LEU2, *TGL3* is N-Term HA tagged and aa L632is replaced by A	This study
pRS315-TGL3-6xHA	CEN, *LEU2 TGL3* is C-Term 6xHA tagged	This study

Figure 1

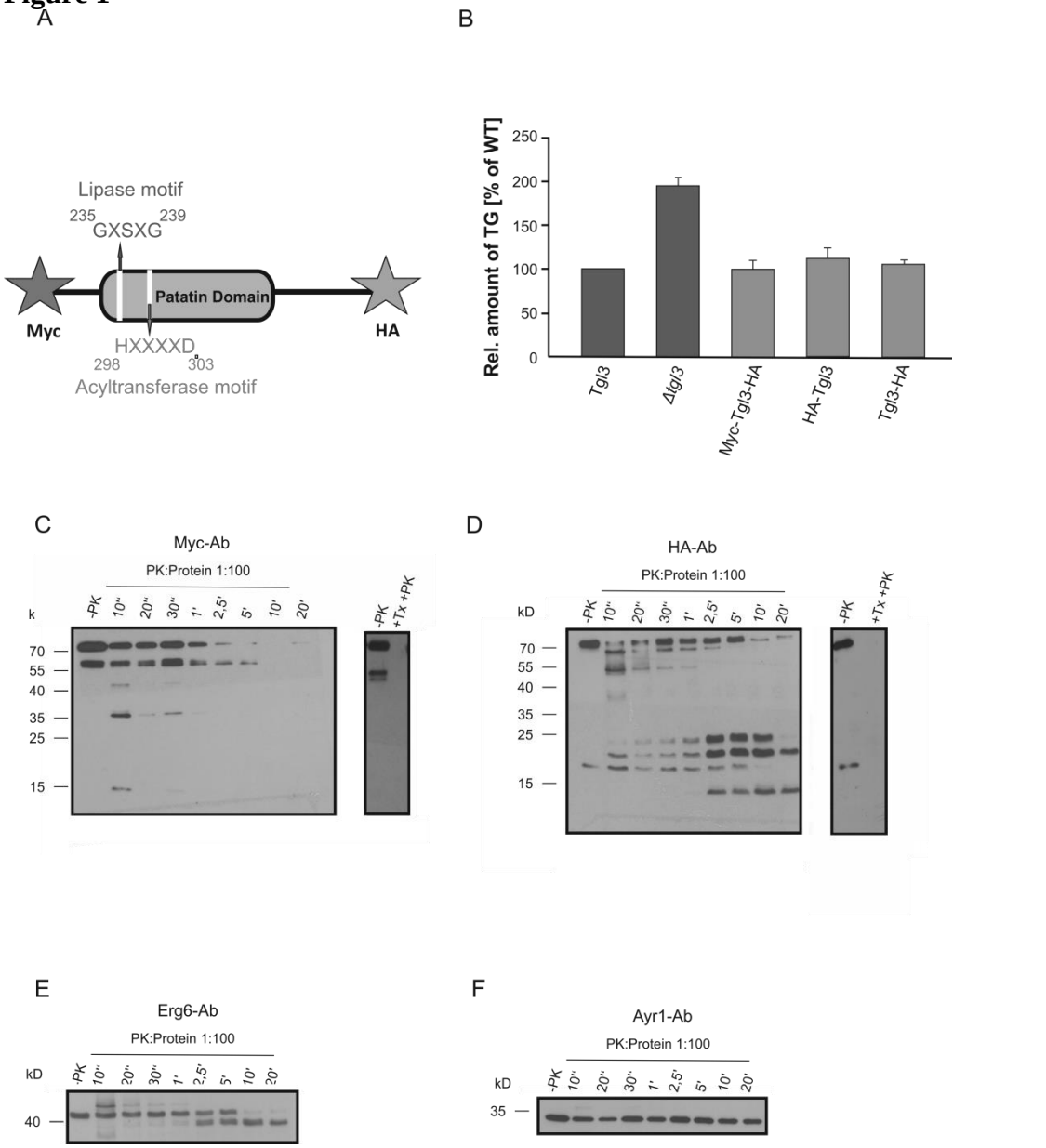

Figure 1. Proteinase K assay to determine the orientation of the N- and C- terminus of Tgl3p. (A) Scheme of Tgl3p showing the patatin domain, the conserved lipase motif and the acyltransferase motif. For detection of the N-terminus and C-terminus, a Myc-tag and an HA-tag were introduced by PCR. (B) Relative amounts of triacylglycerols (TG) in *tgl3*Δ and a *tgl3*Δ strain carrying untagged *TGL3*, the double tagged version of *TGL3*, and N-terminally HA or C-terminally HA tagged *TGL3* are shown. Cells grown to the stationary phase were analyzed. Results are the average from at least three independent experiments with S.D. values (error bars) as indicated. (C and D) Proteinase K protection assays were performed as described in the Methods section with isolated LD from a strain carrying the N-terminal Myc-tagged and C-terminal HA-tagged variant of Tgl3p. Samples were taken at time points from

10 sec to 20 min, and 10 µg LD protein was loaded on each lane. Western blot analysis was performed with antibodies directed against the Myc and HA epitope, respectively. The sample –PK was taken prior to addition of Proteinase K (PK). The sample +Tx +PK was taken after solubilization of LD with 1 % Triton X-100 for 20 min on ice. (E and F) The effect of PK treatment on Erg6p and Ayr1p, two LD resident proteins, was investigated with the respective polyclonal antibodies over the time interval from 10 sec to 20 min.

Figure 2

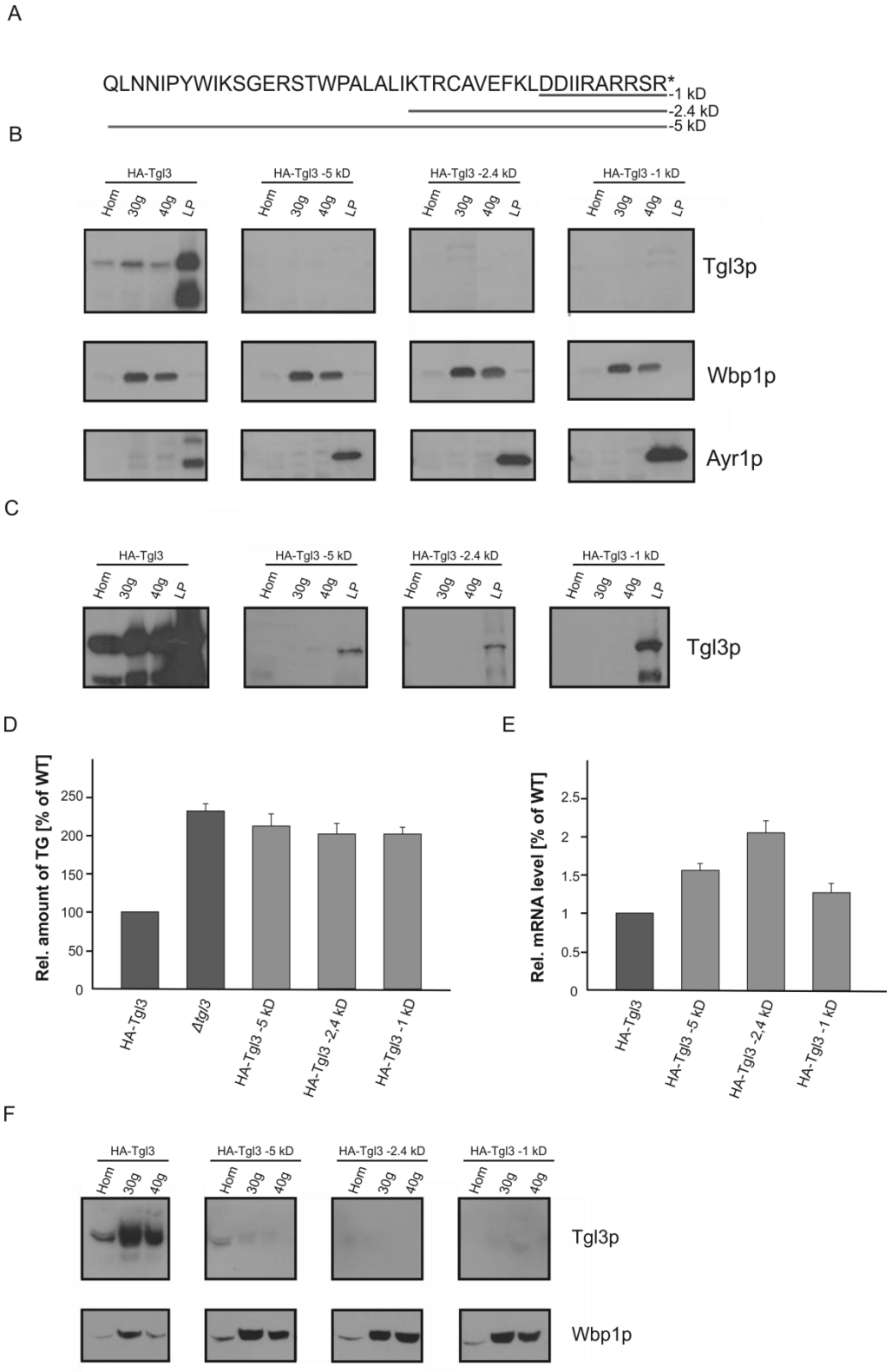

Figure 2. C-terminal truncations of Tgl3p result in protein instability.

(A) Amino acid sequence of the C-terminal 5 kD of Tgl3p and truncations of 1 kD, 2.4 kD and 5 kD are shown. The respective amino acids deleted in each construct are underlined. All

proteins used were N-terminally HA-tagged. (B) Homogenates (Hom); 30,000 x g (30g) microsomes; 40,000 x g (40g) microsomes; and lipid droplets (LD) were isolated from strains carrying full length Tgl3p and C-terminal truncations of -1 kD, -2.4 kD and -5 kD of Tgl3p. Western blot analysis was performed with a primary antibody against the HA tag. Antibodies against Wbp1p (ER marker) and Ayr1p (LD marker) were used for quality control of the subcellular fractions. Ten µg protein were loaded per lane. (C) Western blot analysis of indicated fractions from SDS-PAGE overloaded with ~70 µg protein per lane. (D) Relative TG content of strains grown to the stationary phase was measured to evaluate functionality of Tgl3p *in vivo*. (E) Relative mRNA expression levels of full length *TGL3* (set at 1) and truncated variants of *TGL3*. (F) C-terminally truncated Tgl3p variants in the ER from a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant. Homogenates (Hom); 30,000 x g (30g) microsomes; and 40,000 x g (40g) microsomes were isolated from a QM strain carrying either full length Tgl3p or C-terminal truncations of -1 kD, -2.4 kD and -5 kD of Tgl3p. All proteins are N-terminally HA- tagged. Western Blot analysis was performed with a primary antibody against the HA tag. The antibody against Wbp1p (ER marker) was used as quality control of microsomal fractions. Fifty µg protein were loaded per lane.

Figure 3

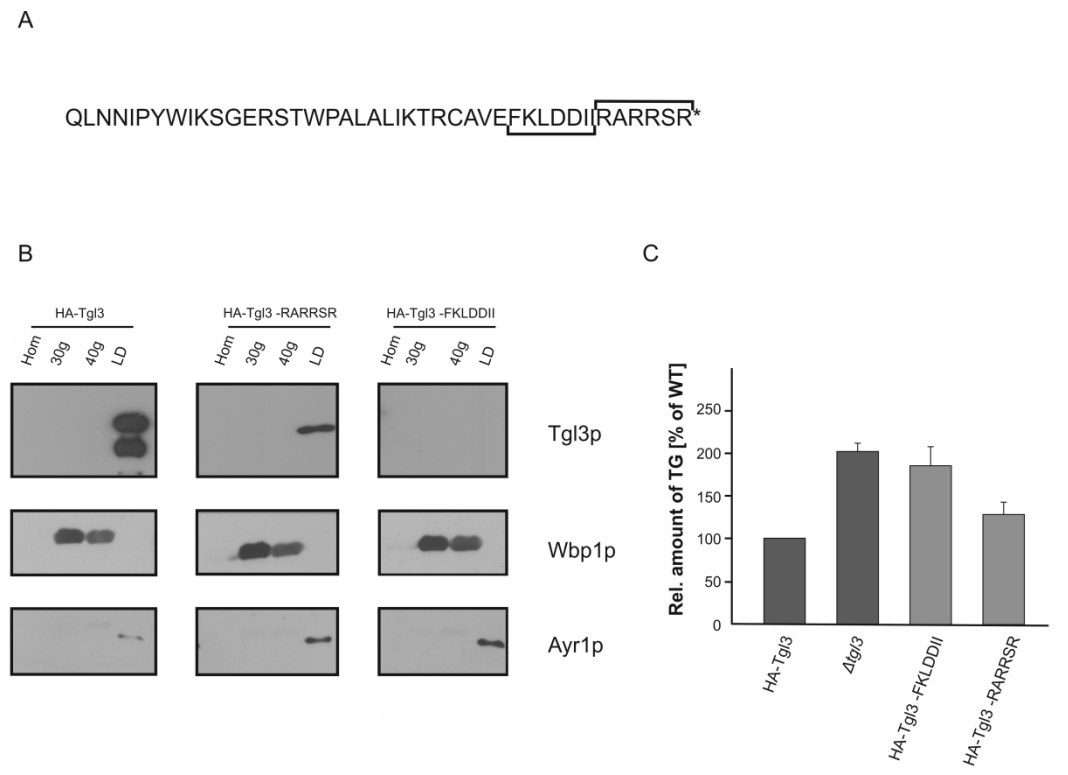

Figure 3. A stretch of seven amino acids within the C-terminus of Tgl3p is responsible for stability.

(A) The C-terminal 5 kD amino acid sequence of Tgl3p is shown. Truncations are indicated by brackets. (B) Homogenates (Hom); 30,000 x g (30g) microsomes; 40,000 x g (40g) microsomes; and lipid droplets (LD) were isolated from strains harboring full length Tgl3p; Tgl3p deleted of FKLDDII; and Tgl3p deleted of RARRSR. All constructs were N-terminally HA-tagged, and Western blot analysis was performed using a primary antibody against the HA tag. Wbp1p (ER marker) and Ayr1p (LD marker) were used for quality control of subcellular fractions. Ten µg protein were loaded per lane (C) Relative amounts of triacylglycerol (TG) from a strain carrying full length N-terminally HA tagged Tgl3p; a *tgl3*Δ deletion mutant; and a *tgl3*Δ strain harboring Tgl3p deleted of FKLDDII or RARRSR, respectively, were measured. Cells were grown to the stationary phase. Results are the average from at least three independent experiments with S.D. values (error bars) as indicated.

Figure 4

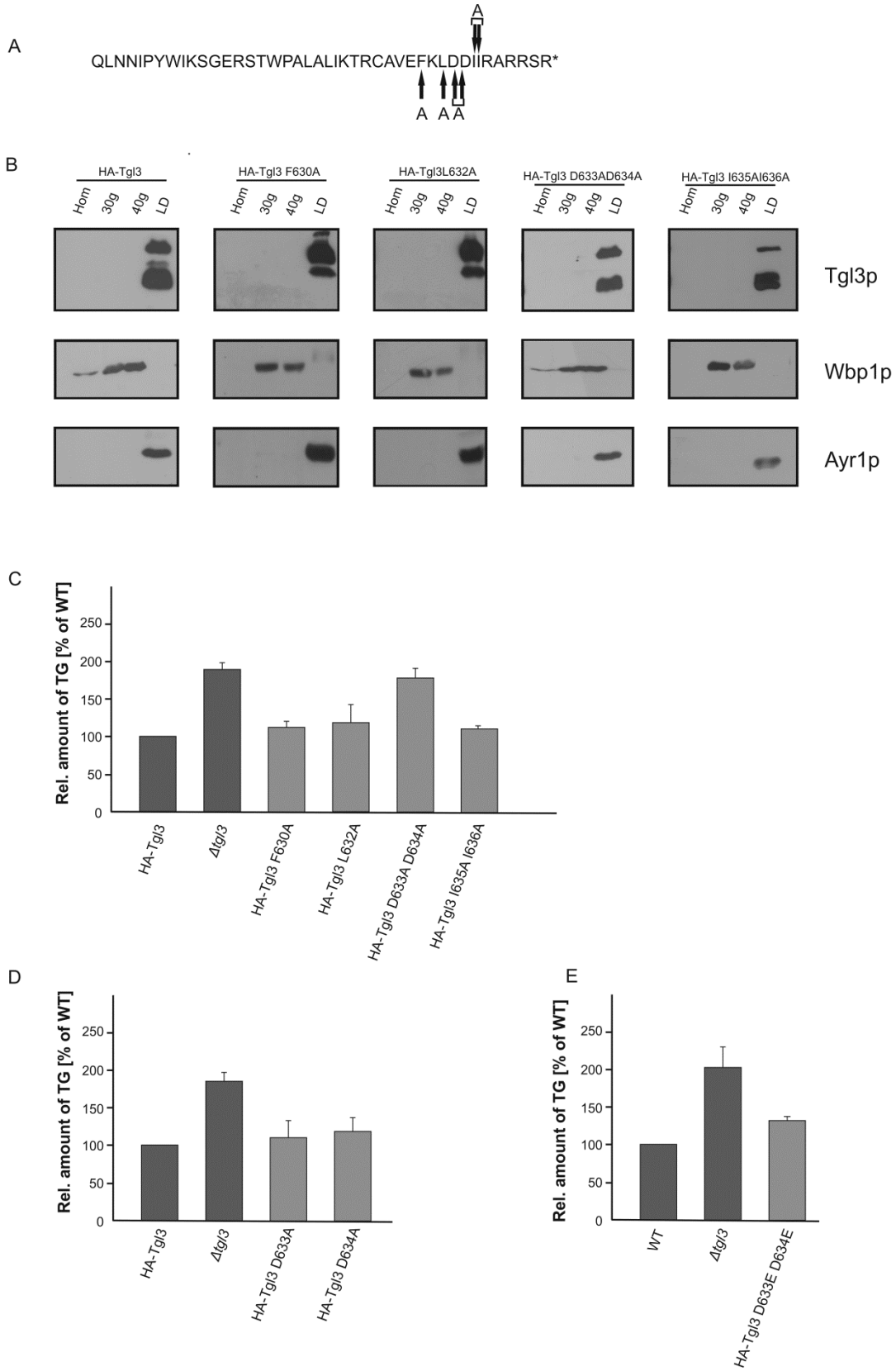

Figure 4. Single and double amino acid exchanges within the C-terminal region affect stability and activity of Tgl3p.

(A) Amino acid sequence of the C-terminal 5 kD of Tgl3p is shown, and positions of single and double amino acid exchanges are marked with arrows. All variants were N-terminally HA-tagged. (B) Homogenates (Hom); 30,000 x g (30g) microsomes; 40,000 x g (40g) microsomes; and lipid droplets (LD) from strains harboring full length Tgl3p; Tgl3p variants with single amino acid exchanges F630A and L632A; and Tgl3p variants bearing double amino acid exchanges D633A/D634A and I635A/I636A were analyzed by Western blotting. Primary antibodies were directed against the HA tag, Wbp1p (ER marker) and Ayr1p (LD marker). Ten µg protein were loaded per lane (C – E) The relative amounts of triacylglycerol (TG) from the respective strains were analyzed from cells grown to the stationary phase. Results are the average from at least three independent experiments with S.D. values (error bars) as indicated

Figure 5

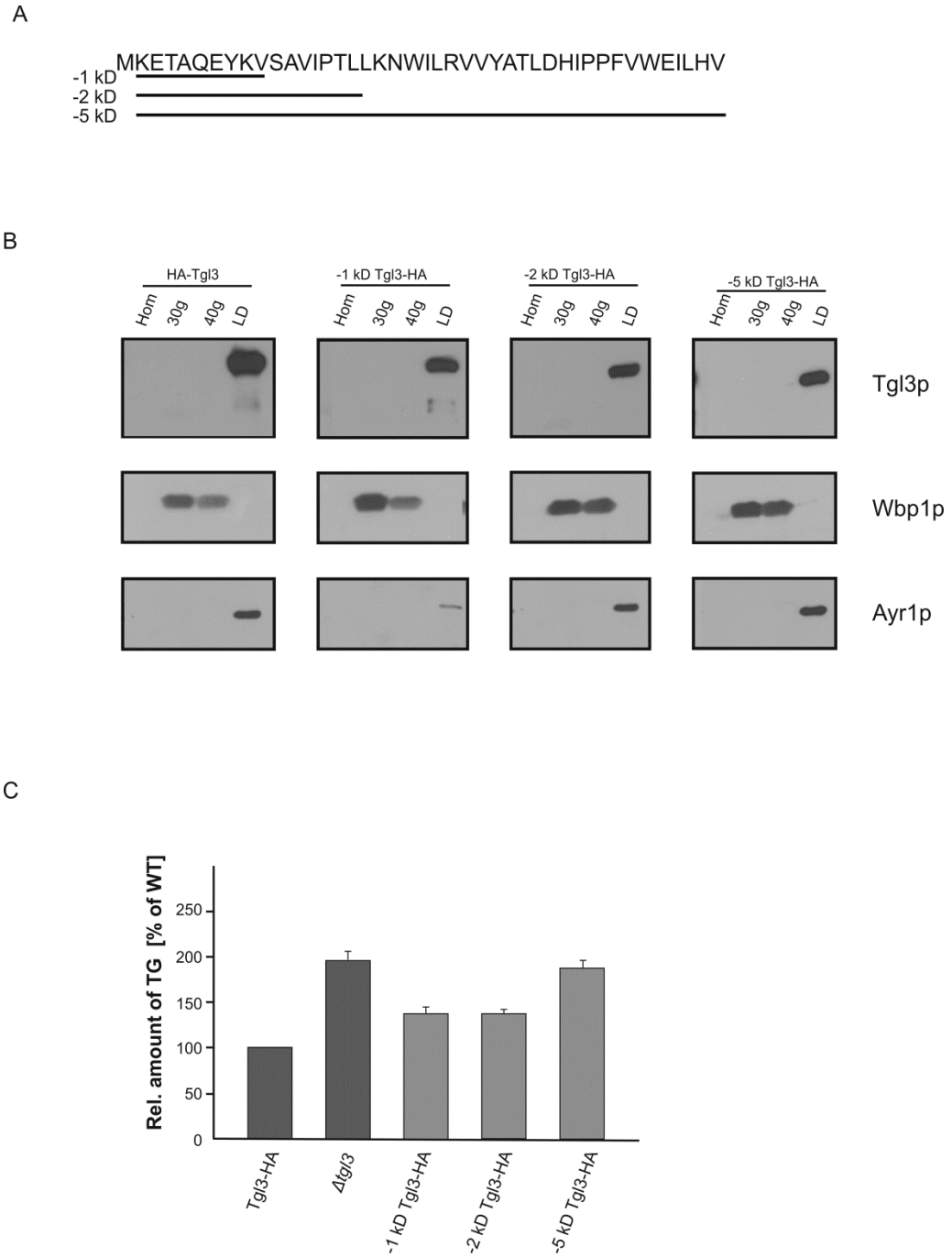

Figure 5. Truncations of the N-terminus of Tgl3p.

(A) The amino acid sequence of the N-terminal 5 kD of Tgl3p is shown, and the amino acids deleted in each construct are underlined. (B) Homogenates (Hom); 30,000 x g (30g) microsomes, 40,000 x g (40g) microsomes; and lipid droplets (LD) were isolated from strains carrying full length Tgl3p; and 1 kD, 2 kD and 5 kD N-terminally truncated variants of Tgl3p. All proteins were C-terminally HA-tagged, and Western blot analysis was performed using primary antibodies against the HA tag, Wbp1p (ER marker) and Ayr1p (LD marker). Ten µg

protein were loaded per lane (C) The relative amount of triacylglycerol (TG) from the respective strains grown to the stationary phase was measured. Results are the average from at least three independent experiments with S.D. values (error bars) as indicated

Figure 6

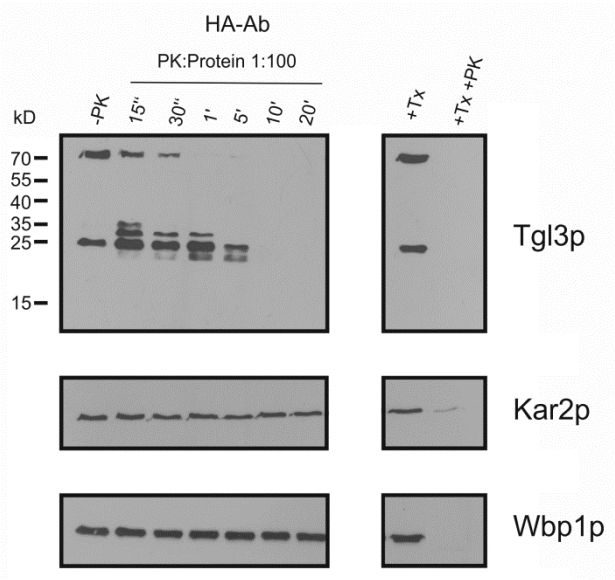

Figure 6. Orientation of the C-terminus of Tgl3p in the ER.
30,000 x g microsomes were isolated from a quadruple mutant (QM) lacking lipid droplets. A C-terminally HA-tagged Tgl3p variant was used to perform Proteinase K (PK) protection assays over a time range from 15 sec to 20 min. +Tx, addition of 1% Triton X-100; +Tx +PK, addition of 1% Triton X-100 and PK; -PK, control without PK. Kar2p served as marker protein for luminal ER; and Wbp1p as an integral membrane protein marker. Thirty µg protein were loaded per lane

CHAPTER IV

Topological Investigation of Lipid Droplet Surface Proteins

Barbara Koch[1], Clemens Gruber[2] and Günther Daum[1]

[1] Institute of Biochemistry, Graz University of Technology, Austria
[2]Department for Chemistry, Division of Biochemistry, University of Natural Resources and Applied Life Sciences, Vienna

[#]To whom correspondence should be addressed: Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria; Tel.: +433168736462; Fax: +433168736952; E-mail: guenther.daum@tugraz.at

Key words: Lipid droplets, topology, *S. cerevisiae*, mass spectrometry

Abstract

Since the last decade, proteome analysis of lipid droplets (LD) from yeast and humans gave important insights in lipid metabolism. LD display a unique membrane environment for proteins as their surface is built from a phospholipid monolayer. Topological investigations of LD surface proteins are just at the beginning and time consuming. Here, we describe a novel strategy to test the membrane topology of LD proteins in a global approach by applying traditional Proteinase K digestion experiments in combination with mass spectrometry. A first evaluation of this method with the LD proteins Erg6p and Ayr1p showed different behaviors of these proteins upon Proteinase K treatment and allowed first conclusions of surface exposed regions of these proteins. This method seems to be an applicable technique to test the protein surface landscape of LD.

Introduction

Lipid droplets (LD) are specialized organelles to store non-polar lipid such as triacylglycerols (TG) and steryl esters (SE). LD of *Saccharomyces cerevisiae* are small, spherical organelles with an approximate diameter of 400 nm (1). TG and SE contribute about 50%, each, to the non-polar lipid content of LD. The highly hydrophobic core of LD is most likely built from TG which is surrounded by several shells of SE. The surface of LD is covered by a phospholipid monolayer (2). Mass spectrometry (MS) proteome analysis of purified LD identified a small but specific set of proteins on the LD, most of them being involved in lipid metabolism (3, 4).

The question how LD proteins reach their destination and how these proteins are inserted into a phospholipid monolayer is still a matter of dispute. Primary amino acid targeting consensus sequences on LD proteins have not yet been identified. However, N-terminal amphipathic helices and hydrophobic stretches of N- and C-terminal domains seem to be important for proper targeting of proteins to LD (5–8). Many LD proteins display a dual localization and are also found in the endoplasmic reticulum (ER). This dual localization raises the question how proteins can cope with two different membrane environments, namely a phospholipid monolayer in LD and a phospholipid bilayer in the ER. The proteins studied best in this respect are Erg1p, Erg6p and Tgl3p from *S. cerevisiae* (8–11). A very recent study of our lab showed that the C-terminus of Tgl3p, the main TG lipase of *S. cerevisiae,* is embedded within the LD whereas the N-terminus faces the cytosolic site of the organelle (11). Contrary, the C-terminus of this lipase faces the cytosolic site of the ER. This result indicates that structural rearrangement of LD proteins might be a possibility to adapt to the two types of membrane environments, but experimental data for such a process are still missing.

As traditional experiments to test the membrane topology of proteins such as Proteinase K treatments are time intensive we designed an experimental procedure to study a large number of LD surface proteins in a more general approach. We combined traditional Proteinase K treatment with MS analysis of isolated LD organelles with the aim to detect “missing” domains of the proteins after protease treatment. We evaluated this approach by bioinformatic analysis to get a proof-of-principle of this new approach.

Materials and Methods

Strains and culture conditions

A *S. cerevisiae* BY4741 wild type strain was used for this study. Cells were grown aerobically to the stationary growth phase at 30°C in YPD media containing 1% yeast extract, 2% glucose and 2% peptone. Growth was monitored by measuring OD_{600}.

Lipid droplet isolation

Yeast LD were obtained at high purity from cells grown to the stationary phase according to published procedures (12, 13). In brief, cells were grown to the early stationary phase. After preparation of spheroplasts 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 $Na_2EDTA{\cdot}2H_2O$), followed by mechanical disintegration using a Dounce homogenizer with a loose fitting pestle. The resulting homogenate was centrifuged at 7,000 rpm 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) and overlaid with LD-A. Ultracentrifugation at 28,000 rpm for 45 min at 4°C using a swing out rotor yielded a white layer on top (crude LD) which 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 and overlaid with LD-B (8% Ficoll 400 in 10 mM MES/Tris, pH 6.9, 0.2 mM $Na_2EDTA{\cdot}2H_2O$). Ultracentrifugation at 28,000 rpm for 30 min at 4°C resulted in a top layer containing LD, which were 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 $Na_2EDTA{\cdot}2H_2O$) was filled into a fresh ultracentrifuge tube. The homogenized LD were 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 yielded a top layer containing highly purified LD. The top layer was collected with a pipette and transferred into a 7 ml Dounce homogenizer. LD were homogenized for further analysis.

Protein determination

Prior to protein analysis, LD fractions were delipidated with 2–3 volumes of diethyl ether. 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 15%

and solubilized in 100 µl of 0.1% SDS, 0.1 M NaOH. Purified proteins were quantified by using the Bio-Rad protein assay based on the method of Lowry et al. (14) and bovine serum albumin as a standard.

Proteinase K treatment

Isolated LD corresponding to 70 µg LD proteins were used for Proteinase K treatment. Proteinase K (Roche) was added to a ratio of 1:100 (Proteinase K:protein; w:w) and the incubation was carried out on ice and at 37°C in a Vortex. The reaction was stopped by adding trichloroacetic acid at a final concentration of 15% and vigorous vortexing. Proteins were precipitated for 1 h and centrifuged in a table top centrifuge at 4°C for 30 min. The protein pellet was washed with ice-cold water and collected by centrifugation again.

LC-ESI-MS Peptide analysis

The protein pellets were re-dissolved in 100 mM ammonium bicarbonate buffer (ABC buffer) containing 0.02% SDS and 15 mM DTT. For effective reduction of disulfide bonds samples were incubated for 45 min at 56°C. Free thiol groups were carbamidomethylated (30 min incubation with iodoacetamide in the dark at room temperature), and samples were acetone precipitated (acetone:aqueous phase; 4:1). The pellet was re-dissolved in 0.1 M ABC buffer and digested with trypsin (37°C) over night.

About 3 µg of each digest was loaded on a BioBasic C18 column (BioBasic-18, 150 x 0.32 mm, 5 µm, Thermo Scientific) using 60 mM ammoniumformate buffer as the aqueous solvent. A gradient from 5 to 80 % acetonitrile was developed over 75 min at a flow rate of 6 µL/min. Detection was performed with a Q-TOF mass spectrometer equipped with the standard ESI source (Bruker maXis 4G) in the positive ion, DDA mode (= switching to MSMS mode for eluting peaks). Instrument calibration was performed using an ESI calibration mixture.

Results

To investigate the effect of a Proteinase K treatment on LD surface proteins, we isolated LD from a *S. cerevisiae* BY4741 wild type strain. Isolated LD were treated with Proteinase K for different time periods and temperatures. The goal of this approach was to achieve a gradual increase of the effect of the Proteinase K treatment to distinguish between differently exposed sites of the proteins on the LD surface. Therefore, LD (70 µg protein) were incubated for 2.5 min and 10 min on ice, and one sample was incubated for 15 min at 37°C on a heating block with shaking. All LD samples were treated with Proteinase K at a ratio of 1:100. The reaction was stopped by addition of TCA to a final concentration of 15% and washed prior to MS analysis. An untreated LD sample was used to compare the effect of the Proteinase K treatment.

The first protein analyzed in detail was Erg6p. This protein was the most abundant protein found on LD (Table 1). Erg6p is a Δ(24)-sterol C-methyltransferase and converts zymosterol to fecosterol in the ergosterol biosynthetic pathway by methylating position C-24 (15) Figure 1 shows an amino acid sequence comparison of the MS analysis obtained from the untreated sample with three different Proteinase K treated samples. Amino acids shown in red are detected with MS/MS, whereas amino acids shown in green are unlikely to be found. Amino acids in light blue are not found and bold underlined dark blue stretches are digested by Proteinase K. This result shows immediately that the untreated sample does not differ from the samples treated with the proteinase on ice for 2.5 min and 10 min. However, comparing the sample which was treated for 15 min at 37°C to the control clearly shows that some fragments of the N-terminus were digested. Furthermore, the last 4 C-terminal amino acids are also missing, indicating that the N-terminal regions and the C-terminus most likely face the cytosolic site of LD. As there is no crystal structure of Erg6p available, we performed a 3D modelling with Phyre2 (16). Figure 2 shows the modeled structure of Erg6p in two different angles. The upper part of the picture shows the untreated samples while the lower part represents the structure after Proteinase K digestion for 15 min at 37°C. In previous experiments of our lab we found that small parts of about 5 kD of Erg6p were digested after Proteinase K treatment for 10 min on ice (11). As this result was obtained with a polyclonal antibody against Erg6p, we did not know which parts of the protein were digested. A comparison of the digested parts obtained with MS analysis suggested that after protease treatment at 37°C for 15 min about 15 kD are missing. According to the modelled structure these parts seem to be surface exposed and easily accessible for digestion. Figure 3 shows the Kyte-Doolittle Hydrophobicity Plot for Erg6p with the red line indicating the hydrophobicity

score for a suitable transmembrane domain (17). The digested parts are marked by brackets. No clear correlation of hydrophobicity and digestion can be drawn.

The second LD protein investigated by our new approach was Ayr1p, which was also a top 10 hit of most abundant LD proteins in our MS analysis. This NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase was recently also identified as TG lipase (18, 19). Previous experiments suggested that Ayr1p is deeply embedded within the LD and Proteinase K does not get access to this protein after a 20 min digest on ice, whereas only a rough treatment of LD with hard vortexing at 37°C showed a small part of Ayr1p to be digested (11). It was not possible to identify these regions as a polyclonal antibody was used. Our MS analysis presents a different picture of Ayr1p. As can be seen in Figure 4 after a 2.5 min Proteinase K digest on ice parts of the protein are already digested whereas different regions are affected after 10 min on ice. The treatment for 15 min at 37°C shows the most pronounced effect and was used in Figure 5 in a comparison with an untreated protein sequence of the modelled structure of Ayr1p. The digestion mostly affects the flexible loop regions of the protein. Additional to α-helical parts present on the outside of the proteins, β-sheets in inner core of the protein seem to be affected. Also the N-terminus was digested indicating a cytosolic localization on LD, whereas the C-terminus was not able to be determined. A comparison with the Kyte-Doolittle Hydrophobicity Plot for Ayr1p does not show a general correlation of digestion (marked with brackets) and non-hydrophobic parts (Figure 6). Interestingly, the conserved GXSXG lipase motif found between amino acids 16 to 20 in the N-terminus of Ayr1p was not affected by digestion.

Discussion

The insertion of proteins to LD is still a matter of dispute. Investigations of membrane topology with tagged problems by Proteinase K experiments followed by Western Blot analysis are time consuming. Therefore, we started a new approach which might allow a "high throughput" screening for the topology of untagged (non-modified) LD surface proteins. For this purpose we tried to combine traditional methods such as Proteinase K treatment of isolated LD with MS analysis. The goal of this strategy was to achieve significant results with reduced working steps.

MS analysis showed that the samples treated at 37°C for 15 min contained a 90% less protein amount than the untreated sample. Only the most abundant proteins seem to be present in significant amounts to be evaluated with this method. The two proteins first investigated with this approach, Erg6p and Ayr1p, showed different behaviors upon Proteinase K treatment.

With our experimental design we tried to achieve a gradually increasing proteolytic effect on LD proteins. While Erg6p seems to be affected only when LD were treated for 15 min at 37°C, some regions of Ayr1p were already digested after 2.5 min on ice. This result is in contrast to traditional Proteinase K experiments which were previously done in our lab, where Ayr1p was not affected by any Proteinase K treatment on ice for up to 20 min. Comparing MS analysis results for the digested regions with the untreated Ayr1p sample may indicate that the fragments missing in the LD samples on ice were too small to be detected in Western Blot analysis as they may be in a 1 to 3 kD range. Interestingly, the N-terminus of both Erg6p and Ayr1p seem to be orientated towards the cytosolic side of the LD. However, the digestion of the N-terminus of Ayr1p stops right before the conserved GXSXG lipase motive located between the amino acids 16 to 20. The last four amino acids of Erg6p were digested, indicating a cytosolic orientation. It was not possible to determine the last C-terminal amino acids of Ayr1p most likely due to technical reasons.

While the orientation of N-and C-termini of proteins seem to be easily examined with this method, digested regions within the proteins are hard to interpret. Crystal structures of LD proteins are not available, and therefore modelled structures have to serve as templates. Hence, any conclusions are currently only hypothetical. Nevertheless, the method of MS analysis of LD surface proteins after proteinase treatment seems to be an applicable technique to test the protein surface landscape of LD.

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Figure1

Figure 1: Sequence analysis of Erg6p obtained by MS analysis

Isolated LD were subjected to increasing Proteinase K treatment starting with an untreated sample, followed by a digestion for 2.5 and 10 min on ice and 15 min at 37°C. LD proteins were precipitated with TCA and analyzed by MS. Sequences shown in red: confirmed by MS/MS; green: unlikely to be found; blue not found with automatic interpretation by GPM. Dark blue and underlined: peptide fragments missing after Proteinase K treatment.

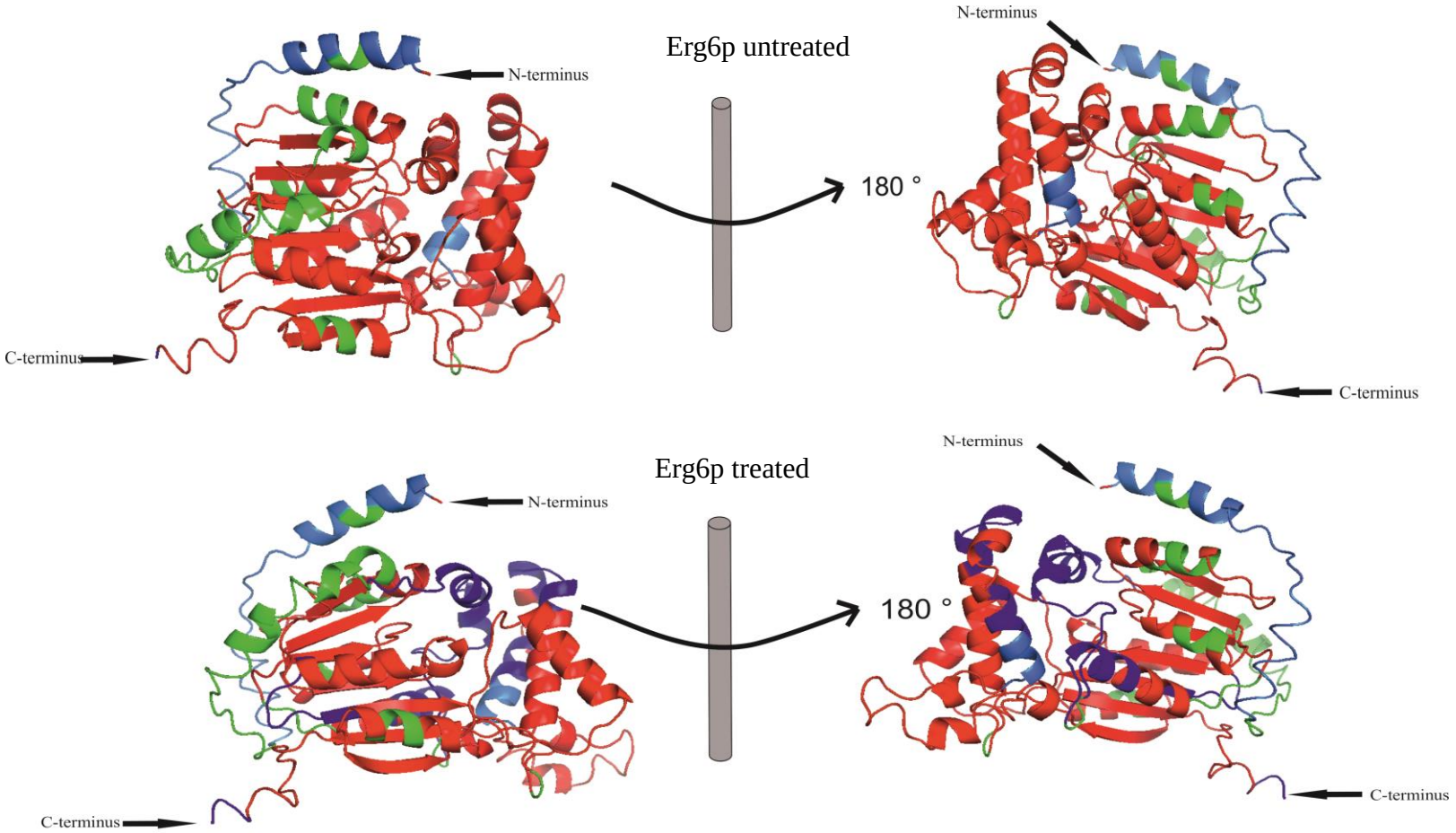

Figure 2: Modelled structure of Erg6p

The 3D structure of Erg6p was modelled with Phyre2. The upper part of the Figure shows the model of the untreated samples in two different angles. Sequences shown in red: confirmed by MS/MS; green: unlikely to be found; blue: black not found with automatic interpretation by GPM. The lower part of the Figure shows the structure after Proteinase K treatment for 15 min at 37°C. Digested parts are marked in dark blue. Sequences shown in red: confirmed by MS/MS; green: unlikely to be found; blue: not found with automatic interpretation by GPM

Figure 3

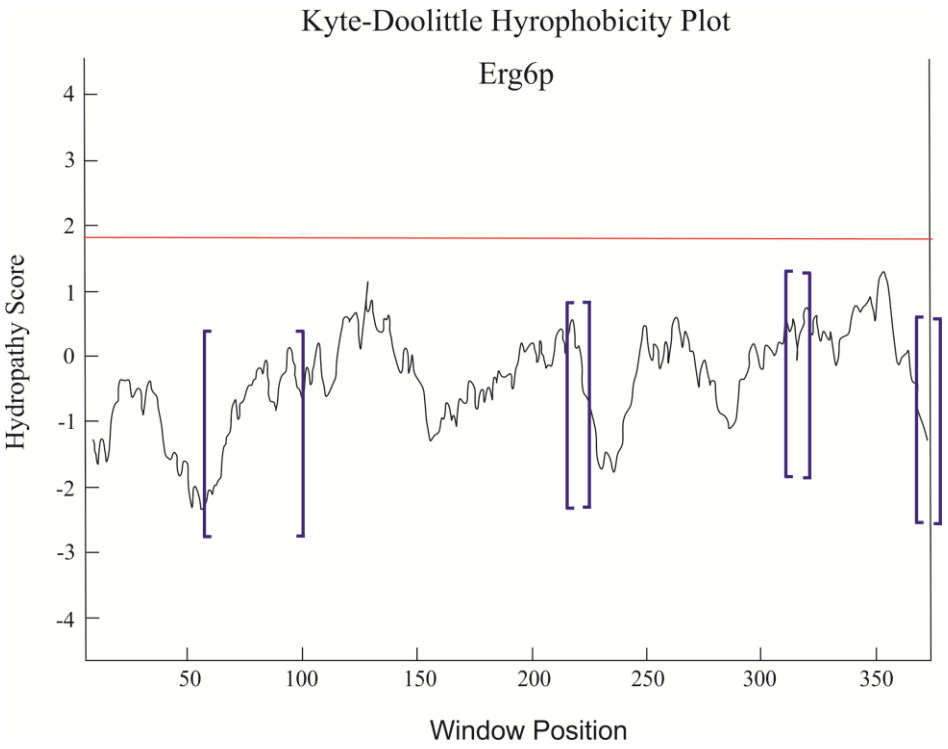

Figure 3: Kyte-Doolittle Blot of Erg6p

The hydrophobicity of Erg6p was analyzed with a Kyte-Doolittle Blot (Window size 19). The red line indicates necessary hydrophobicity for transmembrane domains. Brackets indicate digested peptide regions after Proteinase K treatment for 15 min at 37°C.

Figure 4

Figure 4: Sequence analysis of Ayr1p obtained by MS analysis

Isolated LD were subjected to increasing Proteinase K treatment starting with an untreated sample, followed by a digestion of 2.5 and 10 min on ice and 15 min at 37°C. LD proteins were precipitated with TCA and analyzed by MS. Sequences shown in red: confirmed by MS/MS; green: unlikely to be found; blue not found with automatic interpretation by GPM. Dark blue and underlined: peptide fragments missing after Proteinase K treatment.

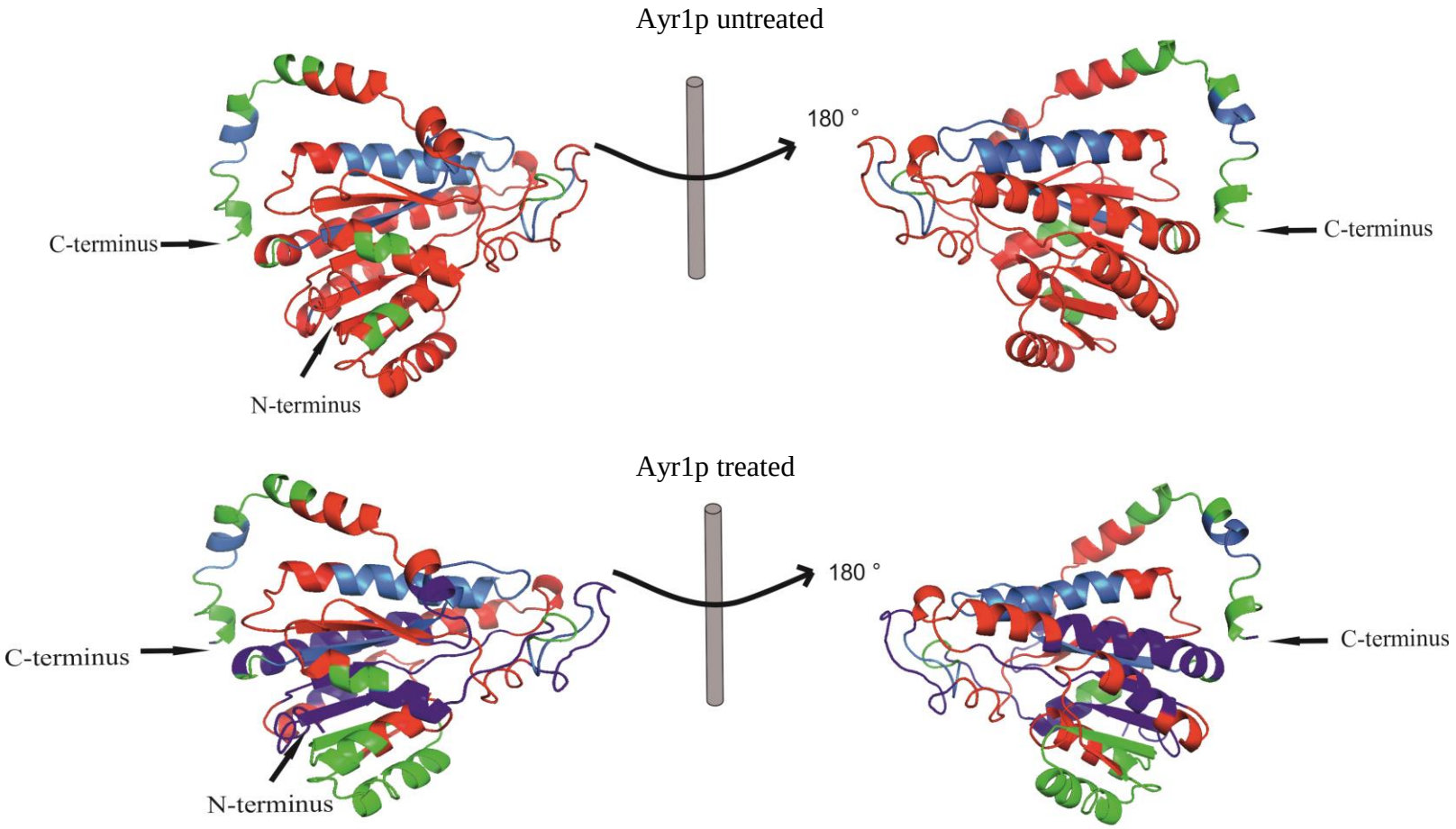

Figure 5: Modelled structure of Ayr1p. The 3D structure of Ayr1p was modelled with Phyre2. The upper part of the Figure shows the model of the untreated samples in two different angles. Sequences shown in red: confirmed by MS/MS; green: unlikely to be found; blue: black not found with automatic interpretation by GPM. The lower part of the Figure shows the structure after Proteinase K treatment for 15 min at 37°C. Digested parts are marked in dark blue. Sequences shown in red: confirmed by MS/MS; green: unlikely to be found; blue: not found with automatic interpretation byGPM.

Figure 6

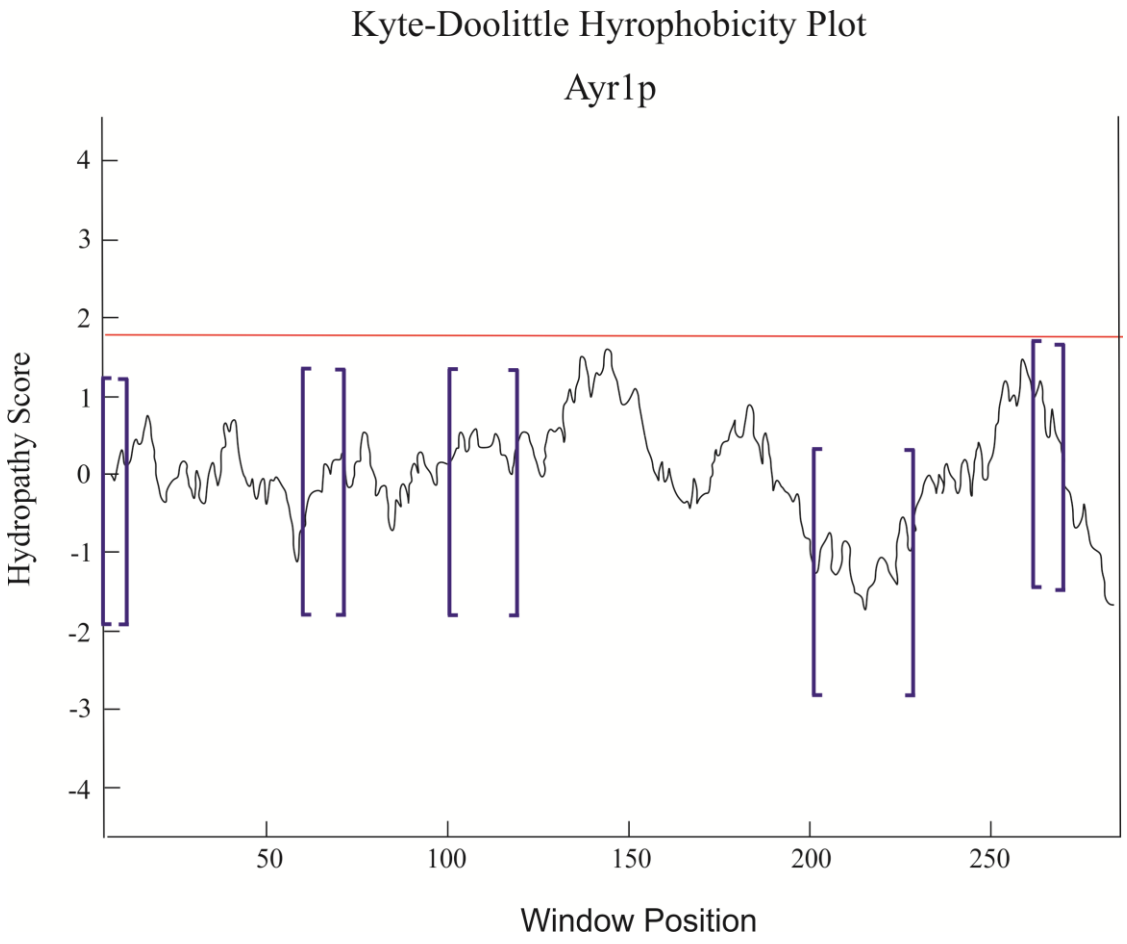

Figure 6: Kyte-Doolittle Blot of Ayr1p

The hydrophobicity of Ayr1p was analyzed with a Kyte-Doolittle Blot (Window size 19). The red line indicates necessary hydrophobicity for transmembrane domain. Brackets indicate digested peptide regions after Proteinase K treatment for 15 min at 37°C.

Table1: LD of a *S.cerevisiae* BY4741 wild type strain were isolated and a proteome analysis was performed by mass spectrometry. The list shows scores with decreasing numberof peptide coverage.

Rank	Accession	Protein	MW [kDa]	Scores	#Peptides	SC [%]
1	ERG6	Sterol 24-C-methyltransferase	43,4	2377.1 (M:2377.1)	38	75,7
2	LCF1	Long-chain-fatty-acid--CoA ligase	77,8	1561.8 (M:1561.8)	30	61,4
3	MCFS2	Medium-chain fatty acid ethyl ester synthase/esterase 2	51,2	1558.2 (M:1558.2)	30	68,3
4	FAT1	Very long-chain fatty acid transport protein	77,1	1506.0 (M:1506.0)	33	56,7
5	ERG1	Squalene monooxygenase	55,1	1479.1 (M:1479.1)	34	65,1
6	ERG27	3-keto-steroid reductase	39,7	1237.8 (M:1237.8)	25	76,4
7	AYR1	NADPH-dependent 1-acyldihydroxyacetone phosphate reductase	32,8	1097.8 (M:1097.8)	21	68
8	VPH1	V-type proton ATPase subunit a, vacuolar isoform3	95,5	1001.7 (M:1001.7)	22	31,5
9	ERG7	Lanosterol synthase	83,4	933.7 (M:933.7)	25	45
10	VATB	V-type proton ATPase subunit B	57,7	752.6 (M:752.6)	16	45,3
11	PET10	Protein PET10 , unknown function	31,2	737.4 (M:737.4)	15	59
12	VATA	V-type proton ATPase catalytic subunit =3	118,6	737.3 (M:737.3)	19	23,8
13	ATPB	mitochondrial ATP synthase subunit beta	54,8	735.3 (M:735.3)	17	53
14	HFD1	Putative fatty aldehyde dehydrogenase	59,9	732.4 (M:732.4)	17	40,2
15	KES1	Protein KES1 oxysterol binding protein family	49,5	727.7 (M:727.7)	16	48,8
16	PDR16	Phosphatidylinositol transfer protein	40,7	717.0 (M:717.0)	19	55,8
17	LCF4	Long-chain-fatty-acid--CoA ligase 4	77,2	705.9 (M:705.9)	17	34
18	VA0D	V-type proton ATPase subunit d	39,8	675.6 (M:675.6)	13	68,4
19	TGL3	Triacylglycerol lipase 3	73,6	642.1 (M:642.1)	14	30,2
20	PDI	Protein disulfide-isomerase	58,2	564.1 (M:564.1)	15	38,3
21	PGC1	Phosphatidylglycerol phospholipase	37	556.5 (M:556.5)	12	46,1
22	GRP78	78 kDa glucose-regulated protein homolog	74,4	544.9 (M:544.9)	12	23
23	TGL1	Sterol esterase	62,9	521.2 (M:521.2)	13	27,4
24	EF1A	Elongation factor 1-alpha	50	507.8 (M:507.8)	12	31
25	PMA1	Plasma membrane ATPase 1	99,6	507.5 (M:507.5)	13	24,2
26	VTC4	Vacuolar transporter chaperone 4	83,1	497.8 (M:497.8)	16	29,5
27	RUVB2	RuvB-like protein 2	51,6	457.5 (M:457.5)	11	27,4
28	APE3	Aminopeptidase Y	60,1	455.4 (M:455.4)	11	35,9
29	VDAC1	Mitochondrial outer membrane protein porin	30,4	454.2 (M:454.2)	10	49,5
30	YJU3	Serine hydrolase YJU3 2	35,5	451.5 (M:451.5)	11	40,9

31	ATPA	ATP synthase subunit alpha, mitochondrial	58,6	448.6 (M:448.6)	12	27,2
32	VATE	V-type proton ATPase subunit E	26,5	429.0 (M:429.0)	10	52,4
33	TSC10	3-ketodihydrosphingosine reductase TSC10	35,9	427.1 (M:427.1)	10	43,4
34	TGL4	Triacylglycerol lipase 4	102,7	414.0 (M:414.0)	11	17
35	RS3	40S ribosomal protein S3	26,5	384.7 (M:384.7)	9	44,6
36	RUVB1	RuvB-like protein 1	50,4	351.8 (M:351.8)	10	33,3
37	VPS66	Vacuolar protein sorting-associated protein 66	33,8	341.2 (M:341.2)	9	36,3
38	ADT2	ADP,ATP carrier protein 2	34,4	339.2 (M:339.2)	9	30,8
39	YO059	Putative lipase YOR059C	51,1	329.9 (M:329.9)	8	24,4
40	YIM1	Protein YIM1, unknown function	41,6	315.0 (M:315.0)	9	36,4
41	GST1	Glutathione S-transferase	26,8	293.3 (M:293.3)	7	32,1
42	RER2	Dehydrodolichyl diphosphate synthase	32,7	282.1 (M:282.1)	7	33,2
43	ENO2	Enolase 2	46,9	261.5 (M:261.5)	5	17,2
44	UPPS	Probable undecaprenyl pyrophosphate synthase	42,5	261.5 (M:261.5)	9	26,1
45	HSP77	Heat shock protein SSC1, mitochondrial	70,6	255.4 (M:255.4)	6	12,5
46	RHO1	GTP-binding protein	23,1	254.8 (M:254.8)	6	34
47	YP147	Uncharacterized protein YPR147C	34,8	244.9 (M:244.9)	5	22
48	PLSC	Probable 1-acyl-sn-glycerol-3-phosphate acyltransferase	33,9	240.9 (M:240.9)	7	26,1
49	CARP	Saccharopepsin	44,5	227.0 (M:227.0)	6	21,7
50	PPB	Repressible alkaline phosphatase	63	224.1 (M:224.1)	5	16,3
51	SEC63	Protein translocation protein	75,3	224.0 (M:224.0)	6	12,4
52	CSR1	Phosphatidylinositol transfer protein	47,4	223.8 (M:223.8)	6	19,9
53	FMP52	Protein FMP52, mitochondrial	25,1	214.9 (M:214.9)	6	30,3
54	RLA2	60S acidic ribosomal	10,7	211.5 (M:211.5)	4	34,9
55	YO246	Uncharacterized oxidoreductase	37,5	208.6 (M:208.6)	6	30,9
56	TGL5	Triacylglycerol lipase 5	84,7	206.0 (M:206.0)	5	9,2
57	LDH1	Lipid droplet hydrolase 1	43,3	205.0 (M:205.0)	5	18,9
58	RLA0	60S acidic ribosomal protein P0	33,7	202.1 (M:202.1)	5	20,8
59	VAC8	Vacuolar protein 8, vacuolar membrane protein	63,2	191.1 (M:191.1)	5	12,1
60	CAB5	Dephospho-CoA kinase CAB5	27,3	187.0 (M:187.0)	5	36,9
61	RLA4	60S acidic ribosomal protein P2-beta	11	186.1 (M:186.1)	4	53,6

62	HSP76	Heat shock protein SSB2	66,6	181.6 (M:181.6)	3	8,6
63	GDI1	Rab GDP-dissociation inhibitor	51,2	180.6 (M:180.6)	5	16,6
64	UBX2	UBX domain-containing protein 2	66,7	172.0 (M:172.0)	5	9,6
65	G3P3	Glyceraldehyde-3-phosphate dehydrogenase 3	35,7	171.6 (M:171.6)	4	19
66	DGA1	Diacylglycerol O-acyltransferase 1	47,7	164.0 (M:164.0)	3	9,1
67	YCFI	Metal resistance protein YCF1	171	160.6 (M:160.6)	5	4,6
68	VATC	V-type proton ATPase subunit C	44,2	157.1 (M:157.1)	4	16,3
69	GDIR	Rho GDP-dissociation inhibitor	23,1	150.9 (M:150.9)	3	29,2
70	CISY1	Citrate synthase, mitochondrial	53,3	131.4 (M:131.4)	4	11,5
71	CYPD	Peptidyl-prolyl cis-trans isomerase D	25,3	126.0 (M:126.0)	3	24
72	ALG11	GDP-Man:Man(3)GlcNAc(2)-PP-Dol alpha-1,2-mannosyltransferase	63,1	123.1 (M:123.1)	3	6,4
73	H4	Histone H4	11,4	119.1 (M:119.1)	3	33
74	YJR1	Cell wall protein YJL171C	42,9	112.7 (M:112.7)	2	7,6
75	VTC3	Vacuolar transporter chaperone 3	96,5	112.5 (M:112.5)	4	5,1
76	SEC4	Ras-related protein SEC4 =1	23,5	107.9 (M:107.9)	3	18,1
77	HSP26	Heat shock protein 26	23,9	106.7 (M:106.7)	4	36
78	YBQ6	Uncharacterized glycosyl hydrolase YBR056W	57,8	105.4 (M:105.4)	4	12,8
79	CH10	10 kDa heat shock protein, mitochondrial	11,4	92.9 (M:92.9)	2	25,5
80	RS5	40S ribosomal protein S5	25	89.6 (M:89.6)	2	19,1
81	RS6A	40S ribosomal protein S6-	27	88.8 (M:88.8)	2	13,6
82	HXT6	High-affinity hexose transporter HXT6	62,7	85.9 (M:85.9)	2	7,4
83	QCR2	Cytochrome b-c1 complex subunit 2, mitochondrial	40,5	84.4 (M:84.4)	2	7,9
84	YN034	Uncharacterized protein YNR034W-A	10,8	84.4 (M:84.4)	2	25,5
85	VATG	V-type proton ATPase subunit G	12,7	82.3 (M:82.3)	2	12,3
86	MAS5	Mitochondrial protein import protein	44,6	81.4 (M:81.4)	2	7,6
87	HSP60	Heat shock protein 60, mitochondrial	60,7	79.3 (M:79.3)	2	5,6
88	RL401	Ubiquitin-60S ribosomal protein L40	14,5	76.8 (M:76.8)	2	19,5
89	H2A1	Histone H2A.1	14	74.8 (M:74.8)	2	28,8
90	H2B2	Histone H2B.2	14,2	73.0 (M:73.0)	2	18,3
91	VATF	V-type proton ATPase subunit F	13,5	71.8 (M:71.8)	2	39,8
92	SSH1	Sec sixty-one protein homolog	53,3	69.5 (M:69.5)	2	7,1

93	RS25B	40S ribosomal protein S25-B	12	68.5 (M:68.5)	2	17,6
94	YL225	Uncharacterized SVF1-like protein YLR225C	46,4	66.6 (M:66.6)	2	5,2
95	SAY1	Steryl acetyl hydrolase 1	48,5	66.6 (M:66.6)	3	11,3
96	ZEO1	Protein ZEO1, peripheral plasmamembrane protein	12,6	62.8 (M:62.8)	2	17,7
97	YPT7	GTP-binding protein YPT7	23	60.6 (M:60.6)	2	11,5
98	VTC1	Vacuolar transporter chaperone 1	14,4	53.1 (M:53.1)	2	10,1
99	IML2	Mitochondrial outer membrane protein	82,5	52.8 (M:52.8)	2	3,3
100	PHO88	Inorganic phosphate transport protein	21,1	52.3 (M:52.3)	2	14,9
101	MSC1	Meiotic sister chromatid recombination protein 1	59,6	43.3 (M:43.3)	2	6,8
102	RRT8	Regulator of rDNA transcription protein 8	39,6	38.8 (M:38.8)	2	6,1

General Discussion

In the last 20 years, the knowledge of the lipid metabolism of *Saccharomyces cerevisiae* increased tremendously. Many key players involved in non-polar lipid synthesis, storage and mobilization not only of the *S. cerevisiae*, but also from *Pichia pastoris* and *Yarrowia lipolytica*, two yeasts with high economic value, were characterized and studied extensively (1, 2). Insights into the marrow of a central organelle in lipid metabolism, the LD, were gained. The protein equipment of LD from these yeasts was identified and characterized in some detail (1–5). However, several questions related to yeast non-polar lipid biology remained open so far. As an example, it is not known how non-polar lipid hydrolytic enzymes locate to LD and get access to their substrates. LD models predict that proteins are embedded in the surface phospholipid monolayer of the organelle. However, the substrates for TG lipases and SE hydrolases form the hydrophobic core of LD, and the access of enzymes to their substrates appears to be a problem. To address this question a more detailed knowledge of the topology of LD proteins is required. Also, little is known about targeting and insertion of LD proteins into the phospholipid monolayer of LD. Moreover, several LD proteins display a dual localization. They are found on LD and in the ER (6–8). Consequently, they are able to integrate into both phospholipid monolayer and bilayer membranes. How the different membrane environments affect the stability and function of these enzymes needs to be investigated. More detailed studies of structural properties of LD proteins will help to answer this question. Therefore we started to investigate the topology of yeast LD proteins in more detail.

In our research we focused on Tgl3p, the main TG lipase of *S. cerevisiae*. As there is not much knowledge of the topology of any LD protein we aimed to get insight how Tgl3p is inserted in the surface phospholipid monolayer of LD. We applied a mild proteolytic digestion assay with Proteinase K to determine the orientation of the N-terminus and C-terminus of this lipase. This approach revealed that the N-terminus is most likely facing the cytosolic site of the organelle whereas the C-terminus seems to be protected by membranes and most likely faces the inside of LD. It was already hypothesized before, that the C-terminus of LD proteins might be important for targeting proteins to their LD destination, although no primary amino acid targeting sequence has been identified (9). In order to test the role of Tgl3p's C-terminus in the process of targeting, we created C-terminal truncations of Tgl3p and investigated the distribution of Tgl3p between the ER and LD. Surprisingly, we

found, that not only a truncation of 50 amino acids but already a truncation of the last 10 amino acids severely affected the protein stability. Only traces of the protein were found on LD. This result led us to draw two conclusions. First, parts within the C-terminus seem to be essential for protein stability, not only on LD but also in the ER, as none of the truncated variants of Tgl3p were detected in this organelle. Second, there seems to be no targeting signal within the last 50 amino acids, as traces of Tgl3p were still found on LD.

The way how LD proteins reach their destination is still unclear. It is an accepted model for LD biogenesis, that LD arise from the ER (for review see reference 10) and proteins destined to LD route via the ER during LD biogenesis. Whether they are translated in ER associated ribosomes or free ribosomes, is not clear. Usually, ER proteins are translated in ER associated ribosomes in a signal recognition particle (SRP) dependent process. The signal peptide for the SRP is mostly found in the N-terminal domain of these proteins. No such signal peptide was found in the N-terminus of Tgl3p and also N-terminal truncations up to 50 amino acids had no impact on targeting of Tgl3p to ER or LD and did not affect protein stability severely. As the C-terminus plays a critical role in stability it might be hypothesized that it is important for translation efficiency. Only little is known at present about the machinery responsible for insertion of proteins to the ER membrane by a single C-terminal transmembrane domain of so called tail anchored proteins. In recent years, a SRP independent pathway of insertion into the ER membrane was discovered by Schuldiner et al. (11). They showed that the GET-Complex mediates the insertion of tail anchored (TA)-proteins to the ER membrane, with Get3p recognizing the TA-protein. Then, both proteins together are recruited to the ER membrane where insertion happens via the Get1p/Get2p receptor. Although it is still pure speculation, it might be taken into account that Tgl3p and other LD proteins may be recruited to the ER via routes different from the classical SRP pathway.

The fact that the loss of 10 amino acids is already sufficient to highly compromise the stability of Tgl3p, led us to define the region which is responsible for this severe effect. During these investigations we narrowed down the region responsible for stability to the seven amino acids FKLDDII, located in the far C-terminus of Tgl3p. The interesting finding was that single or double amino acid exchanges within this sequence did not affect stability. Most surprisingly, however, we found that a double amino acid exchange of D633A/D634A led to an inactive Tgl3p protein and a strain accumulating TG like a *tgl3*Δ strain. This situation is unique as despite correct targeting the lipase activity of this Tgl3p variant was severely compromised. A single amino acid exchange of only D633A or D634A had no effect on the lipase activity of Tgl3p. As an exchange of aspartate to alanine comes along with the

loss of a negative charge, we also tested whether preserving the negative charge by an exchange of both aspartate residues to glutamate rescued the lipase activity. Indeed, we were able to show that Tgl3p is still active with two glutamate residues at these positions. Thus, the negative charge(s) of these two aspartate residues seem to be important for the functionality of Tgl3p. It is tempting to speculate, that one of the aspartates D633 or D634 may be part of the catalytic dyade. However, there are arguments which speak against this view. First, an amino acid exchange of only one aspartate, either D633 or D634, respectively, had no effect on the lipase activity as TG accumulation was not observed in these strains. Secondly, D633 and D634 are not located within the predicted patatin domain where one would usually expect the catalytic active serine – aspartate dyad. As none of the aspartate residues seems to be part of the active center, we wondered about their role for lipase activity. It might be possible that D633 and D634 are important for the interaction with a protein partner. Until now, however, the interaction of Tgl3p with other proteins has not been shown. It is well known that the adipose triglyceride lipase (ATGL) of humans and mice, the homologue of yeast Tgl4p, needs CGI-58 to be fully active (12). It is tempting to speculate, that such a partner protein also exists for yeast Tgl3p, but evidence is still missing.

It is also possible, that an interaction of Tgl3p with other proteins concerns anchoring Tgl3p to LD. Although we found that the C-terminus of Tgl3p is deeply embedded within the LD, it seems that this part of the protein is not explicitly hydrophobic as one would expect. This finding raises the question how such a non-hydrophobic part of the protein can be inserted into membranes. One explanation might be anchoring via another, deeply embedded protein partner. In an interactomic study with a split GFP screen Ayr1p turned out to be an interaction partner of Tgl3p (13). Ayr1p was recently also identified as TG lipase, although with only minor lipase activity (14). Our results for Ayr1p obtained with Proteinase K experiments suggested that this protein is deeply embedded in the LD. PK got only access to Ayr1p when LD were incubated at 37°C and vigorously vortexed during the treatment. Thus, the possibility remains that Tgl3p might be associated with a partner protein which allows assembly in the form of a complex into the LD surface. Currently, however, there is no further experimental evidence supporting the view of an interaction of Tgl3p with Ayr1p, and formation of LD surface complexes containing Tgl3p remains pure speculation at present. Interestingly, *TGL3* was also shown to be synthetic lethal with *GAS1* in two high throughput screenings (15, 16). Gas1p is a β-1,3-glucanosyltransferase which is required for cell wall assembly and is anchored to the cell surface with a glycosylphosphatidylinositol (GPI) anchor (17). This fact is interesting insofar, as the whole non-polar lipid biosynthetic as well

hydrolytic pathway can be deleted in yeast cells with no severe phenotypes although there might be some links to cell wall assembly which have not yet been taken into account. Further characterization of protein interactions with Tgl3p will be a prerequisite to explain the highly connected network of lipid pathways and interactions.

As already mentioned before, Tgl3p and also other LD protein show a dual localization. They can be found on LD and in the ER. This is an intriguing observation and raises the question how proteins can adapt to two different membrane environments. A previous study from or lab had shown that the half-life of Tgl3p is dramatically reduced when the protein was located to the ER and the enzyme lost its lipase as well as the acyltransferase activity (6). We found that the C-terminus of Tgl3p faces the cytosolic side of the ER. Given the fact that the C-terminus of Tgl3p is very important for its stability, exposure of this part of the protein at the cytosolic side of the ER may be the reason for the instability of Tgl3p in this compartment. The membrane bilayer environment of the ER might cause a different topology of Tgl3p in this organelle, whereas the phospholipid monolayer of the LD surface seems to provide the more appropriate surrounding for the enzyme. It is not yet clear, how Tgl3p is inserted into a bilayer membrane, but taking into account that the C-terminus is facing the cytosolic site it might as well be that Tgl3p is not an integral membrane protein in the ER but rather associated on the surface of the ER membrane. Tgl3p seems to undergo structural rearrangements during LD maturation. Whether these arrangements are actively driven by other proteins or passive movements is not known.

This detailed molecular analysis of Tgl3p provided some insight into the membrane topology of Tgl3p and elucidated functionality of this lipase. The link between topology, targeting and function of LD proteins will be a challenge for further research in this field.

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Barbara Koch

Personal Inforamtion

Date of Birth: 22.04.1980

Gender: female

Nationality: Austrian

University Education

Feb 2011-July 2014	Working on PhD thesis in Molecular Biosciences (Topology of yeast lipases) under the supervision of Prof. Dr. Günther Daum at Graz University of Technology	PhD
2008-2010	Masters study Molecular Microbiology and Master thesis "Yar1 – a chaperone for the ribosomal protein Rps3" under the supervision of Dr. Brigitte Pertschy at the Institute of Molecular Biosciences, Karl-Franzens-University of Graz completed with high distinction	MSc
2005-2008	Bachelor level study of Molecular Biology Bachelor thesis: „Cellcycle and Speedy/Ringo proteins". "Transgenic animals – methods and prospects".	Bakk. rer. nat
1998-2005	Completed the basic and preclinical sections of a degree in medicine at the University of Graz	

Publications

Koch, B., Schmidt, C. Ploier, B. and Daum, G. (2014) Modifications of the C-terminus affect functionality and stability of yeast triacylglycerol lipase Tgl3p. *J. Biol. Chem May 20 [Epub ahead of print]*

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Talks and Poster presentations

41st Annual Conference on Yeast, Smolenice, Slovakia 20th – 23rd May 2014,
Talk: To be or not to be: minimal changes in the C-terminus affect functionality and stability of yeast triacylglycerol lipase Tgl3p

Keystone Symposium: Lipid Pathways in Biology and Disease, Dublin, Ireland 19th – 24th March 2014
Poster presentation: *Topological investigations of Tgl3p – New aspects of the main triacylglycerol lipase of Saccharomyces cerevisiae*

30th International Specialised Symposium on Yeast – Cell Surface & Organelles in Yeast: from Basics to Applications Stara Lesna, Slovakia, 18th -22nd June 2013
Poster presentation: Topological investigations of Tgl3p – New aspects of the main triacylglycerol lipase of Saccharomyces cerevisiae

5th ÖGMBT Annual Meeting, Innsbruck, Austria, 25th -27th September 2013
Poster presentation: Topological investigations of Tgl3p – New aspects of the main triacylglycerol lipase of Saccharomyces cerevisiae

11th Euro Fed Lipids Congress: Oils, Fats and Lipids: New Strategies for a High Quality Future Antalya, Turkey, 27th -30th October 2013
Poster presentation: Topological investigations of Tgl3p – New aspects of the main triacylglycerol lipase of Saccharomyces cerevisiae

FEBS Workshop on Lipids: from Lipidomics to Disease and Green Energy Spetses, Greece, 23rd -29th August 2012
Talk and poster presentation: Topology of triacylglycerol lipases of the yeast Saccharomyces cerevisiae

Skills

Research:

- Expertise in working with recombinant DNA molecules (amplification of challenging DNA molecules, plasmid construction for different host strains, construction of *Saccharomyces cerevisiae*.

- Expertise in expression and purification of recombinant proteins from different host strains (bacteria and yeasts) for the purpose of protein crystallography and functional characterisation (protein-protein interaction studies and *in vitro* enzyme assays).

- Expertise in isolating different organelles from yeast cells (mitochondria, microsomes, lipid droplets, peroxisomes, ribosomes)

- Expertise in characterisation of yeast genes (involved in ribosome biogenesis and lipid metabolism)

- Expertise in analysis of non-polar lipids and phospholipids.

- Scientific writing skills demonstrated in research papers and reviews recently published.

- Experience in presentation of scientific work at international conferences (posters and talks).

Teaching:

- Organisation and supervision of lab courses for undergraduate students (including topics such as western blotting, ELISA and protein purification).

- Evaluating student performances in laboratory experiments and report writing.

- Supervision of project lab students and exchange students.

Communication/Teamwork

- Ability to work independently and as an active team member.

- Passion for guiding and teaching students.

- Organisation and enforcement of lab rules and regulations and maintenance of lab equipment.

Prizes and awards

FEBS Youth Travel Grand 2012

Interests

Outdoor sports (hiking, skiing, running, climbing)
Travelling (including long distance hiking trips)